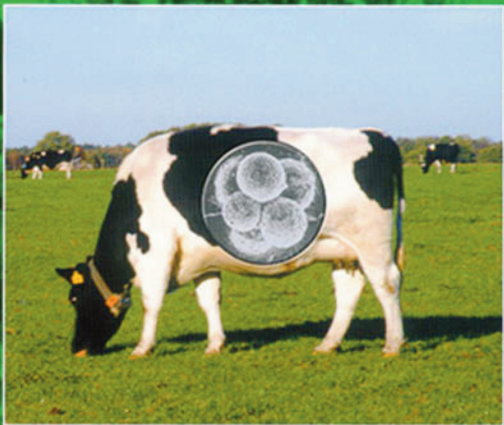
Biotechnology in Agriculture Series, No. 27

Laboratory Production of Cattle Embryos, 2nd Edition



I. Gordon

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*Out of print

Biotechnology in Agriculture No. 27

Laboratory Production of Cattle Embryos

Second Edition

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CABI Publishing

CABI Publishing is a division of CAB International

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A catalogue record for this book is available from the British Library, London, UK.

Library of Congress Cataloging-in-Publication Data

Gordon, Ian R.
  Laboratory production of cattle embryos / I. Gordon. -- 2nd ed.
    p. cm.
  Includes bibliographical references and index.
  ISBN 0-85199-666-3 (alk. paper)
  1. Livestock--Embryos. 2. Livestock--Embryos--Transplantation. I. Title.

SF887.G59 2003
636.2′0898178059--dc21

2003043498

ISBN 0 85199 666 3

Typeset by AMA DataSet Ltd, UK.
Printed and bound in the UK by Cromwell Press, Trowbridge.

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Preface

This monograph is primarily aimed at meeting the needs of technical and scientific personnel interested or engaged in the laboratory production of cattle embryos; such personnel also include the growing numbers using *in vitro* embryo production in conjunction with oocyte recovery from live cattle. The present work also seeks to provide information of general interest to those who wish to follow developments in reproductive biology. The book draws on literature running to more than 3000 reports that have appeared since the first edition was published in 1994. Although designed to be self-contained, the work contains no more than a passing reference to much historical information; those interested in a fuller story may find such facts in the 1994 edition.

The application of reproductive biology to commercial farm practice over the past 50 years, in the form of artificial insemination (AI), has proved to be an outstanding success, with the technique being applied to dairy cattle in most countries around the world. Without detracting in any way from the efforts of pioneering researchers, it was fortunate that bull sperm could be frozen and stored and still achieve conception rates little different from fresh semen without too much science being involved. If bull sperm had behaved like ram or boar sperm, the history of cattle AI might have been rather different, simply because the science of the times might have been inadequate. Likewise, with conventional cattle embryo transfer (ET) in the last three decades of the 20th century, embryos could be subjected to a great many insults during collection, storage and transfer and still produce perfectly normal and healthy young at the end of the process. Again, the science and understanding of the 1970s was sufficient to permit commercial application without raising concerns.

Not so with *in vitro*-produced (IVP) embryos, which, initially, when it was a matter of artificial maturation and fertilization and culture in the sheep oviduct, seemed to be in a similar category to conventional *in vivo*-derived cattle embryos. Their fall from grace came when the transition was made from the sheep oviduct to the co-culture system – the blastocysts produced simply would not withstand freezing; add to this the complications of the large offspring syndrome (LOS) and associated events, and the whole basis of commercial application – low-cost cattle embryos in large numbers – was undermined. Alongside such challenges in the laboratory, down on the farm, market forces from the 1990s onwards in Ireland and Britain ensured that novel techniques for increasing production efficiency in livestock became much less appealing. Laboratory embryo production came to be viewed as a research tool rather than a practical means of improving the lot of the cattle producer. Today's commercial use of *in vitro* embryo production is almost exclusively confined to obtaining young from problem cows – those that have failed to produce embryos by conventional ET means.

None the less, whether talking of bovine sperm or oocytes, the main objective of modern reproductive technologies in cattle reproduction is to increase reproductive efficiency and the

quality of the young that cows produce, whether in terms of milking ability or in the carcass quality of the beef animals that are born. Today's reproductive technologies in cattle also offer the potential for greatly extending the range of genetic material that may be stored for possible future use. The development and refinement of these novel technologies is likely to concentrate on IVP of embryos, culturing, manipulation of embryos (splitting, nuclear transfer, establishing embryo stem cells and gene transfer) and ET. The development of these new techniques is likely to be greatly facilitated by the availability of increasingly sophisticated equipment for ultrasonography, for embryo micromanipulation and cryopreservation, for sperm sorting by high-speed flow cytometry and for DNA microarray technology. The real impact on cattle productivity will doubtless come from combining the new reproductive techniques with powerful DNA technologies. The new reproductive techniques will facilitate a rapid turnover of generations, while DNA technology will permit selection of cattle based on genetic rather than phenotypic information.

In research, there is need to achieve a much fuller understanding of ovarian and follicular events in the cow as the dominant follicle progresses towards ovulation; the development rate of *in vitro* cattle embryos can only be high when the bovine oocyte is fully competent. In the live animal, as shown by researchers in Canada, it is possible to influence oocyte quality markedly by stimulating the ovaries with exogenous hormones before oocyte recovery. Once the mechanisms by which the oocyte attains full competence in the ovarian follicle are better understood, it should be possible to re-create such conditions in the laboratory; this is likely to require arrested meiosis culture conditions for the oocytes and the appropriate signal(s) to mimic follicular maturation. In the laboratory itself, it seems likely that gene-expression profiling will become increasingly effective in identifying those genes that are involved in normal embryo development and permit a much more accurate prediction of embryo survival and normality. It is clear from the literature that a clean bill of health cannot be given to IVP cattle embryos for commercial use until methods are available to minimize or entirely eliminate the possibility of abnormalities, such as LOS. As part of the crucial body of evidence required to deal with the LOS problem, it is likely that the knowledge necessary to effectively cryopreserve the IVP embryo will also be available.

In drawing attention, albeit in summary form, to various aspects of human assisted reproduction, one is mindful of the gulf that has existed between those working in animal and human embryology, even though they have much in common and have great opportunities to work as allies in gaining knowledge and understanding of mammalian reproductive processes. For many years, sex preselection has been an objective of the dairy and beef industry, as a means of increasing the rate of response to selection, to reduce the cost of progeny testing for genetically superior bulls and to produce desired specialized and high-quality calves. At this point in time, *in vitro* embryo production may be the means of allowing breeders to use sexed semen, whereas sorted semen quality may not be sufficiently high to use in superovulated donor cattle.

The subject-matter covered in ten chapters includes the recovery and maturation of the secondary bovine oocyte, the preparation and capacitation of bull sperm, the fertilization of the oocyte and the subsequent culture of the early embryo to the blastocyst stage, at which it can either be transferred or stored for later use. This is followed by a consideration of the options available for the cryopreservation of embryos and oocytes. The final chapters are given over to discussing methods used in establishing pregnancies with IVP embryos and the use of such embryos and oocytes for research purposes and in commercial applications. Since the primary concern is with the production of embryos, rather than the rapidly expanding areas of cloning and transgenesis, the sections of the final chapter dealing with these topics are not intended to be exhaustive but more to provide a broad indication of the problems that are under investigation. At the same time, these are areas that could not be readily contemplated but for the developments that have already occurred in the IVP of cattle embryos.

Acknowledgements

The author wishes to express grateful thanks to the many editors and publishers who have given permission to reproduce illustrations and tables from books and original articles. I must also extend sincere thanks to the staff of CAB International for their excellent help in the preparation and production of this work.

The author is also most appreciative of the office and other facilities provided by University College Dublin at their Lyons Research farm and to staff members of the Department of Animal Science and Production for help on many occasions.

Dedication

To Joan: wife, friend and mother, who made life a happy experience.

1

Developments in Embryo *In Vitro* Production (IVP) Technology

1.1. Historical Aspects

More than a century ago, there were those who recognized that knowledge of mammalian fertilization would be greatly aided if the process could be observed under the microscope *in vitro*. Towards that end, workers started developing culture systems to simulate conditions within the oviduct that would permit the egg (oocyte) and sperm to be maintained in a fully functional state.

1.1.1. Early IVF reports

Sixty or so years ago, much of our understanding of fertilization was based on the study of sea urchin eggs, although early experiments to provide information on mammals were reported by Gregory Pincus and colleagues in the 1930s. Greater appreciation of the basic mechanisms involved in mammalian fertilization grew in the 1950s, with the recognition of the phenomenon of sperm capacitation (Austin, 1951; Chang, 1951) and the first comprehensive account of *in vitro* fertilization (IVF) in the rabbit, including the birth of young (Chang, 1959). For the first time, a repeatable technique for fertilizing mammalian eggs *in vitro* was available, using sperm recovered from the rabbit uterus 12 h after mating.

Before that time, and going as far back as 1878, studies with rabbits and other laboratory animals had failed to demonstrate that egg cleavage was due to sperm penetration rather than activation of the egg by some other means. Extensive experiments were carried out by Gregory Pincus and colleagues during the 1930s in which rabbit eggs were exposed to sperm and maintained in culture; in some instances, the birth of young was recorded after the transfer of eggs exposed to sperm in the oviducts of rabbits in which ovulation had been induced. Such results, however, were not regarded as convincing because the experimental procedures did not always preclude other possibilities of fertilization.

The mouse was the second mammalian species in which IVF was successfully accomplished, the event being reported by Whittingham in 1968. Another landmark was the work of Chang and Yanagimachi in the early 1960s, in which they were able to demonstrate sperm capacitation *in vitro* in the hamster, thereby providing much of the basic data that were to lead to the first successful test-tube fertilization of the human egg, reported by Edwards in 1969 (see Table 1.1 and review by Bavister, 2002).

1.1.2. Cattle IVF

One of the earliest attempts to fertilize artificially matured cattle oocytes *in vitro* was reported by Sreenan in Ireland, using bull sperm preincubated in a medium containing the enzyme α-amylase, then being employed by some to partially capacitate sperm destined for artificial insemination (AI). The first genuine success in

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fertilization *in vitro* of an artificially matured bovine oocyte was to await the efforts of Iritani and Niwa (1977) in Japan. A few years later, the first IVF calf (Virgil) was born in the USA, the result of the pioneering work of Brackett and his associates at the University of Pennsylvania Veterinary School (Brackett *et al.*, 1982). Further north, in Canada, using a laparoscopic technique to recover ovulated oocytes, Lambert *et al.* (1983) produced six IVF calves; the oocytes were fertilized *in vitro* soon after ovulation and the rabbit oviduct was used as an *in vitro* embryo culture system. Earlier work in Ireland by Sreenan and Scanlon (1968) had shown that the rabbit oviduct could be usefully employed in this way.

The first calves born after IVF of artificially matured oocytes were those reported by Hanada *et al.* (1986) in Japan; as in the Canadian work, the embryos were cultured to the blastocyst stage in the rabbit oviduct before transfer. The first twin pregnancy achieved by totally *in vitro* procedures (*in vitro* maturation (IVM), IVF and *in vitro* culture (IVC) of the early embryo) was that reported in Ireland by Lu and co-workers (Fig. 1.1).

Table 1.1. Milestones in mammalian *in vitro* fertilization.

Year	Event	Researcher(s)
1951	Recognition of sperm capacitation	Austin; Chang
1959	*In vitro* fertilization in the rabbit	Chang
1963	IVF with sperm capacitated *in vitro*	Yanagimachi and Chang
1969	Fertilization of human ova *in vitro*	Bavister *et al.*; Edwards *et al.*
1977	Fertilization of bovine ova *in vitro*	Iritani and Niwa
1978	First human birth with IVF embryo	Steptoe and Edwards
1982	First calf born after IVF in cattle	Brackett *et al.*
1986	Cloning in sheep by nuclear transfer	Willadsen
1986	Calves born after IVF of IVM ova	Hanada *et al.*
1988	Cattle twins after transfer of IVP embryos	Lu *et al.*

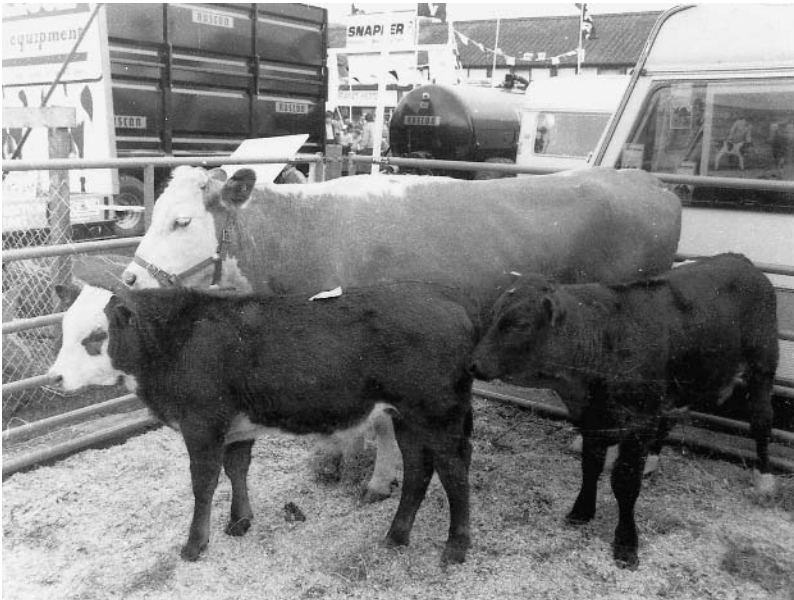

Fig. 1.1. Beef-cattle twins produced by totally *in vitro* procedures (from Lu *et al.*, 1988).

1.2. Cambridge Contributions

The development of reproductive technology in mammals in the 20th century will always be associated with the many valuable contributions made by workers in the Downing Street Cambridge School of Agriculture and at the Animal Research Station on the Huntingdon Road. The application of reproductive biology to dairy cattle, in the form of AI, owes much to the pioneering efforts of Hammond, Walton, Rowson and Polge at these locations; a recent survey by Thibier and Wagner (2002) has shown how widely the technique is used around the world.

1.2.1. School of Agriculture

Marshall's book *The Physiology of Reproduction*, first published in 1910, was to be widely acclaimed; it came to be regarded as a masterpiece, not only because it contained information on practically every known aspect of reproduction, but for clearly pointing the way forward research-wise on several fronts, As well as being notable for the publication of Marshall's classic on reproduction, 1910 was the year in which the new School of Agriculture building was opened. Along its corridors in due course would stride the giants of reproductive biology, including Marshall, Hammond, Walton, Pincus, Asdell, Parkes and Chang. The first edition of Marshall's book was dedicated to Walter Heape. Heape had written a book on *The Breeding Industry* in 1906, in which he drew attention to the considerable economic importance of the British livestock industry and the heavy losses that occurred annually because of a failure to apply existing scientific knowledge. However, Heape's name will for ever be associated with the first successful egg transfer in the rabbit (Heape, 1890). It was also Heape who, in 1905, first proposed that the gonads were dependent for their activity and function on a generative ferment produced elsewhere in the body in minute quantities; 20 years later, John Hammond was to adopt and expand on this hypothetical substance to great effect. In his book *Reproduction in the Rabbit*, published in 1925, Hammond developed the view that this 'generative ferment' was responsible for ovarian activity; it was not long after the publication of the book that work in other lands revealed the crucial role of anterior pituitary gonadotrophins in controlling ovarian function.

It is worth noting that the first comprehensive treatise on reproduction was Aristotle's *Generation of Animals* and that 2000 years were to pass before the next great work, Marshall's *Physiology of Reproduction*, would appear in 1910. Up to that time, it was not uncommon for physiology textbooks to relegate discussion of the reproductive organs to a few final pages. In his treatise, Marshall was to bring forward all the relevant information on the breeding season, fertilization and other aspects of reproduction. Although most discussion was on mammals, Marshall did not hesitate to draw from his knowledge of the reproductive processes in invertebrates and other forms of animal life.

The appearance of the expanded second edition of Marshall's book in 1922 was to act as an important stimulus to research activities pursued in many laboratories between the two world wars. With Marshall as the biologist and John Hammond as the agricultural scientist, the Cambridge School of Agriculture was to become a renowned international centre for reproductive research.

1.2.2. Animal Research Station

The Animal Research Station, 307 Huntingdon Road, Cambridge, was founded by John Hammond as part of the Cambridge School of Agriculture in 1932 on a small acreage of land allocated from the University farm. Overseas visitors to the Station after the Second World War were often to express astonishment that so many significant findings should emerge from a facility of such limited resources. Funds for the buildings and laboratories were provided mainly through the good offices of Walter Elliot and the Empire Marketing Board. In 1932, Elliot had attained cabinet rank as Minister for Agriculture and was making good use of the funds he had available for agricultural research. As the field station for his many research activities in fertility and growth, the Huntingdon Road Station became increasingly important to Hammond, providing the facilities to accommodate the large

farm animals in which he was so interested. After the Second World War, in 1949, the government-sponsored Agricultural Research Council (ARC) established the Unit of Animal Reproduction at the Animal Research Station, with John Hammond as director. The new unit's title ominously omitted any reference to 'growth' and the ARC's restructuring efforts of the day effectively closed down a substantial amount of Hammond's research. There was an almost complete cessation of the work on farm-animal growth and development that had previously been so vigorously pursued by Hammond and graduate students.

John Hammond's interest in embryo transfer (ET) in farm animals, especially cattle, was a natural follow-on to his pioneering efforts with AI, much of that being in concert with Arthur Walton; it was a matter of trying to do for the female what had been done for the male. His interest in this field went back many years. As early as 1929, Gregory Pincus, then a visiting scholar from Harvard, was superovulating rabbits and culturing their embryos *in vitro* in the School of Agriculture; egg transfers were carried out by Hammond and Walton, because they had the appropriate Home Office licences to carry out the required surgical interventions. Around that same time, Asdell and Hammond were also reporting three litters of pups from a series of 21 transfers carried out in rabbits.

Although the possibilities of ET in cattle were foreseen by Hammond back in the 1930s his first writings on what he termed 'artificial pregnancy' date from around 1950. At that time he suggested that embryo transfer could be the means 'whereby females of high genetic value could be enabled to produce more offspring by transplanting their eggs into inferior cows'. He was also to mention that 'beef cattle required for fattening could be provided by transplanting beef-bred Aberdeen-Angus eggs into dairy-bred Jersey cows, so reducing the costs of beef production'. Nor did he confine remarks to farm animals; he was among the first to suggest the 'possibility that women with blocked Fallopian tubes might be able to have an artificial pregnancy'.

Embryos across the Atlantic

Among the bright international stars to emerge from Hammond's Cambridge School was Min-Chueh Chang, who came to the Animal Research Station in 1939; he was destined to make several outstanding contributions to the development of IVF technology. After completing his doctorate in 1941, Chang was to spend a further 4 years in Cambridge, mainly working on IVF/ET procedures in the rabbit, before moving to the Worcester Foundation of Experimental Biology. When he decided to spend a year with Gregory Pincus in the USA at the end of the war, it was to work on IVF; Pincus had received funds at that time for work in cattle, but Chang convinced him that IVF should first be tried on small animals. The question of 'test-tube fertilization' of mammalian eggs was to remain controversial for many years, the final unequivocal proof of success, in the form of healthy newborn rabbit pups, finally being provided by Chang in 1959 (see Chang, 1968). Along the way, with Marden in Cambridge and Chang in the USA, the first trans-Atlantic shipment of fertilized eggs was achieved in the early 1950s (Chang and Marden, 1954); two white Californian rabbits were born to a black foster-mother, the embryos having been shipped from Boston to London by Trans World Airlines and then by train to Cambridge, a journey of some 27 h.

Using rabbits to good effect

Among Australian graduates working with Hammond in the late 1940s was Dowling, who examined various aspects of ET in the rabbit (Dowling, 1949). In that same year, John Hammond Junior described how it was possible to achieve consistent *in vitro* early embryo development in the mouse using a simple, defined medium (Hammond, 1949). In the 1947/48 period, Hammond Junior had worked in the Strangeways laboratory, an Edwardian mansion named after its founder, Thomas Strangeways (1866–1926), a pioneer in tissue-culture techniques. Established in 1905 as an independently funded research institution, Strangeways at one time was to number such luminaries as Francis Crick and Peter Medawar among its staff. Crick came to Cambridge in the autumn of 1947 to work in biology at Strangeways, but moved on 2 years later to join Max Perutz and John Kendrew in the Cavendish laboratory.

Notable achievements in the mid-1950s at the Animal Research Station included the work

of Bob Averill and colleagues in showing that sheep embryos could be safely stored in the rabbit oviduct for several days (Averill *et al.*, 1955). By that time, the efforts of Rowson and associates had laid the foundations for successful ET in farm ruminants. Much of their work was with sheep, which were both more readily available and easier to handle than cattle. There was also a great deal of valuable research with the rabbit, in the hands of Cyril Adams, who was probably the first to attempt shipping mammalian (rabbit) embryos from one country to another in 1950; two decades and more down the years, Dub Adams was to report the first successful ET in mink (in 1975).

Dawn of cattle ET industry

By the late 1960s, Rowson was able to report that acceptable pregnancy rates could be achieved in cattle using surgical approaches. This provided much of the stimulus to commercial concerns far and wide to develop ET technology. Across the Irish Sea, Sreenan and Beehan (1974) in Galway were among the first to confirm the effectiveness of Rowson's procedures. Up until retirement in 1979, Rowson continued to make valuable contributions to ET knowledge and became rightly regarded as the father-figure in cattle ET technology.

In the 1970s and early 1980s, the work of Moor and colleagues at the Animal Research Station on follicular function led to the identification of many factors involved in follicular growth and atresia and in developing methods of maturing oocytes *in vitro*. It became all too clear that normal embryonic development was greatly dependent on successful maturation of the oocyte. In a series of important contributions Moor and co-workers stressed the importance of the interactions between the oocyte and the surrounding follicular cells. It was evident that follicular somatic cells have both supportive and regulatory roles; Moor was probably the first to suggest that a relatively simple IVM procedure would be effective for cattle oocytes (Moor *et al.*, 1984).

Another landmark event at the Station, in June 1973, was the birth of Frosty II, the first calf to develop from a frozen and thawed embryo; this was the result of the efforts of Ian Wilmut, in association with Polge and Rowson. During the 1970s, Willadsen was at the forefront in developing effective freezing procedures for cattle and sheep embryos and with Trounson made many valuable contributions to reproductive biology, culminating in the mid-1980s, when Willadsen was responsible for the first report of successful cloning in sheep by nuclear transfer (Willadsen, 1986); this was a key event and ultimately led to the birth of Dolly in the closing years of the century (Wilmut *et al.*, 1997).

1.2.3. Cambridge, Babraham and beyond

With the retirement of John Hammond in 1954, the ARC in 1956 redesignated the Huntingdon Road Station as the Unit of Reproductive Physiology and Biochemistry, under Thaddeus Mann and with Rowson as Deputy Director. Dr Mann's books, *The Biochemistry of Semen*, published in 1954, and *The Biochemistry of Semen and of the Male Reproductive Tract* published 10 years later, were long to remain standard texts. After Mann's retirement in 1976, the Animal Research Station became part of the Babraham-based Institute of Animal Physiology; Rowson was to spend his remaining years as Officer-in-Charge of the Station until retirement in May 1979. The Station continued to operate at Huntingdon Road under Chris Polge until 1986, when it was finally closed by the Agricultural and Food Research Council (AFRC) in response to the savage cutbacks in research funding in the 1980s by Margaret Thatcher's government. The Station staff moved across to the opposite side of Cambridge and into new laboratories at the AFRC Physiology Institute in Babraham, in what was to become the Department of Molecular Embryology. The Huntingdon Road Station, however, was almost immediately to reopen as a commercial entity, Animal Biotechnology Cambridge, Ltd, under the direction of Philip Paxman and Chris Polge.

What started as the Institute of Animal Physiology at Babraham after the Second World War, and which was long regarded by farmers as a white elephant, with many highly qualified scientists busily investigating obscure non-agricultural topics, was eventually to become the Cambridge Research Station in 1986. The Station's remit was to advance knowledge of the

molecular, cellular and systematic processes of the animal; particular attention is paid to the mechanisms controlling the development, fertility, growth, behaviour, health and welfare of animals. In line with increasing emphasis on molecular biology, what was the AFRC became the Biotechnological and Biological Sciences Research Council. Notwithstanding new terms and titles, the activities of Moor and his colleagues in the Station's Department of Molecular Embryology continued to be especially relevant to all aspects of *in vitro* embryo production.

The Cambridge scene would not be complete without recalling that Bob Edwards in the university's Physiology Laboratory, located a few steps away from the Downing Street School of Agriculture, did much useful collaborative work with Rowson and Polge at the Animal Research Station in the mid-1960s, investigating various aspects of sperm capacitation and artificially maturing the oocytes of various mammals, including cattle, sheep and pigs. In his research in the capacitation of human sperm, Edwards was to use the cow as his animal model, because bovine oocytes could be matured *in vitro* and bull semen could be collected from Rowson's nearby AI station. Seminal plasma was removed from bovine sperm using mild centrifugation and was used to inseminate matured cow oocytes; several oocytes were found to be undergoing fertilization (Edwards, 1973), indicating that the problem of sperm capacitation had been overcome; the use of washed ejaculated human sperm to achieve fertilization *in vitro* was an important milestone in human IVF (Edwards, 2000). Although his strictly agricultural interests did not extend beyond first-year agriculture at University College Bangor, Bob Edwards's pioneering efforts with farm-animal gametes contributed in no small measure to the formidable body of expertise located in Cambridge at that time.

Further afield, across the Atlantic in the USA, Brackett at various locations, First in Wisconsin, and Foote in Cornell, together with their many able students and associates, are justly held in high regard for having pioneered many valuable developments in reproductive biotechnology as it relates to cattle (see Brackett, 1998; First *et al.*, 1999; Foote, 1999a,b). North of the USA, Sirard, Lambert and colleagues in Canada at Quebec pioneered the use of laparoscopy to recover *in vivo*-matured oocytes from the ovaries of donor cows; after capacitation of fresh semen with high ionic strength (HIS) medium and IVF, the rabbit oviduct was used as the *in vivo* culture system to obtain transferable embryos (Lambert *et al.*, 1986).

Elsewhere, in Japan, Akira Iritani and associates were able to report successful fertilization *in vitro* of artificially matured cattle oocytes with ejaculated sperm capacitated in chemically defined medium in the early 1980s; a year or two later, in that same country, Hanada and colleagues reported the birth of the first calves derived from embryos produced by IVM and IVF (Iritani *et al.*, 1984; Hanada *et al.*, 1986). Across the world in Europe, Charles Thibault at Jouy-en-Josas in France was to make major contributions to knowledge in many areas of oocyte and embryo culture and sperm capacitation (Thibault, 1977; Thibault *et al.*, 1987). In Denmark, Torben Greve and associates were responsible for many valuable contributions to the development of effective cattle reproductive technology (Greve *et al.*, 1995, 1996a,b). Synthetic oviductal fluid, widely used today in the culture of cattle embryos, immediately brings New Zealand's Robin Tervit's pioneering reports on embryo culture to mind (Tervit *et al.*, 1972).

1.3. Irish Contributions

In the 30-year period, 1960–1990, as a dazzling array of quality produce grew on the supermarket shelves of Ireland, the UK and other countries in the European Union (EU), consumers were to see the proportion of their household income devoted to food expenditure almost halved. On the farm in Ireland, milk and sheep output within this same period almost trebled, cattle and pig output doubled and grain output increased substantially, despite reductions in cereal acreage. In the countryside, the red and white of the Dairy Shorthorn was to be almost completely replaced by the black and white of the Friesian. On the beef front, Continental bulls (Charollais, Limousin, Simmental) came to dominate the production scene, breeding twice the number of cows exposed to the traditional beef sires (Hereford, Aberdeen Angus, Beef Shorthorn). By the mid-1980s, however, Irish and British livestock producers were to see their

working environment change from one of encouraging greater production to one of positive discouragement; by the 1990s, many on the land were to feel that the incentives that had previously guided their efforts were fading fast. The changing international scene was to witness the crumbling of the Berlin wall in 1989 and the British farming industry losing its long-held priority as a security consideration.

The world of animal science in Ireland and the UK has undergone dramatic changes in the past half-century. In the context of reproductive science, the 1940s and 1950s saw the estalishment of AI and the introduction of frozen semen in cattle breeding; in the 1960s, accurate control of the oestrous cycle in sheep and the menstrual cycle in women were among the major leaps forward. Towards the end of the 1960s came a revolution in the measurement of hormones in body fluids with the introduction of the exquisitely sensitive radioimmunoassay techniques; the 1970s saw ET in cattle becoming a commercial reality and its incorporation into increasingly effective breeding-improvement programmes: the same period saw the emergence of IVF procedures in the human; the 1980s saw the first steps towards mammalian cloning and the ability to produce cattle by totally *in vitro* methods in the laboratory; the 1990s have witnessed cloning by way of adult somatic cells and transgenic sheep and cattle capable of producing valuable human proteins; the same period has seen sexed bull semen introduced into commercial farm practice; such advances would have been viewed with incredulity back in the early 1950s. None the less, particularly in the wake of the bovine spongiform encephalopathy (BSE) outbreak in the UK and elsewhere, optimism and hope derived from such advances in reproductive technologies must be tempered with caution to ensure that progress is not at the expense of consumer confidence in farm practices; careful thought on the scope and nature of regulatory measures to be adopted in dealing with new developments is clearly essential (Evans, 1999).

Despite changes in the emphasis placed on production agriculture, there remains endless scope for improving the efficiency of livestock production in a way fully compatible with the sensibilities of the general populace. In that context, research programmes in reproductive physiology have been conducted in the Irish Republic for many years. The starting-point of such research is certainly the fact that the biological and economic efficiency of Ireland's beef and dairy cattle is limited in various ways by their reproductive performance and by the quality of the young they produce. Part of the incentive for conducting research in bovine reproductive technology in Dublin over the 1963–1993 period lay in the hope that such technology could be used commercially to produce high-quality sexed beef embryos from the country's dairy cattle population. In the event, the value of the work carried out in this 30-year period was to relate more to those in the laboratory than on the land and to practitioners of conventional ET technology who wished to explore new avenues in producing genetically superior embryos for breeding-improvement purposes. Before the late 1980s, the only certain way to obtain cattle oocytes and zygotes was by flushing them out of the oviducts of the live cow, a tedious and costly procedure. As a result of the extensive studies conducted in Ireland with oocytes recovered from the ovaries of slaughtered cattle to produce viable blastocysts, workers elsewhere were encouraged to explore this approach to low-cost embryo production.

1.3.1. Early studies in cattle

In Aldous Huxley's *Brave New World*, published in 1932, the author provides a vivid description of the Central London Hatchery, where human sperm and eggs were stored in test-tubes; after IVF, the embryos were kept under development in an artificial enviroment in the laboratory. More than half a century further on, something akin to Huxley's hatchery was to be found, not in London, but on a farm near Dublin. Hundreds of cattle eggs, obtained some hours previously from deceased donor animals were being exposed to sperm and fertilized, using semen from a bull that might already have been dead for years. This form of posthumous assisted reproduction was the subject of research by Sreenan in Dublin and was reported both at the International Reproduction Congress in Paris in 1968 and later in the scientific press (Sreenan, 1968, 1970). Despite such efforts, viable embryos were not obtained and almost 20 years

were to pass before Dublin workers caught sight of a possible solution. This insight took the form of Moor's report in Cambridge on the successful maturation of sheep eggs *in vitro* (Moor *et al.*, 1984); the Cambridge workers were to fertilize these oocytes by placing them in the oviducts of previously inseminated sheep, and the outcome was several normal healthy lambs.

Elsewhere, this time in the USA, workers at Wisconsin were to show that bull sperm could be prepared very effectively for IVF (i.e. capacitated) using the glycosaminoglycan heparin (Parrish *et al.*, 1984). It fell to Lu in Dublin to blend the Cambridge and Wisconsin techniques together with special reference to the cow; within 3 years, he was able to report a successful IVM/IVF procedure (Lu *et al.*, 1987) and a few months later an effective IVM/IVF/IVC method, using bovine oviductal cells in the co-culture of the embryos (Lu *et al.*, 1988). Steps in the production process are depicted in Fig. 1.2; a patent covering Lu's protocol was duly granted to University College, Dublin, by the European and Australian Patent Offices.

1.3.2. Cattle twins by embryo transfer

For embryos to be used in commercial practice, whether they were produced by conventional superovulation and recovery procedures or came from an embryo production unit in the laboratory, it was necessary to have a reasonably simple and effective non-surgical ET technique. The excellent ET results achieved in the 1950s and 1960s by Rowson and colleagues at Cambridge in sheep, where the standard medium for recovering and transferring embryos was homologous blood serum, were in marked contrast to their efforts to establish pregnancies by similar methods in the cow. In Ireland, Sreenan was to find that the medium employed in the handling and storage of cattle embryos was a major factor influencing their viability. In bovine follicular fluid the embryos survived; in homologous serum they did not (Sreenan and Scanlon, 1968). In Cambridge, Rowson compared homologous serum and tissue-culture medium 199 (TCM-199) for the collection and surgical transfer of cattle embryos; there was no success with serum but with TCM-199 the pregnancy rate was 91% (Rowson *et al.*, 1969). The use of an appropriate medium for handling cattle embryos was clearly a major factor.

Although that work of Rowson in 1969 marked an important turning-point in cattle ET prospects by showing that an acceptable pregnancy rate could be achieved, for any thought of extensive commercial application of ET the surgical transfer approach was clearly unacceptable. Although numerous instruments designed specifically for the non-surgical transfer of cattle embryos were described in the literature during the 1960s and early 1970s, it was the successful application of the Cassou AI gun that eventually proved to be the answer to this particular problem (Boland *et al.*, 1975; Sreenan, 1975)

A number of studies have shown that the biological and economic efficiency of certain cattle production systems may be substantially improved (by 20–25%) by increasing the twinning rate. This was the incentive for research into a method of producing twins by ET in Ireland. The method developed was based on the non-surgical transfer of a single embryo to the empty (contralateral) horn of the uterus of a cow that had been bred (by AI or natural service) a week earlier. By the early 1980s, farm trials with several hundred cattle in Ireland and elsewhere had shown that a combined AI and ET technique was capable of consistently producing a twin calving rate of 40–50% in pregnant cows (see Gordon, 1996; Fig. 1.3); it remains today the most effective method of producing cattle twins. For the farmer, however, the economics of twinning using embryos from superovulated donor cattle was not attractive and several countries concentrated their attention on increasing the ovulation rate in the cow by immunization procedures. In Dublin, the emphasis was on producing embryos by a novel low-cost procedure.

1.3.3. Low-cost embryos

As a result of the expanding knowledge of the essential factors influencing oocyte maturation (Staigmiller and Moor, 1984) and sperm capacitation (Parrish *et al.*, 1984), a method of maturing and fertilizing cattle oocytes *in vitro* was developed by Lu in Dublin. This led to the birth of

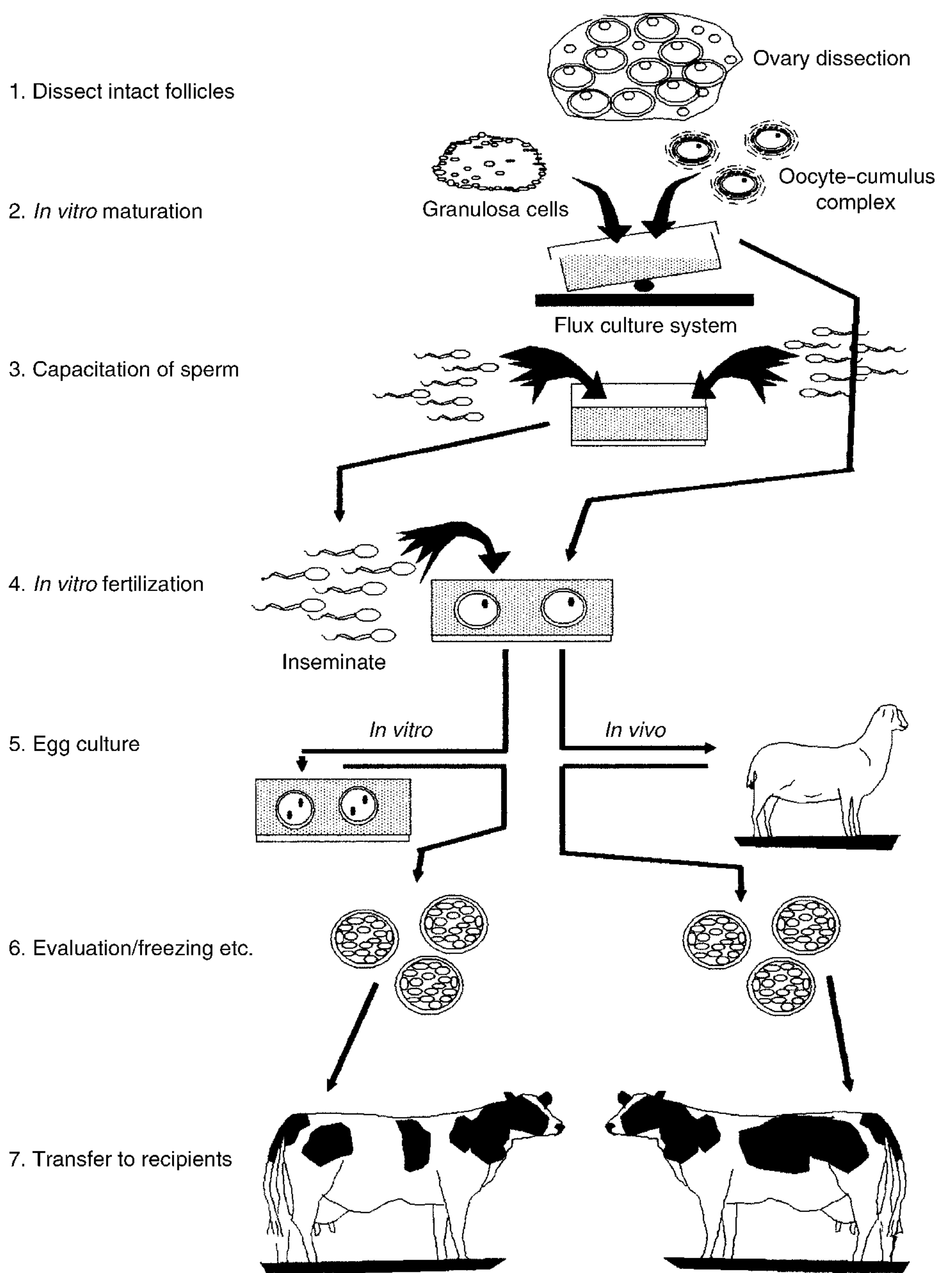

Fig. 1.2. Steps in Lu's IVP embryo production process.

18 calves to 13 foster-mothers in the autumn of 1987 and a considerable body of evidence supporting the view that the embryos produced were capable of establishing normal pregnancy rates in cattle. Initially, an *in vivo* culture period in the sheep oviduct was employed to enable embryos to reach the blastocyst stage for transfer (Lu *et al.*, 1987), but late in 1987 the first calves were born from a totally IVP system (Lu *et al.*, 1988), the culture of embryos being by

Fig. 1.3. Cattle twins born after one-embryo transfer to a bred recipient.

way of the oviductal cell method that had recently been reported in Cambridge (Gandolfi and Moor, 1987).

1.3.4. Commercializing the embryo production procedure

Towards the end of 1987, a commercial company (Ovamass Ltd) was established in Ireland, having its base first in Dublin and later in County Tipperary. During 1988, Ovamass carried out large-scale field trials in the Republic and Northern Ireland in which trained and experienced inseminators non-surgically transferred IVM/IVF embryos to more than 1000 recipient cattle in 58 separate herds. Embryos were produced by IVM in an undefined medium with fertilization in a modified Tyrode/albumin/sodium lactate/sodium pyruvate (TALP) medium; presumptive zygotes were cultured from 20 h onwards *in vivo* in the sheep oviduct to reach the blastocyst stage. After freezing, the IVM/IVF embryos yielded results little different from those commonly experienced at that time with *in vivo*-derived embryos (pregnancy rates of 50.3% for 803 'fresh' transfers vs. 43.1% for 308 frozen ETs). Following the change to a totally IVC system, initially with bovine oviductal cells and subsequently with granulosa cells, there came the somewhat unexpected evidence that the freezability of such IVP embryos was markedly below that found with the sheep-cultured blastocysts.

The general efficiency of the Ovamass IVP embryo production system was recorded in a report by Lu and Polge (1992). In the 2-year period (1989–1991), the company fertilized 709,333 oocytes from abattoir ovaries, producing 222,089 blastocysts, of which 165,928 were considered freezable. The Company's average of 4.6 freezable embryos per slaughterhouse heifer was little different from that normally found after conventional superovulation and embryo recovery in the live animals (see Fig. 1.4); in their fresh state, the IVP embryos showed survival rates comparable to those achievable with *in vivo* embryos.

1.3.5. Commercial unacceptability

Among the factors influencing the commercial acceptability of Ovamass-produced embryos for cattle twinning in Ireland was the occasional birth of heavy singleton calves, some weighing 70 kg rather than the expected 40–50 kg. Although the recorded incidence of such calves was low (2%: 13/644 births), they did little to reassure farmers that this novel form of

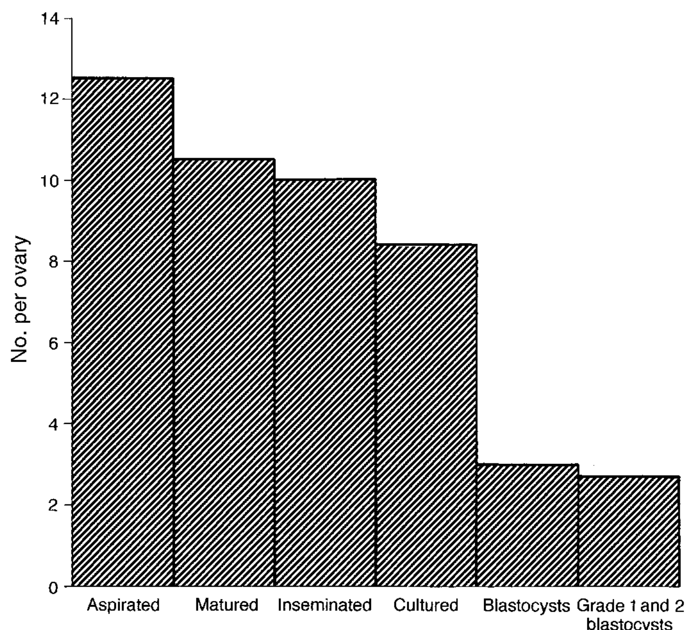

Fig. 1.4. Production of cattle IVP embryos in Ovamass laboratories in 1989–1991. Data expressed in terms of yield of oocytes and embryo per abattoir ovary.

twinning by laboratory-produced embryos was necessarily trouble-free. Around this time, press reports were starting to surface of dramatically oversized offspring being born after cloning in sheep and cattle; this was a further factor influencing farmer acceptability of the Ovamass technique. The restructuring of Ovamass in 1989 was to see the Company concentrating on research and development, aided by a 3-year research grant from the European Commission (EC); later in the year, Ovamass became part of Animal Biotechnology Cambridge (ABC), with the Tipperary laboratory researching embryo production techniques and ABC in Cambridge devoting attention to embryo sexing and cloning technology.

The 3-year EC-sponsored research programme did much to improve the efficiency of embryo production technology and the way in which IVP embryos could be used on Irish farms. A particular difficulty had been the need to thaw and remove cryoprotectant from cryopreserved embryos before transfer to bred recipient cattle; this limited the application of IVP technology on the farm by requiring prior involvement of skilled personnel and a laboratory setting. A freeze–thaw procedure was developed by Lu and associates in the Ovamass laboratory which enabled cryopreserved IVP embryos to be stored until the moment of transfer, using a method akin to that employed in conventional AI.

As a follow-up to the Brussels-sponsored project, a study by a market and business analyst was commissioned to investigate the market for IVP/ET in the Irish Republic; it was concluded that at least 0.5 million of the total population of 2 million cows in the country could be used as potential recipients in attempts to increase calf numbers. It may be noted that this was still at a time in Irish livestock farming when calves for beef rearing were both scarce and costly; the view still held that it was an opportune time to increase the country's beef-calf population. Validation of the 3-year EC research programme was considered to be essential before recommending widespread commercial exploitation of the technique.

To that end, Ovamass and the State Agricultural Research Organization (Teagasc) were funded by a further EC programme to carry out an extensive field trial using the Company's freeze–thaw technology. In this, IVP embryos were transferred to 1400 bred recipient cows on beef and dairy farms by the commercial AI service. Beef embryos were produced by recovering

follicular oocytes from Limousin, Simmental and Charolais-cross heifers that had been graded with high carcass scores at the abattoir. The oocytes were fertilized using sperm from a bull that had a proven record for ease of calving and good growth rates. Suitable recipient animals were selected on farms already achieving good results with AI; the belief was that producers on such farms were those most likely to benefit from the twinning application. The recommended procedure was to select cows that would normally be identified as suitable for breeding to a Continental bull, taking account of the body size and condition of the recipient animal. The results are in Table 1.2; those conducting the study recorded no evidence of 'large calf syndrome' among the young born on farms.

Despite the fact that IVP technology had been greatly strengthened by the research and trials conducted in Ireland in the early 1990s, when it came to applying the technology serious and insurmountable difficulties were to arise. ABC terminated its Irish connections and relocated its research staff to Cambridge, so there was no longer a company to promote the technology in the Republic. The fateful announcement in the House of Commons by the British Health Secretary in March 1996 of a possible link between BSE and a new variant of Creutzfeld–Jakob disease (vCJD) was to have a devastating effect on consumer confidence in the safety of beef products and on beef sales in the EU. The climate in Ireland's farming industry for twinning innovations in beef production systems was to change irrevocably.

1.3.6. Towards sexed semen on the farm

In the UK, ABC concentrated attention on the semen-sexing side of their research and development activities (Cran *et al.*, 1993a,b, 1994, 1995). On a historical note, it may be noted that semen sexing by flow cytometry was actively researched in the early 1980s at the National Institute of Medical Research (NIRM) in London and a semen-sexing patent was granted to that institution in 1986, covering Europe and Canada. Subsequently, the US Department of Agriculture (USDA) filed a patent for semen sorting in 1989 for the USA and a year later extended its claim to another 14 countries around the world.

The US sexing (Beltsville technology) was that devised by Johnson, working at the US Agricultural Research Service at Beltsville; the technology has been licensed by the USDA for use in mammals in two categories: humans and animals.

In humans, the Genetics and IVF Institute in Fairfax, Virginia, was granted an exclusive licence for the sexing patent in 1994 and workers were soon to use the technology in human assisted reproduction (Fugger *et al.*, 1998; Stern *et al.*, 2001)). The animal licence was granted to ABC (later to Mastercalf, a subsidiary of ABC Ltd); some 4 years later, a second animal licence was granted to Colorado State University Foundation. The company formed to develop the technology for the commercial animal market was XY Inc. of Fort Collins (Seidel, 1999b; Seidel and Johnson, 1999). Since Mastercalf was purchased by XY Inc., the American-based company is currently the commercial entity that handles the Beltsville technology for animals.

In the UK, one of major cattle-breeding companies (Cogent) signed a collaboration agreement with XY Inc. and in 1999 started farm AI trials with sorted semen. In July 2000, Cogent started making limited amounts of sexed semen commercially available to its members with the expectation that sorted semen would become available for more widespread use on British and Irish farms in the course of the next few years. As with many other farming activities, the widespread 2001 foot-and-mouth outbreak

Table 1.2. Effect of IVP embryo transfer on calf output per cow (from Bourke *et al.*, 1995).

	Embryo transfer	Control	
No. of cows	469	858	
Calves born per cow	1.35 ± 0.02	1.02 ± 0.01	$P < 0.001$
Calves alive at 48 h, per cow	1.25 ± 0.02	0.99 ± 0.01	$P < 0.001$
% Multiple births	34%	2%	$P < 0.001$

interrupted many of Cogent's plans for farm applications of its sexed semen in Britain.

Of historical interest is the fact that the first calves born from semen sexed by the Beltsville technique were derived from IVP cattle embryos (Cran *et al.*, 1993a, 1995); clearly, far fewer sperm are required for fertilization when IVF rather than AI is employed, although account had to be taken in initial studies of the reduced motility and viability of sorted sperm. In some of the initial studies, it was estimated that sufficient sperm for 100 oocytes could be sorted in about 1 h (Cran *et al.*, 1994). In due course, sexed semen will presumably become available to the cattle ET industry; this may involve bull sperm being sorted at a central facility for use by ET practitioners. For some in the cattle ET industry, there may be attractions other than the provision of sorted semen for cattle IVF; sexed semen may have a role to play in breeding donor animals after conventional superovulation treatments. Workers in Colorado recorded the recovery of cattle embryos from superovulated beef heifers inseminated with low doses of sexed sperm; 93% of embryos recovered were of the intended sex (Chung *et al.*, 1998).

1.4. Developments in ET Technology

Although the golden anniversary of the first ET to produce a calf in the USA was celebrated a couple of years ago (see Betteridge, 2000; Table 1.3), it was to be two decades beyond 1950 before Rowson and colleagues at Cambridge were able to demonstrate a surgical recovery and transfer technique capable of achieving commercially acceptable results. Landmark developments in the past half-century leading to gamete biotechnology as currently practised have been ably covered in several reviews (see Brackett, 1998; First *et al.*, 1999; Foote, 1999a,b).

1.4.1. Thirty years of progress

Commercial cattle ET started in North America and elsewhere during the early 1970s, primarily as a means of multiplying the number of young produced by exotic breeds of beef cattle. In the early years, embryo recoveries usually involved midventral laparotomy with the donor animal under halothane anaesthesia. ET was not widely attempted with dairy cattle at that time because the udder of the milking animal made mid-ventral laparotomies rather difficult. However, by the mid-1970s, workers in several laboratories had devised non-surgical recovery methods to the point where they matched the efficiency of surgical interventions.

Around that same time, non-surgical transfer techniques, most based on the Cassou inseminating gun (Instruments de Médecine Vétérinaire (IMV)) were developed. The availability of non-surgical recovery and transfer procedures opened the way to exploitation of cattle ET under farm conditions. An effective freeze–thaw protocol, which permitted embryos to be shipped to the furthest points of the globe, completed the requirements for commercial exploitation of cattle ET at that time.

Table 1.3. Milestones in farm-animal embryo transfer and related techniques.

Year	Species	Event	Researcher(s)
1890	Rabbit	Birth of young as a result of embryo transfer	Heape
1949	Sheep/goat	Birth of a lamb and kid from embryo transfer	Warwick and Berry
1951	Pig	Birth of piglets from embryo transfer	Kvansnickii
1951	Cattle	Birth of calf from embryo transfer	Willett *et al.*
1971	Cattle	First commercial cattle ET company formed	Alberta Livestock
1973	Cattle	Birth of calf after frozen stage (Frosty II)	Wilmut and Rowson
1974	Horse	Birth of a pony after embryo transfer	Oguri and Tsutsumi
1982	Cattle	Birth of calf after IVF	Brackett *et al.*
1983	Buffalo	Birth of calf after embryo transfer	Drost *et al.*
1986	Sheep	Lamb born via nuclear transfer	Willadsen
1988	Cattle	Cattle twins by IVP embryo transfer	Lu *et al.*
1997	Sheep	Birth of lamb cloned from adult cell	Wilmut *et al.*

1.4.2. Current cattle ET activity

Currently, cattle ET technology is used in many countries around the world, with an estimated 530,000 embryos being transferred annually. Data for cattle ET activity worldwide in 2000 were reported by Heyman (2001). Details in Table 1.4 show that some 110,000 cows were flushed in 2000, yielding more than 650,000 embryos of transferable quality (mean yield of 5.9 transferable embryos per collection), of which 528,000 were transferred. Much the same numbers of fresh and frozen embryos were transferred (47% fresh, 53% frozen).

Cattle ET trends in European countries were reported by Thibier (2001a), with numbers transferred in Germany, the Netherlands, Italy, Ireland and Sweden shown to be increasing. Elsewhere, a large-scale study (14,699 animals) of factors affecting pregnancy rates in cattle after ET by Hasler (2001) reported no evident decrease after transition from surgical to non-surgical transfer in a commercial ET programme; there was a loss of about 10–13 percentage points between fresh and frozen–thawed embryos of comparable grades. The same study showed dairy cows sustaining a lower pregnancy rate than dairy heifers or beef heifers or cows; there was no evident effect when cycle synchrony between donor and recipient was within 24 h plus or minus of zero. According to a survey of 26 ET companies in the USA, non-surgical recovery and transfer plus embryo filters have reduced the 'flush-to-transfer interval' to a few hours (Nelson and Nelson, 2001); this is a considerable improvement from the routine of the early 1970s when flushing one cow and transferring the embryos was likely to take 1 whole day. The culture medium of the early days was usually a bicarbonate-buffered medium (Ham's F-10 or TCM-199) maintained in a CO_2 incubator; in due course such media were replaced by phosphate-buffered saline (PBS) media to avoid the need for incubators in the field. The most commonly used formulation in the period up to 1990 was modified Dulbecco's PBS (D-PBS), supplemented either with 10–20% bovine serum or 0.4% bovine serum albumin (BSA); in the recent decade, the D-PBS medium has been further supplemented with glucose and pyruvate.

The commercial availability of improved media for embryo development may now permit embryos that are normally not acceptable for freezing (grades 1 and 2) to be utilized. In Texas, Romo *et al.* (2002) cultured their grade 3 embryos in new and improved culture media (modified BMOC solution, MB1 (ViGro Holding Plus, AB Technology, Pullman)); these media promoted continued development and improved morphological quality of such embryos within

Table 1.4. Data for worldwide cattle ET activity in 2000 (from Thibier, 2001a).

Continents	Flushes	Transferable embryos	Number of transferred embryos		
			Fresh	Frozen	Total
Africa	1,205	7,049	3,566	3,197	6,763 (1.3%)
North America	50,527	287,460[a]	102,285	122,166	224,451 (42.5%)
South America	9,327[a]	56,645	45,679	38,842	84,521 (16.0%)
Asia	12,225	89,063	15,046	43,925	58,971 (11.1%)
Europe[b]	22,734	125,035	47,270	58,698	105,968 (20.1%)
Oceania[c]	17,040	99,068[a]	32,410	15,456	47,866 (9.0%)
Total	113,058	664,320	246,256	282,284	528,540[d]

[a]Data extrapolated for one country of the group.
[b]The European data are derived from the statistics of AETE, (2000).
[c]Due to the low number of teams that responded in Australia, in accord with the AETA we have extrapolated the data from those few teams to all the member teams of that association, so as to give sense to the 2000 results.
[d]A rough estimation of the numbers of ETs that were not officially recorded and hence not incorporated in the table above leads to about 11,500 more embryos transferred in Asia (Bangladesh, India, Pakistan), which leads to the total number of *in vivo*-derived embryos transferred in the year 2000 = 539,000, rounded up for official records to 540,000.

24 h. Although more data are required, the practical implications of such work may lie in permitting embryos that were hitherto discarded to be frozen or used in transfers.

ET is a technology that relies heavily on the stimulation of selected donor cattle to produce large numbers of embryos after the induction of multiple ovulations by gonadotrophin treatment (superovulation). Despite extensive research efforts over many years, superovulation remains one of the weakest links in ET technology (Boland and Roche, 1993; Kanitz *et al.*, 2002; Mapletoft *et al.*, 2002). Factors known to be crucial in determining the effectiveness of superovulation treatments are: (i) the particular gonadotrophin preparation chosen and its method of administration; (ii) adjunct treatments aimed at controlling follicle growth and ovulation; (iii) the genetics of donors and the environment in which they are kept; and (iv) bull effects. In recent years, particular attention has been focused on methods of eliminating the suppressive effect of the dominant follicle in donor animals and in gaining more precise control of the timing of ovulation. The availability of transrectal real-time ultrasonic technology now permits accurate evaluation of follicular dynamics before and during superovulation. In Ireland and some other countries, increasing attention has been devoted to elucidating nutritional effects as they influence ovarian function, superovulatory response and embryo quality in cattle (Armstrong *et al.*, 1997; O'Callaghan *et al.*, 2000; Boland *et al.*, 2001b).

1.4.3. Commercial advantages of cattle ET

The improvement of the genetic quality of cattle has traditionally been in the hands of the pedigree breeder and in the past half-century by way of AI, using progeny-tested and performance-tested bulls. The advent of an effective cryopreservation technique was a particularly valuable milestone in the development of cattle ET technology. It meant that embryos could now enter international trading, procedures having been agreed between countries to ensure that ET would not result in the transmission of pathogenic agents. On some occasions, ET may be used to obtain offspring from seriously disabled cows; one such study, reported by Krolinski *et al.* (1994), used the technique to produce normal, healthy calves from cattle suffering from leukaemia. In terms of the genetic gains made possible by ET in cattle, these are primarily the result of increased selection intensity in females and a reduction in the generation interval. Where young donor animals are used, phenotypic records, such as lactation records for dairy heifers, may not be available; for such reasons, selection tools that can identify superior animals in the absence of phenotype records could play an important role. To that end, efforts to characterize the genomes of cattle and other farm livestock are particularly important, such characterization enabling appropriate molecular tools to be developed for the improvement of animal performance (Beever, 1998; Kappes, 1999). In countries such as the USA, the private sector is playing an increasingly important role in advancing livestock-breeding improvement; specialized breeding companies supply virtually all commercial poultry breeding stock and the trend is towards doing the same with pigs, beef and dairy cattle (Narrod and Guglie, 2000).

1.5. Laboratory-produced Embryos

Over the next decade, the Irish agricultural and food industry will have to compete in a rapidly changing world environment; such changes stem from increased competitiveness, decreased world market prices and increased consumer demands for higher-quality, healthier and safer animal products. The same is true of the agri-food industries in other countries of the EU. Biotechnology has an important role to play in ensuring that the reproductive efficiency of farm animals is maintained in the years ahead at the highest levels compatible with animal welfare and protection of the environment (Morris and Sreenan, 2001). As well as their use in commercial breeding programmes, *in vitro* techniques are routinely used in many laboratories for research purposes. Table 1.5 is an example of embryo production in the course of a year, reported by Krauslich *et al.* (1997) in their laboratory in Germany, using TCM-199 supplemented with 10% oestrous cow serum as the medium for maturation and subsequent embryo culture.

A wide range of factors influence the success of *in vitro* embryo production, varying from the bulls used for IVF to the technicians in the laboratory (Yang, X. *et al.*, 1995). Reviews of such factors as they influence the competence of IVP embryos have been provided by workers in Australia (Trounson *et al.*, 1994, 1998a,b), Germany (Lucas-Hahn and Eckert, 1996), New Zealand (Tervit, 1997), the UK (Polge, 1997; McEvoy *et al.*, 2001), the USA (Leibfried-Rutledge, 1999; Foote, 1999a,b; Hasler, 1994, 1998, 2000a,b, 2001, 2002) and Ireland (Boland *et al.*, 1999, 2001a; Lonergan *et al.*, 2001c). As well as producing embryos for research or commercial use, the *in vitro* technology has relevance to assessing bull fertility (Rodriguez-Martinez and Larsson, 1999). Conventional assessment of sperm quality in AI bulls can now be aided by tests such as the induction of the acrosome reaction (AR), zona pellucida-binding assays and IVF.

1.5.1. Current level of activity

According to data reported by Thibier (2001a), there was a significant increase in the numbers of cattle IVP embryos transferred in the year 2000 over those previously recorded. Almost 42,000 embryos were reported in the various continents (see Table 1.6). In Europe, 14,079 embryos derived from live donor animals and 12,441 produced from abattoir ovaries were transferred.

In recent years, IVP of cattle blastocysts has become a routine research tool in many laboratories; on the farm, it is being used increasingly to obtain embryos from infertile donors. The laboratory production of cattle embryos may often be regarded as the final option in getting calves from genetically élite cows; this is likely to apply to donors in which embryo recovery by normal means is impossible or where it is necessary to recover numerous embryos in a relatively short time-span.

Ovum pick-up (OPU)

The linking of low-invasive ultrasound-guided transvaginal oocyte retrieval, referred to by some as ovum pick-up (OPU) and by others as transvaginal recovery (TVR), with *in vitro* embryo production proved to be a valuable new development to many in the cattle-breeding and ET industry (Taneja and Yang, 1998; Duszewska and Reklewski, 2000). In contrast to oocytes recovered from abattoir ovaries, acquiring oocytes from live cows and heifers means that the genetic merit and health status

Table 1.5. IVP cattle embryo production during 12 months in a German laboratory (BFZF) (from Krauslich *et al.*, 1997).

	Oocytes developed to:		
No. oocytes	2 to 4 cells (day 2)	Morula and blastocysts (day 7)	Blastocysts (day 9)
40,301	31,716 (78.7%)	15,596 (38.7%)	12,815 (31.8%)

Table 1.6. Cattle IVP embryos transferred worldwide in 2000 (from Thibier, 2001a).

	Transferable embryos collected	Transferred embryos		
		Fresh	Frozen	Total
Africa	975	1	21	22
Asia	97,011	6,680	5,684	12,364
North America	1,741	1,382	533	1,915
South America	12,667	12,527		12,527
Europe	26,520	6,377	7,426	13,803
Oceania	1,358	930[a]	130	1,060
Total	139,372	27,967	13,794	41,761

[a]Only one country from this region has reported this figure.

of the donor animal is precisely known. In Canada, for example, Bousquet *et al.* (1999) found IVP to be an effective alternative to conventional superovulation in their quest to produce numerous female embryos within a short time span; OPU and IVP resulted in an average of 3.8 recipients becoming pregnant with heifer young as compared with 1.2 pregnancies following conventional practice. Ultrasound-guided transvaginal aspiration protocols usually involve once- or twice-weekly aspirations, in which donors are either follicle-stimulating hormone (FSH)-stimulated or unstimulated. In the USA as an example, Hasler (1998) reported an 8-year-old cow producing 176 transferable embryos in a period of 3 years by way of a once-weekly OPU programme.

The way is also open to procuring blastocysts from prepubertal heifers and young calves, which is difficult, if not impossible, using conventional procedures. In dairy-cattle breeding there is much to be gained by reducing time between generations. Workers have developed OPU devices that are suitable and economical for routine use in oocyte retrieval, without having negative effects on ovarian structure or adversely affecting subsequent ovarian function. As well as recovering oocytes from young cattle, it is also possible to obtain oocytes from cows in the early months of pregnancy and from cattle during the early post-partum period (Perez *et al.*, 2000).

1.5.2. Research with bovine IVP embryos

The extensive use of *in vitro* embryo production in cattle has enabled large numbers of embryos to be generated for research. This, in turn, has had a significant effect on the accumulation of biological knowledge on oocyte maturation, fertilization and early embryonic development in this species. There are also those who believe that, because of such knowledge and because the rate of early embryonic development is similar to that of humans, the bovine model may supplant the mouse as the model for early mammalian development (see Niemann and Wrenzycki, 2000). A continuing source of concern relates to bovine embryo quality after IVP. It is clear from numerous reports that IVP embryos are much more sensitive to chilling and cryopreservation than embryos produced in the live cow or in the oviductal environment of the ewe. This is a serious limiting factor to the development of international trade in IVP embryos, which for animal-health reasons is wholly based on the frozen embryo. There are several clear-cut characteristics distinguishing IVP from *in vivo*-produced cattle embryos, the main one being the presence of an excessive amount of lipid material in their cells.

A further worrying problem has been the occasional birth of seriously overweight but otherwise apparently normal calves; this has already been alluded to in an earlier discussion of Irish trials (see section 1.3.5 above). The concern here is that certain culture conditions may allow the expression of some genes that are normally inhibited. As noted by Betteridge (2001), a century of investigation has brought reproductive biologists to a clear recognition that the conditions under which the mammalian embryo is conceived and incubated may well have permanent effects on its development before and after birth. Although the incidence of 'large calf syndrome' is low and in some laboratories not recorded at all, it is clearly essential to have an adequate explanation of the phenomenon so that culture conditions are adjusted to eliminate or greatly restrict its occurrence.

On the question of overweight calves, there is probably a good case for making a distinction between calves produced by standard IVM/IVF/IVC technology and those derived from nuclear transfer. Producing cattle clones by nuclear transfer relies upon the reprogramming of the donor nucleus so that it functions in the same way as a zygotic nucleus; such reprogramming constitutes an essential element in the initiation of normal embryonic development. However, nucleus reprogramming does not necessarily direct embryogenesis in a manner identical to that of normal bovine embryos. Studies by Smith *et al.* (1996) have shown that the gene expression pattern for a number of growth factors may differ for blastocysts derived from cloning when compared with that found in either *in vivo*-produced or IVP cattle blastocysts. It would not seem unreasonable to anticipate a greater incidence of anomalies in gene expression in embryos after nuclear transfer than in blastocysts exposed to a routine culture environment.

Lonergan *et al.* (2001c,d) reviewed factors examined in their laboratory influencing oocyte and embryo quality; they concluded that events prior to ovulation determined the ultimate fate of the oocyte but events occurring between the one-cell and the blastocyst stages determined the quality of the blastocyst. They agreed with workers elsewhere that bovine oocytes with identical developmental conditions up to the occurrence of the luteinizing hormone (LH) surge (start of meiosis) differed in their ability to reach the blastocyst stage according to whether they were matured *in vivo* or *in vitro*; this agreees with those who hold that IVM methods are capable of further improvement. As discussed elsewhere (see Section 4.10), one way of improving oocyte quality is likely to be by way of a prematuration treatment that brings germinal vesicle (GV) oocytes to the same common denominator, quality-wise, before they embark on the conventional 24 h maturation journey. As to ensuring high-quality blastocysts, the authors note that it is possible to do this in various ways. IVM oocytes can give high-quality blastocysts when incubated in the sheep oviduct from the one-cell stage; what can be achieved in the sheep oviduct should be capable of simulation under laboratory conditions, given the requisite information about the factors involved.

1.5.3. Commercial use of IVP embryos

There is a considerable body of evidence dealing with trials in which IVP embryos have been used to produce singleton or twin beef calves from dairy cattle. In the UK and Ireland, the majority of calves reared for beef have originated in the dairy herd, and their suitability for beef has often been questioned, given the trend in both countries towards the greater use of the more extreme dairy blood-lines in the national herd. As the dairy herd becomes increasingly efficient in milk-producing terms, the beef-rearing industry is likely to suffer more from a lack of suitable calves. One means of ensuring high-quality beef from dairy cattle, as attempted in Ireland in the early 1990s, is by producing embryos from suitable beef-cattle oocytes fertilized with Continental bull sperm. In Scotland, Sinclair *et al.* (1994) used IVP beef embryos produced by ABC Ltd as a means of establishing pregnancies and improving productivity in beef cows. The advent of semen sexing should also enable IVP embryos to have even greater appeal. Beef producers prefer steers rather than heifers for rearing, primarily because heifers are some 10% less efficient in feed conversion, gain 10% less weight than steers and occasionally run the risk of becoming pregnant. The biological efficiency of beef suckler cattle could be significantly enhanced by the availability of a reasonably low-cost sexing procedure.

Among the more novel uses of IVP embryos would be in the provision of zebu cross-bred embryos (*Bos taurus* × *Bos indicus*) for transfer to suitable foster-mothers in tropical and subtropical lands. The F1 hybrid is known to be more productive than other possibilities in a difficult environment and embryos could be made available in numbers and at a cost that might be attractive in certain regions. A further novel area worth exploring would be using ET and IVP embryos to bypass problems arising from heat stress in dairy cattle. It is well known that pregnancy rates in cattle bred by AI or natural service can fall dramatically due to heat stress during periods of hot weather, particularly if the animals are high-yielding dairy cows. Encouraging results have been reviewed by Rutledge (2001), who suggests that this form of reproductive technology (establishing pregnancies by embryos rather than spermatozoa) may eventually be of some practical relevance in dairy enterprises located in difficult environmental conditions.

On the topical subject of cloning and Dolly the sheep, it may be noted that, within 3 years of Dolly's appearance, several laboratories had proved the feasibility of using similar technology in cattle. Initially, there was commercial interest in using cloning technology to produce genetically élite cattle for farming, but this quickly lost appeal, and for good reason. Biotechnology companies in the USA and elsewhere financed ambitious research and development programmes only to face problems of extremely poor pregnancy rates and dramatically increased birth weights in some calves and neonatal anomalies and poor survival rates in others. A growing body of evidence shows that much of the severe fetal loss observed in the early months of pregnancy can be traced to abnormal placental function, particularly the function of the allantois

(McMillan *et al.*, 1999a,d). The fact that many losses occur around the time of placental attachment, which occurs in the fourth week of gestation, has indicated a possible alteration in the immunological status of the cloned conceptus. In the USA, Hill *et al.* (2002) reported abnormal expression of trophoblast major histocompatability complex class I antigens in cloned cattle pregnancies, which was associated with a marked endometrial lymphocytic reasponse.

Not all reports on cattle cloning dwell on a catalogue of faults and problems. In Wisconsin, Pace *et al.* (2002) report data that probably represent the most comprehensive information on cattle derived from nuclear transfer, using a variety of non-embryonic cell lines. Nearly 25% (535/2170) of recipients receiving reconstructed embryos became pregnant; about 20% (106/535) of those pregnancies resulted in live births, with 77% (82/106) of the clones remaining healthy and productive. Although the authors recorded wide variation in birth weights, the growth rates and reproductive and lactation characteristics were no different from those found in non-cloned dairy cattle.

Despite occasional glimpses of what the future may hold, current commercial interest in cloning is likely to continue emphasizing its role in producing transgenic animals. In this regard, it should be noted that, until recently, the only proved procedure for producing transgenic cattle was by way of pronuclear gene injection. Advances demonstrated by Roslin workers, which combine cell culture and nuclear transfer techniques (somatic cell cloning using transfected cell stocks), are likely to be of great value in the production of transgenic animals, where success is not necessarily limited by current inadequacies in the outcome of the nuclear transfer procedure. Of the various possibilities for farm livestock, the transgenic modification of cow's milk is likely to be of greatest commercial interest, particularly in terms of producing high-value casein proteins (Zuelke, 1998, 1999). Only a small number of transgenic founder animals are required, and these can be produced, given a sufficient number of attempts. Cloning is also capable of eventually bringing substantial benefits to society at large, including new forms of cell therapies for use in human medicine, animals for pharmaceutical protein production and organs for xenotransplantation (Stice *et al.*, 1998; Murray, 1999; Stice, 1999). It is not necessarily a question of adding genes to an animal genome. In farming, as noted by Suraokar and Bradley (2000), there may be a case for removing undesirable genes, such as *PrP*, to produce sheep flocks that are resistant to scrapie (or cattle resistant to BSE).

While acknowledging the great potential of cloning and transgenic technology, it is essential to ensure that adequate welfare regulations are in place (Broom, 1998; Kruip and van Reenen, 2000); there are also those who note that communication among researchers in this fast-developing area of biotechnology may pose questions of concern to the wider scientific community (Piedrahita, 2000). Currently, there are probably more laboratories engaged in cattle cloning worldwide than in all the other species combined. The success of those groups who have produced cattle clones at this time is partly due to the considerable base of knowledge in cattle embryo production built up over the past 20 years.

1.5.4. Pathogen-free IVP embryos

Methods of producing high-quality bovine IVP embryos must take due account of the risks of disease transmission; it is clear from various reports that the efficiency of decontamination treatments designed for *in vivo* embryos may not hold good for laboratory-produced embryos (Stringfellow and Wrathall, 1995; Stringfellow and Givens, 2000a,b). Although the long-term solution lies in the employment of pathogen-free chemically defined culture media at all stages of embryo production, there are various interim measures worthy of mention. In France, Guyader-Joly *et al.* (1996) suggested that Vero cells (derived from green monkey kidney) could be employed in co-culture systems; they come from a well-established cell line and are highly controlled to be free of viruses and other contaminants. In Belgium, Van Langendonckt *et al.* (1997a) produced their cattle embryos using media supplemented with rabbit serum and serum albumin rather than bovine products. In the UK, Fray *et al.* (1998) dealt with methods that would facilitate the screening of donor cattle for freedom from bovine viral diarrhoea virus (BVDV).

As noted by Thibier (2001b), as soon as the first IVP embryos became available in the late 1980s, research on embryo–pathogen interaction indicated that this was somewhat different from that observed in embryos produced *in vivo*, an apparent consequence of morphological differences in the zonae pellucidae of the embryos. Despite the more adhesive properties of the zona pellucida of IVP embryos, tens of thousands of such embryos have been transferred during the past 5–10 years under procedural guidelines laid down in the International Embryo Transfer Society manual without a single instance of recipient contamination being reported. It would appear that procedures used and control measures applied to the IVP embryos have reduced the risks of disease transmission to negligible proportions.

1.5.5. Animal health and welfare considerations

Good health and freedom from physical and psychological stress are of paramount importance to acceptable animal welfare on the farm. The welfare implications of novel forms of embryo biotechnology must be kept constantly under review (see Christiansen and Sandoe, 2000). In contrast to ET in the smaller farm animals, which usually requires surgery for oocyte recovery and transfer, a great advantage with cattle is that relatively uncomplicated non-surgical procedures can be employed. There is an obvious need to accept the fact that increasing numbers of people in the developed countries are expressing their aversion to what is described as intensive farming. Although much of this emotion has been directed towards the pig and poultry scene, on the farm the need is for the livestock producer and his advisers to respond in a positive manner to the challenge posed by such views. In the context of IVP embryos, their use must inevitably require transfer; this transfer need be no more stressful than the many routine husbandry activities in which cattle are handled, treated, dosed or injected. In an animal-welfare context, ET should be regarded in the same light as routine AI, although the fact that it is performed several days after the cow has been in oestrus usually means that the manipulation is carried out under a local anaesthetic.

What happens at the other end of pregnancy, when the cow is delivering its calf, is likely to require much more careful thought and preplanning. Appropriate measures for the selection of suitable recipient cattle, together with adequate attention to feeding and management during pregnancy, must always be regarded as an integral part of reproductive technology on the farm. This could be all the more important as IVP embryos – previously restricted in the main to high-value pedigree cattle, which are usually maintained in higher than average farm conditions – become more commonplace in the normal run of commercial beef and dairy herds.

1.6. Embryo Production in Other Farm Mammals

Although cattle remain at the forefront of many new developments in reproductive biotechnology, what can be achieved in cows today may well be possible in other farm species tomorrow. Nevertheless, there are often profound differences between species in what can be achieved in embryo production, even within cattle. Zebu cattle (*B. indicus*) make up most of the cattle population in tropical and subtropical countries, such as Brazil, where the Nellore breed and its cross-breds account for some 100 million animals. Studies in the early 1990s in that country with zebu cattle suggested that there may be marked differences in the effectiveness of IVP techniques compared with that observed in European cattle (*B. taurus*). This would not have come as any great surprise to Brazilian veterinarians, who have also reported an apparent higher variability in the outcome of superovulation treatments compared with results achieved with European cattle (Barros and Nogueira, 2000b). The causes of the greater variablility are said to be numerous, including a higher susceptibility to stress and physiological and anatomical peculiarities when compared with European breeds.

Although a rarer and much more localized cattle species, the yak (*Bos grunniens*) should not be entirely overlooked in a mention of *in vitro* embryo production. One report, dealing with eight yak and 50 cattle ovaries, led Chinese workers to conclude that, using methods

developed for cattle, IVF in yaks gave results as good as or better than those for cattle (Chen *et al.*, 1997); it would obviously be wise to treat such results with caution.

1.6.1. Buffaloes

Worldwide, there is a growing awareness of the importance of the water-buffalo (*Bubalus bubalis*), which has an estimated population of about 151.5 million (Cruz, 2000) and plays a prominent role in rural livestock production, particularly in Asia, where some 97% of the buffalo population is found, and in certain Mediterranean countries; factors affecting the productivity of the species have a considerable bearing on the agricultural output in such areas. Certainly, there are those who regard the buffalo as being neglected for much too long as a valuable source of animal products for human consumption in many countries; it is encouraging that the world population of buffaloes has grown by 86% in the past 40 years compared with a 34% increase in cattle (Gasparrini, 2002). As noted by Singh *et al.* (2000), reproductive efficiency in female buffaloes is severely restricted by: (i) inherent late maturity; (ii) poor oestrus expression in summer; (iii) distinct seasonal reproductive patterns; and (iv) a prolonged intercalving interval. However, demand for milk and meat from buffaloes has led to breeding programmes involving ET and *in vitro* embryo production in this species.

It is generally accepted that the application of ET in the buffalo has so far met with limited success; response to superovulation treatments in conventional ET operations is usually markedly below that found in cattle, resulting in a low yield of transferable embryos. According to Madan *et al.* (1996), an early recovery rate of 0.15 transferable embryos increased to 2.0 with the use of more effective superovulation protocols. A paper by Misra *et al.* (1999) suggested that an inadequate luteal phase might account for some embryo losses in recipients after transfer. In a review of reproductive biotechnology in water-buffaloes, Cruz (2000) estimated an average yield of 1.8–2.1 transferable embryos per collection after superovulation; the same author noted that the production of IVP embryos may be an alternative to embryos produced by conventional ET technology.

It has to be recognized, however, that *in vitro* embryo production in buffaloes for research and commercial application has shown much slower progress than in cattle (Madan *et al.*, 1994, 1996; Chauhan *et al.*, 1997, 1998a,b,c; Datta and Goswami, 1998, 1999a,b; Palta and Chauhan, 1998; Taneja and Yang, 1998; Abbasi and Sadrekhanloo, 2000). The birth of the first IVP calf, ‘Pratham’, was reported in 1991 in Karnal, India, in the laboratory of Madan and colleagues. A smaller follicle population in the ovaries and a much lower rate of *in vitro* oocyte maturation has made this form of embryo technology difficult to apply in the species (El-Gaafary *et al.*, 1997). However, what can be done in cattle can usually be achieved, albeit less successfully, in the buffalo. In China, Tan *et al.* (1998) recorded that buffalo ovaries contained five oocytes per ovary compared with 14.3 oocytes per ovary for cattle; maturation and cleavage rates were both lower in buffaloes than the equivalent values in cattle. In Thailand, Pavasuthipaisit *et al.* (1995) and, in Italy, Galli *et al.* (1998) are among workers reporting repeated follicle aspiration and OPU in the swamp buffalo. In Thailand, Techakumphu *et al.* (2000) have demonstrated the possibility of harvesting oocytes from buffalo calves and maturing them successfully *in vitro*. A paper by Kumar *et al.* (1997) dealt with chronological studies of meiotic events during the maturation of buffalo oocytes. Indian workers have examined the effect of environmental temperature on the quality and developmental competence of buffalo oocytes (Nandi *et al.*, 2001); high temperatures had a detrimental effect. This agreed with an earlier report by Singla *et al.* (1999), in the same country, who recovered poorer-quality oocytes from abattoir ovaries during summer than during winter.

Supplementation of the IVM-199 maturation medium with FSH significantly increased the oocyte maturation rate in studies reported by Sachan *et al.* (1999) in India; the use of LH alone or in the presence of FSH and oestradiol decreased the maturation rate. In Italy, Gasparrini *et al.* (2000) have shown that adding a thiol compound (such as cysteamine) to their maturation medium improved the efficiency of IVP production. In Egypt, Abdoon and Kandil

(2001a,b) and Abdoon *et al.* (2001) examined various factors affecting embryo production and concluded that maturation of good-quality buffalo oocytes in Charles Rosenkrans 1 with amino acids (CR1aa) or CR2aa medium, with FSH or pregnant mare serum gonadotrophin (PMSG) added, resulted in the most satisfactory outcome, in terms of cleavage and embryo development rates. TCM-199 as an IVM culture medium for buffalo oocytes was superior to Ham's F-10 and Ham's F-12 in studies reported by Singh *et al.* (2001).

The general consensus among workers is that much more research is required before IVP buffalo embryos can be produced efficiently for commercial use. Looking towards other innovations, such as cloning, a study by Parnpai *et al.* (2002) has compared the cloning efficiency in bovine and swamp-buffalo embryos using fetal fibroblasts, ear fibroblasts and granulosa cells; as shown in Table 1.7, development of reconstructed cattle embryos was higher than with buffalo embryos.

There is an obvious need to employ procedures that will yield higher numbers of oocytes from buffalo ovaries. Follicle aspiration by OPU at regular intervals in the meantime and perhaps at some point in the future culture techniques enabling the growth of preantral follicles may have some relevance. In the experience of Gasparrini (2002) in Italy, the OPU technique has proved much superior to the superovulation approach in their studies with buffaloes in yielding more transferable embryos per donor on a monthly basis (2 vs. 0.6, respectively); the author suggests that the technology has considerable potential for improving genetic improvement in the buffalo through the maternal lineage.

1.6.2. Horses

The first foals from ET were born as a result of the efforts of Oguri (1973) in Japan and Allen and Rowson (1975) in Cambridge. A paper by Squires *et al.* (1999) estimated that about 1500 foals are produced by ET each year in the USA. According to Jasko (2002), the non-surgical transfer of equine embryos has gained widespread popularity over surgical transfer in recent years (see Fig. 1.5). In Brazil, Jacob *et al.* (2002) noted that equine ET had become widely used in that country; they estimated that more non-surgical transfers were carried out annually in Brazil than in any other country. The same authors presented data showing that recipient mares could be used with greater flexibility, in terms of synchrony between donor and recipient, than those currently used; this could be useful in reducing costs and facilitating animal management. Elsewhere in the same country, Peres *et al.* (2002) reported evidence showing that technician experience and the uterine condition of recipient mares were more important in influencing the outcome of non-surgical ET in the mare than the equipment used in performing transfers. In France, Ponsart *et al.* (2002) concluded that a difficult cervical transfer was a major factor influencing the success of ET and showed that the stud farm itself (management of donors and recipients) was associated with success rate. A paper by Carnevale *et al.* (2001) in Colorado has noted that the transfer of sperm and oocytes (gamete intra-Fallopian transfer (GIFT)) is a successful and repeatable assisted reproductive technique in the horse.

It is possible that the production of horse embryos by *in vitro* procedures can find a useful

Table 1.7. Comparison of cloning efficiency in cattle and buffalo embryos (from Parnpai *et al.*, 2002).

Species	Donor cell types	Fused (%)	Embryos cultured	Cleaved (%)	8-Cell (%)	Morula (%)	Blastocyst (%)
Cattle	Fetal fibroblasts	91/102 (89)	91	80 (88)	59 (65)	40 (44)[a]	36 (40)[a]
	Ear fibroblasts	94/103 (91)	94	82 (87)	60 (64)	42 (45)[a]	37 (39)[a]
	Granulosa cells	91/101 (90)	91	81 (89)	58 (64)	41 (45)[a]	37 (41)[a]
Swamp buffalo	Fetal fibroblasts	89/101 (88)	89	76 (85)	54 (61)	23 (26)[b]	17 (19)[b]
	Ear fibroblasts	87/100 (87)	87	73 (84)	52 (60)	21 (24)[b]	18 (21)[b]
	Granulosa cells	90/103 (87)	87	76 (87)	54 (62)	22 (25)[b]	19 (22)[b]

[a,b] $P < 0.01$ (chi-square).

Fig. 1.5. Foal born in 1984 at University College Dublin after non-surgical embryo transfer.

place in the study of many aspects of reproduction in this species, as well as enabling progress to be made in several areas of commercial interest. The problems associated with *in vitro* oocyte maturation, semen capacitation and *in vitro* fertilization have been reviewed by Plscopo (2000). The availability of live equine embryos less than 6 days of age, a requirement for various lines of research (e.g. embryo cryopreservation and splitting), is limited unless there is recourse to costly surgical intervention. The laboratory production of horse embryos could be a useful solution to such problems; several reviews of problems and progress in this area have appeared (Bezard, 1997; Hinrichs, 1998). Compared with cattle, progress in producing IVP embryos has been much slower in the horse. This is due to difficulties in obtaining abattoir ovaries as a source of oocytes and problems in one or other of the several steps in embryo production. As yet, the number of foals born after IVF has been limited; the first two foals were produced using *in vivo*-matured oocytes (Palmer *et al.*, 1991; Bezard, 1992).

Primary equine oocytes have been recovered from abattoir ovaries (Alm and Torner, 1994; Brinsko *et al.*, 1995; Del Campo *et al.*, 1995; Dell'Aquila *et al.*, 1996a,b; Guignot *et al.*, 1996; Sosnowski *et al.*, 1997) and by ultrasound-guided transvaginal follicular aspiration and other means in the live animal (Bruck *et al.*, 1996a,b, 1997; Squires and Cook, 1996; Kanitz *et al.*, 1997; Alm *et al.*, 1999). In contrast to follicle aspiration in the cow, the oocyte recovery rate from non-ovulatory follicles in the mare is low. Those working with abattoir ovaries have found that they may be stored after excision from the mare for at least 6–8 h at temperatures of 27–37°C during transport to the laboratory (Guignot *et al.*, 1999a). The results of a study by Hinrichs *et al.* (2002b) showed that immediate oocyte recovery and rapid placement into medium significantly increased maturation rate; time of oocyte recovery after slaughter did not, however, affect the developmental competence of those oocytes that reached metaphase II.

The collection of horse oocytes by transvaginal ultrasound-guided OPU from mares in different phases of reproduction was reported by Colorado workers (Franz *et al.*, 2000); after IVM, preovulatory follicles from oestrous mares yielded a higher percentage of matured oocytes than follicles of dioestrous and pregnant animals. However, there were indications that it might be worth pursuing studies with mares during dioestrus. In Italy, Galli *et al.* (2002b) collected oocytes from donor mares by OPU and, after maturing them *in vitro*, fertilized them by sperm injection and cultured zygotes either *in vivo* (sheep oviduct) or *in vitro*; their results

showed that the efficiency of embryo production was much higher using the sheep oviduct than *in vitro* (see Table 1.8).

A study by Bezard *et al.* (1998) unsuccessfully attempted to increase the population of small follicles available for *in vivo* aspiration during early dioestrus using crude equine gonadotrophin; such results confirmed the gonadotrophin-independence of small follicles observed by Goudet *et al.*, 1998a,b,c). In Louisiana, Cochran *et al.* (1999) reported a method that showed promise of becoming an effective method of repeatedly producing IVP embryos; using a protocol involving progestogen treatment of mares with oocyte collections performed every 10 days, they recorded cleavage rates of up to 58% by way of intracytoplasmic sperm injection (ICSI). Somewhat similar findings were reported by Guignot *et al.* (1999a,b); a weekly ultrasound-guided follicle aspiration routine enabled them to collect 2.1 oocyte per mare per week, which yielded about 1.5 cleaved embryos per cycle after sperm injection in comparison with 0.6 embryos after *in vivo* fertilization.

At one time, there was uncertainty as to how long the primary equine oocyte takes to mature to the metaphase II stage in the mare's preovulatory follicle. Unlike farm ruminants and pigs, the oestrous mare shows much less evidence of a discrete preovulatory surge of LH prior to release of the oocyte at ovulation. According to Allen and Antczak (2000), equine LH possesses a high content of sialic acid, which gives it a much longer half-life than in the farm ruminants (3–5 h rather than the 20 min found in sheep); the LH molecule also possesses considerable FSH activity, the hormone reaching its peak level in the mare's circulation 1–3 days after rather than before ovulation. Such differences may have a bearing on why IVM of equine oocytes appears to be so difficult (Bruck *et al.*, 1994). It is known, however, that ovulation will occur some 36 h after injecting an ovulation-inducing hormone (e.g. human chorionic gonadotrophin (hCG)) where a preovulatory follicle of appropriate size is present. In the maturation of horse oocytes, regardless of what culture conditions are employed, the outcome remains relatively inefficient (with < 70% of oocytes reaching metaphase II).

Studies by Alm and Hinrichs (1996) in Germany led them to suggest that newly synthesized proteins are necessary to permit progression from metaphase I to metaphase II; in France, there is also evidence indicating that the failure of oocytes to resume and complete meiosis may be due to a deficiency of regulators of maturation-promoting factor (MPF) and/or an inability to phosphorylate mitogen-activated protein kinase (Goudet *et al.*, 1998c). Others have suggested a possible link between the acquisition of oocyte competence and the expression of certain follicular cell proteins (Goudet *et al.*, 1999) and the possibility that the addition of equine oviductal-conditioned medium during incubation might improve the development of oocytes after sperm injection (Maclellan *et al.*, 2000a). In California, Carneiro *et al.* (2001, 2002) found that the addition of insulin-like growth factor I (IGF-I) to the IVM medium containing hormones and fetal calf serum (FCS) had a positive effect on nuclear maturation; in the Netherlands, Tremoleda *et al.* (2001) used fluorescence and confocal microscopy to show how the microfilament and microtubular elements of the equine oocyte cytoskeleton reorganized dramatically during IVM. In Texas, Hinrichs *et al.* (2002a) found no effect of duration of oocyte maturation (24 vs. 42 h) on embryo production and demonstrated that maturation of equine oocytes in follicular fluid increased fertilization rate *in vitro*; it was concluded that further work is needed to determine the optimum environment for sperm

Table 1.8. Equine embryo development: sheep-oviduct vs. *in vitro* culture (from Galli *et al.*, 2002).

Number OPUs	Number oocytes (per OPU)	Number metaphase II (% of oocytes) (oocytes per OPU)	Number cleaved (% of metaphase II) (embryos per OPU)	Number compacted morulae or blastocysts (% of cleaved) (embryos per OPU)
20 (sheep-oviduct culture)	60 (3.0)	46 (76.7) (2.3)	41 (89.1)[a] (2.1)	23 (56.1)[a] (1.2)
12 (*in vitro* culture)	49 (4.1)	36 (73.5) (3)	25 (69.4)[a] (2.1)	5 (20.0)[b] (0.4)

Student's *t*-test. Numbers within columns with different superscripts are significantly different ($P < 0.05$).

capacitation and IVF in the mare. An earlier paper by Gadella *et al.* (2001) reviewed the processes that occur at the sperm plasma membrane before and during penetration of the zona pellucida of the equine oocyte; they noted that relatively little has been documented for stallion sperm on the changes that occur during capacitation and the acrosome reaction. The same authors stress the need to develop and implement new assays for the detection of the capacitation status of live, acrosome-intact and motile stallion sperm.

The difficulty of obtaining an acceptable fertilization rate in the mare by IVF turned the attention of researchers to ICSI, a procedure used with great technical success in human assisted reproduction. In France, Guignot *et al.* (1995) reported that ICSI may be a useful alternative to conventional IVF in the mare. In Italy, the studies of Cho *et al.* (1995) indicated that sperm injection may be successfully employed, despite the very dark ooplasm of the oocyte (high lipid content). Other work in that country demonstrated that sperm injection was much more effective than conventional IVF, particularly when oocytes were matured in the presence of follicular fluid (Dell'Aquila *et al.*, 1996a,b); similar views were expressed in Colorado, where a successful pregnancy was established after ICSI (Squires *et al.*, 1996). Other reports dealing with ICSI include those of Galli *et al.* (2002b), dealing with the subsequent development of equine oocytes in different media, and Choi *et al.* (2002a) who dealt with the developmental competence of *in vivo*-matured and IVM equine oocytes injected with fresh or frozen–thawed sperm.

In the culture of horse zygotes and embryos, information remains limited. Those IVF- and ICSI-derived foals that have been born have usually followed the transfer of early embryos to the Fallopian tubes of surrogate mothers. In Colorado, Choi *et al.* (2000, 2001a) compared a trophoblast-conditioned medium with a chemically defined medium in culturing zygotes; although some viable blastocysts were produced, the yield was very low (<5%). Some workers have attempted to assess the quality of horse embryos on the basis of their metabolism (Lane *et al.*, 2001); it is suggested that nutrient uptake (determined by non-invasive microfluorometry) may provide a useful viability marker of morulae and blastocysts. The storage of equine embryos in HEPES-buffered Ham's F-10 with 0.4% BSA at 15–18°C for up to 18 h was studied by Fleury *et al.* (2002); they found this formulation to be very effective for embryo storage and transport under field conditions. It enabled specialized recipient farms to be utilized by horse owners and veterinarians who did not have their own recipients. In long-term oocyte preservation, Maclellan *et al.* (2002) reported the first foals to be produced from the transfer of vitrified and thawed equine oocytes to inseminated recipient mares; although the viability of vitrified oocytes was significantly decreased in comparison with untreated controls, the establishment of pregnancies and live births was regarded as a significant advance in the cryopreservation of equine gametes.

In the area of cloning, studies reported by Li *et al.* (2002a) have demonstrated a very limited potential for *in vitro* development of horse embryos after nuclear reprogramming following the transfer of nuclei from either fetal or adult fibroblasts into recipient enucleated oocytes. It is clear from their results that much remains to be done in improving the *in vitro* developmental potential of equine embryos derived from nuclear transfer and in achieving full-term development of cloned horses. As in many aspects of equine reproduction, methods that work in rodents and ruminants may not necessarily be applied with similar effectiveness to the horse. Among the possible reasons advanced by the Cambridge workers for limited cloning success was the fact that there was an apparent failure of the 'blastocyst capsule' to form in the reconstructed embryo. The capsule, which develops between the zona pellucida and trophectoderm in the late morula/early blastocyst, is regarded as essential for the survival of the horse embryo in the early stages of pregnancy. In the USA, Choi *et al.* (2002b,c) examined the reconstruction rate of equine oocytes using direct nuclear injection with a piezo-driven pipette and activation using a stallion sperm extract; they were able to demonstrate cleavage and development of embryos up to the ten nuclei stage.

Apart from ET, IVF and related techniques, there is also much interest in horses in the development of techniques for low-dose AI. A report by Lindsey *et al.* (2001) demonstrated that hysteroscopic insemination in the mare is a practical technique for achieving pregnancies with low numbers of fresh or frozen–thawed

sperm. Mares are usually inseminated with 500 million progressively motile fresh sperm or about double that dose with cooled or frozen sperm. Low-dose AI would enable more mares to be bred by a given stallion, permit the use of stallions with poor semen quality, extend the use of frozen sperm, enable mares to be bred with sexed semen and possibly reduce the incidence of postbreeding endometritis. The accurate assessment of stallion sperm quality is also a research area relevant to both ET and AI in horses; as evident in a report by Graham (2001), it is possible for multiple sperm attributes, including cell viability, acrosomal integrity and mitochondrial function, to be measured by way of flow-cytometric assays simultaneously in sperm cells.

1.6.3. Pigs

There are those who believe that techniques, such as ET, which permit the simultaneous control and manipulation of the genetic quality and health status of selected animals will become increasingly important for internationally based pig-breeding companies (Garcia, 2000). The development of IVM and IVF techniques in the pig is likely to be important in facilitating the application of this technology in several areas (see Hazeleger and Kemp, 1999; Brussow *et al.*, 2000; Day, 2000); for commercial application, the combination of IVP embryos with successful non-surgical transfer techniques would have great potential. Instrumentation for non-surgical embryo transfer has recently been developed (Hazeleger, W. *et al.*, 1995; Li, J. *et al.*, 1995; Prather *et al.*, 1998; Kano *et al.*, 2000; Riha and Vejnar, 2000; Martinez *et al.*, 2002) and there has been considerable progress in the cryopreservation of porcine embryos by vitrification (Dobrinsky, 1999; Gajda and Smorag, 2000; Berthelot *et al.*, 2001). However, despite intensive research, the successful production of IVP pig embryos has lagged some way behind that of cattle (Funahashi *et al.*, 1994a,b,c, 1995a,b; Nagai, 1994; Funahashi and Day, 1995; Hyttel *et al.*, 2000a,b; Swain *et al.*, 2000; Niemann and Rath, 2001; Abeydeera, 2002; Coy *et al.*, 2002; Stroble *et al.*, 2002).

Although the cytoplasmic maturation of oocytes has been improved by modifications to the culture procedure, the problem of polyspermic penetration remains a major issue. According to Abeydeera (2002), the IVM of pig oocytes in the presence of gonadotrophins, cysteine, thiol compounds and growth factors, together with the replacement of caffeine with adenosine and a short IVF interval, will help to ensure developmentally competent zygotes; the type of IVF medium and certain modifications to that medium can reduce the incidence of polyspermy. A report by Funahashi *et al.* (2000) has shown that supplementation of IVF media with adenosine rather than caffeine may help to minimize the problem. Elsewhere, Martinez-Madrid *et al.* (2000), using a modified Tris-buffered medium rather than a modified Tyrode's medium, reported a significant decrease in the incidence of polyspermy. There is also evidence in pigs that the development of fully effective mechanisms to prevent polyspermy may only be complete after oocytes are exposed to the oviduct; this would agree with reports indicating the beneficial effects of oviductal cells and/or conditioned media in reducing the problem (see Dubuc and Sirard, 1994, 1995). In the Netherlands, some workers have shown that the stimulatory effect of porcine follicular fluid on oocyte developmental competence can be influenced by follicle size; Algriany *et al.* (2002) found that the addition of follicular fluid from large (5–8 mm) rather than small (2–4 mm) follicles to their IVM medium significantly increased nuclear maturation rate and blastocyst yield.

In Missouri, a paper by Lai *et al.* (2002) suggested that preactivation of the porcine oocyte might be an efficient way of reducing the polyspermy rate; they also found that the polyspermy rate was significantly reduced when oocytes were fertilized 6 h after activation rather than at 2 and 4 h after activation. In Spain, Martinez-Madrid *et al.* (2002) used a two-step sperm–oocyte co-incubation method (oocytes exposed to sperm in a droplet for 5 min and then transferred to a second, sperm-free droplet for 4 min); the two-step procedure greatly increased the monospermic fertilization rate (64% vs. 36%). In the same country, Matas *et al.* (2002) reported on the effect of different sperm treatments prior to IVF; they recorded various factors that resulted in a higher rate of sperm penetration. In the USA, McCauley *et al.* (2002) demonstrated that porcine oviduct-specific

glycoprotein improved the development of zygotes to the blastocyst stage and found evidence suggesting that this effect was distinct from modulation of the sperm–oocyte interaction at the time of fertilization.

In Japan, Tatemoto *et al.* (2000) examined the role of cumulus cells in protecting porcine oocytes against apoptotic cell death caused by oxidative stress during IVM; their results suggested that the protective effect was by way of enhancement of the glutathione (GSH) content in oocytes. Subsequent work by this group examined the developmental competence of pig oocytes after IVF when matured with a medium (North Carolina State University 37 (NCSU-37)) containing ascorbic acid-*O*-α-glucoside (AA-2G); they found that blastocyst yield was significantly increased by the addition of the enzyme (Tatemoto *et al.*, 2001). The authors suggest that a critical level of intracellular L-ascorbic acid, supplied by AA-2G during maturation, plays an important role after fertilization in alleviating the adverse effects of oxidative stress.

One problem of concern in pig IVF relates to the quality of available semen. The difficulty in freezing boar sperm effectively has meant that fresh ejaculated semen has usually been employed in attempts to fertilize oocytes. Results presented by Cordova *et al.* (2001) suggested that frozen boar semen was adequate for IVF, whether packaged in 0.5 or 5 ml straws. It is known that large variations exist among boars in the ability of their sperm to achieve penetration of the oocyte; the polyspermy problem is also greater with some boars (Xu *et al.*, 1995); other studies have shown that sperm penetration, monospermy and male pronuclear formation may all be significantly affected by the male factor (Foxcroft *et al.*, 1995).

In Germany, oocyte aspiration was investigated in gilts by follicular puncture via laparoscopy by Brussow and Ratky (1996); they reported that follicular and oocyte development was not influenced by repeated follicular puncture. In further studies, Brussow *et al.* (2000) recovered pig oocytes by endoscopy after oestrus synchronization with progestogen (altrenogest), followed by PMSG and with hCG administered after a further 80 h; follicles were aspirated at varying intervals after the ovulating injection. The results showed a high proportion of immature oocytes (germinal vesicle stage) 2 h before and 10 h after hCG; the number of mature (metaphase II) oocytes increased thereafter according to the time of recovery.

In the IVC of pig embryos after maturation and fertilization, the medium commonly used is NCSU-23). In Indiana, Swain *et al.* (2001) examined Gardner's sequential growth medium (G1.2/G2.2), a medium designed to meet the changing energy demands of developing embryos associated with such changes as the maternal zygotic transition, compaction and blastocoel formation and expansion. Although they found that the sequential culture system supported embryo development, it was not as effective as NCSU-23; it is concluded that porcine embryos require a medium formulated specifically for that species. In Japan, Kikuchi *et al.* (2002) sought to establish reliable IVP methods, maturing oocytes for 44 h in NCSU-37, exposing them to frozen–thawed epididymal sperm for 3 h in a modified fertilization medium and culturing embryos subsequently in NCSU-37 in a two-stage IVC sytem; for the first 2 days, NCSU-37, conditioned by porcine oviductal cells, was supplemented with pyruvate and lactate and for the following 3–4 days with glucose. The authors achieved pregnancies with embryos transferred to recipients and suggest that the system is capable of producing high-quality blastocysts. A paper by Callesen *et al.* (2002) showed that low oxygen tension during embryo culture increased cell number without altering blastocyst yield.

As in other farm mammals, comparisons have been made in pigs between oocytes recovered from young and old animals. In Australia, O'Brien *et al.* (2000) reported a comparison of IVM, IVF, metabolism and ultrastructure of oocytes from prepubertal and adult sows; they showed evidence of a different *in vitro* developmental capacity between the two categories, although metabolic activity and ultrastructure was similar.

The cryopreservation of pig embryos has posed particular problems that have yet to be adequately resolved; vitrification has been the approach that has yielded the most promising results (Dobrinsky, 2002), but it has usually involved hatched porcine blastocysts that have been manipulated to remove lipid droplets. However, because such blastocysts are no longer surrounded by the zona pellucida, they do not

satisfy the requirements for international trade. Of practical interest, for such reasons, is a paper by Beebe, L.F.S. *et al.* (2002) in the USA reporting the birth of healthy, normal piglets born from zona-intact early blastocysts, vitrified without the need for micromanipulation to remove lipid droplets; in France, similar evidence came from Berthelot *et al.* (2002), who have showed that the open pulled straw (OPS) vitrification method can be useful for cryopreserving zona-intact morulae. These are useful steps towards the commercial development of an international trade in pig embryos.

In cloning, somatic-cell nuclear transfer has been used successfully to produce live piglets. In Australia, Boquest *et al.* (2002) produced cloned pigs from cultured fetal fibroblast cells, exposing the donor cell nuclei to oocyte cytoplasm some 3 h before activation by chemical (ionomycin) means. In Korea, Cheong *et al.* (2002) studied factors affecting the development of reconstituted embryos derived from clonal lines of fetal fibroblasts; they found evidence suggesting that clonal lines with different population doubling times and sizes could result in different development after nuclear transfer. There is much interest in pigs in the possibility of using gene-targeted cells for nuclear transfer in order to remove genes, such as the α-(1, 3)-galactosyltransferase gene, which codes for xenoreactive transplantation antigens; this is regarded as a crucial step in making pig organs and tissues suitable for human transplantation.

1.6.4. Sheep and goats

In many countries around the world, sheep and goats feature prominently in the livestock industry. ET technology has been useful in enabling the importation of animals in the form of frozen embryos and appreciable numbers of sheep and goat embryos have been transferred in countries such as Australia and South Africa (Thibier, 1996). Sheep were the first of the farm mammals to be used in IVF studies by Dauzier and Thibault in France during the 1950s; reports of lambs born after IVF of artificially matured ovine oocytes were to come much later. Czlonkowska *et al.* (1991) in Poland were among the first to report lambs born after IVM, IVF and IVC of early-stage embryos with oviductal cells. Elsewhere, in New Zealand, Pugh *et al.* (1991) were also to report the birth of lambs after IVM and IVF, using frozen–thawed sperm and oocytes collected during the non-breeding season; such results showed the possibility of year-round production of IVM/IVF embryos. A paper by Baldassarre *et al.* (1994) dealt with the recovery of sheep oocytes by laparoscopy. In Fargo, Stenbak *et al.* (2001) were to report decreased cleavage rates in their IVP sheep embryos during anoestrus compared with those recovered in the normal breeding season.

According to Cognie (1999), ovulated sheep oocytes fertilized and cultured *in vitro* can be expected to yield between 60 and 70% blastocysts, which is close to the development rate found in the live animal. Using IVM oocytes, the developmental rate is approximately halved. The author notes that the major problem lies in the selection of oocytes competent for IVM, IVF and subsequent development. A typical medium used in IVM would be one based on bicarbonate-buffered TCM-199, supplemented with 10% FCS and other additives (gonadotrophins, oestradiol, glutamine, sodium pyruvate, cysteamine, trace amounts of antibiotics); the oocytes are incubated for 24 h under a humidified 5% CO_2 atmosphere at 39°C. A further supplement to the maturation medium, epidermal growth factor (EGF) has been reported to increase blastocyst formation in the sheep (Guler *et al.*, 2000; Choi *et al.*, 2001); such evidence agrees with that provided in similar studies with other species. In Ireland, the effect of ram semen fertility and dietary energy and urea supplements on oocyte and embryo quality in sheep have been examined, using *in vivo* and *in vitro* experimental models (Byrne *et al.*, 2000; Papadopoulos *et al.*, 2001a).

Workers in Italy have shown that it is possible to obtain repeated embryo production and normal births in sheep using an OPU technique, Ptak *et al.* (1999a) in Sassari report aspirating 11.5 oocytes per ewe and producing 2.7 blastocysts per ewe after processing in the laboratory. Other work by this same group led to the first report of births after the collection and processing of oocytes from 1-month-old lambs (Ptak *et al.*, 1999b). A study by Ledda *et al.* (2001) in Italy sought to analyse differences between prepubertal (month-old) and adult sheep; they

recorded a decreased presence of transzonal processes and defective coupling between cumulus cells and oocytes. The same authors found a significantly lower level of MPF activity at the metaphase III stage than in adult oocytes; such deficiencies could help to explain the lowered developmental capacity observed in the lamb oocytes. In cattle, it is well established that IVP embryos show increased sensitivity to chilling and cryopreservation in comparison with those produced *in vivo*; similar evidence is available in sheep (Dattena *et al.*, 2000; Papadopoulos *et al.*, 2001a,b). Some details of the Irish work are given in Table 1.9.

Although Hanada in Japan reported the birth of a kid after IVF of ovulated oocytes in 1985, the first birth after IVM/IVF appears to be that reported by Crozet *et al.* (1993) in France, using the sheep oviduct for the *in vivo* culture of the caprine embryos. In goats, unlike cattle, one of the limiting factors in acquiring caprine oocytes from abattoir sources is likely to be the lack of commercial enterprises capable of providing consistent supplies of ovaries. Research in maturation and fertilization of oocytes has been reported in several European countries, including Spain (Mogas *et al.*, 1995a,b,c), France (Crozet *et al.*, 1995a,b) and Germany (Pereira *et al.*, 1995). The development of IVP embryo technology in species such as the goat would be valuable in facilitating certain biotechnology programmes, such as that involving gene transfer. The goat would permit more rapid progress in the production of transgenic animals than would be possible in cattle and other livestock with longer generation intervals. Cloning by way of somatic cells for the production and propagation of transgenic goats was described by Keefer *et al.* (2002); transgenic goats could be cloned at similar efficiencies using either transgenic somatic cells obtained by adult cell biopsy or *in vitro* transfection. Elsewhere in the USA, Reggio *et al.* (2002) took advantage of nuclear-transfer technology to produce cloned goats transgenic for recombinant proteins that may be useful as human pharmaceuticals.

Methods of recovering goat oocytes vary from the aspiration of abattoir ovaries to collection from live animals by way of laparotomy and occasionally ovariectomy (Cognie *et al.*, 1995a,b; Samake *et al.*, 2000). A paper by Graff *et al.* (1995) dealt with ultrasound-guided transvaginal oocyte recovery from FSH-treated goats. According to Baldassarre *et al.* (2002a,b), OPU by way of laparoscopy is very reliable and enables a predictable number of oocytes to be recovered during each session; it also has the advantage of being less invasive than laparotomy, thereby allowing multiple recoveries to be made from the some donor goat. In Zaragoza, Aguilar *et al.* (2002) found that repeated OPU in unstimulated sheep and goats yielded sufficient oocytes for *in vitro* techniques while maintaining donors alive and well. As with sheep, oocyte maturation is usually based on TCM-199, supplemented with FCS, FSH, LH and oestradiol, and incubated for 24–27 h. A report by Rho *et al.* (2001) shows that a maturation period of 27 h results in a significantly higher proportion of oocytes reaching maturity than does one of 24 h; in their studies on the preparation of goat semen for IVF, the same authors found Percoll density-gradient centrifugation to be about four times more efficient than swim-up and about two times better than glass-wool filtration for recovering sperm from a freeze–thawed semen sample. Studies by Cognie *et al.* (2001) compared the survival of *in vivo* and *in vitro* embryos after transfer to recipients. As shown in Table 1.10, with IVP embryos the kidding rate and embryo survival

Table 1.9. Pregnancies after transfer of *in vitro*- vs. *in vivo*-produced sheep embryos (from Papadopoulos *et al.*, 2001b).

	Embryo source	No. transfers	Pregnant (%)	Singles (%)	Twins (%)	Embryo survival (%)
IVP	Fresh	35	19 (54.3)[a]	15 (79.0)	4 (21.0)	23 (32.8)[a]
	OPS	40	2 (5.0)[b]	2 (100.0)	0	2 (2.5)[b]
In vivo	Fresh	10	9 (90.0)	3 (33.3)	6 (66.7)	15 (75.0)
	OPS	10	5 (50.0)	3 (60.0)	2 (40.0)	7 (35.0)

$P < 0.05$.

Table 1.10. Pregnancies after transfer of *in vitro*- vs. *in vivo*-produced goat embryos (from Cognie *et al.*, 2001).

Embryo production method	Recipients *n* (embryos)	Pregnancy rate % (*n*) day 21	Pregnancy rate % (*n*) day 41	Kidding rate % (*n*)	Embryo survival % (*n*)
In vitro	18 (36)	83 (15)	61[a] (11)	61[a] (11)	47[a] (17)
In vivo	19 (38)	89 (17)	89[b] (17)	89[b] (17)	71[b] (27)

[a,b]Values differ significantly ($P < 0.05$ chi-squared test).

were significantly below that recorded with *in vivo*-produced embryos.

In Korea, Lee, C.S. *et al.* (2000) examined the effect of GSH on the development of early goat embryos *in vitro*; results clearly showed that GSH improved development markedly by specifically acting on the 8–16-cell-block stage; the authors suggest that GSH may be one of the important regulators of goat embryo development in the live animal. In Canada, Bhatia *et al.* (2002) evaluated two different methods of sperm capacitation, using both fresh and frozen–thawed sperm; according to the authors, the study established protocols that could be successfully applied to the large-scale production of goat IVP embryos.

1.6.5. Deer

The rapid growth in deer farming in countries such as New Zealand, where 2 million animals are currently farmed for venison and velvet production, together with the growth in interest in the preservation of various endangered members of the deer family, which stretches all the way from the small Chinese water-deer to the large North American wapiti, has seen the development of ET technologies suitable for such animals. A comprehensive discussion of deer reproduction and approaches for improving the success of controlled breeding programmes has been provided by Jabbour *et al.* (1997). There are those who believe that deer offer an ideal model for the development of *in vitro* technologies for endangered-species conservation (Bainbridge and Jabbour, 1998). Most work has been with red deer (*Cervus elaphus*) and wapiti (*Cervus canadensis*). Methods of management that will enhance the reproductive performance of deer have been discussed by Wilson *et al.* (1998). Superovulation procedures are based on those used in sheep and cattle and embryo recovery is by surgical intervention; ET is usually by way of a technique involving laparoscopy or by a Cassou pipette as used in AI (Fennessey *et al.*, 1994; Jabbour and Bainbridge, 1996; Asher, 1998).

According to Fukui *et al.* (1991), the development of effective IVP techniques in deer could be valuable in the *in vitro* hybridization of different deer species that do not readily hybridize naturally (e.g. Père David's and red deer). Working at Ruakura in New Zealand, these workers reported successful maturation and fertilization of red deer oocytes, some cleaving subsequently to the two- to eight-cell stage. In Canada, the IVP of wapiti and red deer embryos was reported by Pollard *et al.* (1995); their results, in terms of oocytes collected per ovary (>20) and embryos produced (25–30%), were equal, if not superior, to those often obtained with cattle oocytes. The first pregnancies from the maturation and fertilization of red deer oocytes were recorded in New Zealand (Berg *et al.*, 1995). The development of ultrasound-guided transvaginal oocyte pick-up for farmed red deer has been reported by Berg *et al.* (2000); their data showed that weekly OPU can be used to recover oocytes in both the breeding and the non-breeding season. In further studies, the New Zealand group reported that red deer oviduct epithelial-cell monolayers were capable of sustaining sperm motility at levels comparable with initial sperm motility over a 24 h period (Berg *et al.*, 2002a). They also confirmed that *in vitro* red deer oocyte maturation follows a similar time course (24 h) to that in cattle and sheep (Berg *et al.*, 2002b) and that procedures developed in their laboratory were capable of leading to the birth of normal calves after transfer of IVP embryos (Berg *et al.*, 2002c).

In oocyte maturation, methods are usually based on those developed and used in cattle, with oocytes either recovered from ovaries recovered

at abattoirs or collected by the laparoscopic aspiration of follicles in hormone-treated females (Bainbridge *et al.*, 1999); a typical medium would be TCM-199, supplemented with FCS, gonadotrophins, oestradiol and additional granulosa cells. In red deer, Bainbridge *et al.* (1999) have shown that sperm can be capacitated *in vitro* by supplementing the fertilization medium with heparin, as in cattle; the same authors were also to show that epididymal sperm could be successfully employed in IVF. Elsewhere, however, both in New Zealand and France, it has been shown that 20% of oestrous sheep serum was an efficient supplement for the *in vitro* capacitation of red deer ejaculates and for IVF of red deer oocytes (Berg *et al.*, 1995; Comizzoli *et al.*, 2000a,b). The fertilizing ability of different deer semen samples, including epididymal sperm, in various supplemented IVF media was examined by Comizzoli *et al.* (2001b), using zona-free bovine oocytes to decondense deer sperm chromatin; the authors suggest that heterologous IVF may be a useful tool in permitting a clearer understanding of unsuccessful homologous IVF in deer; another report by Comizzoli *et al.* (2001a) attempted to define the conditions for IVM and IVF of oocytes in two common deer species (red and sika deer) as models for endangered related subspecies.

1.6.6. Camelids

Some 30–40 million years ago, as noted by Skidmore *et al.* (1999), the camelid family originated in North America; some members of the family migrated across the Bering Straits and gave rise to the Old World camelids (Bactrian and dromedary camels), while others went south and gave rise to the smaller New World camelids (llama, guanaco, alpaca and vicuña). In the UK, there is a growing population of New World camelids, which are currently kept as pets, as producers of fine fibre and as pack-animals for mountain-trekking holidays (Davis *et al.*, 1998; Wright *et al.*, 1998). Reproduction and reproductive management of New World camelids are dealt with in several reviews (Sumar, 1996; Del Campo and Del Campo, 1998; Davis and Knight, 1998). Techniques that may be employed to improve the reproductive performance of both camels and the smaller camelid species include AI, oestrus-control techniques, pregnancy diagnostic techniques, ET and *in vitro* embryo production. Such technologies and the prospects for their application in dromedaries and Bactrian camels and South American Camelidae have been reviewed by Purohit (1999) in India; in the same country's National Centre for Camel Research, Vyas *et al.* (1998) reported on superovulation and non-surgical ET in dromedary camels. Although, due to their marked differences in size, Old and New World camelids are not able to mate naturally, with modern reproductive technology this is not an insurmountable problem. Already a viable hybrid (dromedary × guanaco) has been reported from the Dubai Camel Reproduction Centre (Skidmore *et al.*, 1999). Other work at that Centre has also seen the first pregnancy in a camel resulting from the transfer of three cryopreserved and cultured embryos (Skidmore and Loskutoff, 1999).

The reproductive activity of camelids has several unique features, and information on oocyte physiology is limited. Bou *et al.* (1993) in China described small-scale studies on IVF in the dromedary camel; follicular oocytes were recovered from abattoir ovaries and matured in TCM-199 supplemented with FCS and gonadotrophin. After maturation, oocytes were exposed to epididymal sperm that had been capacitated in medium containing caffeine and BSA for 2 h; after 6–7 h, oocytes were transferred to TCM-199 containing FCS and sodium pyruvate. The Chinese workers recorded a maturation rate of 47% and a fertilization rate of 43%, with some embryos developing to the 8–16-cell stage *in vitro*. The morphology of cumulus–oocyte complexes (COCs) in the dromedary camel was examined by Torner *et al.* (1999), who found most oocytes at the time of recovery (after slicing abattoir ovaries) to be in a state of resumed meiosis; significant differences in COC morphology were observed between pregnant and non-pregnant animals. The same workers suggest that the maturation period for camel oocytes should be 36 h. In Egypt, Abdoon (2001) reported that the growth and development of ovarian follicles in the dromedary camel and the yield of good-quality oocytes were greater during the breeding season; oocyte maturation rate in CR1aa medium was not improved by prolonging

IVC beyond 36 h. Semen biology and assisted reproductive technology in dromedary and Bactrian camels are among various topics reviewed by Hafez and Hafez (2001) in the USA.

According to Taylor *et al.* (2000), establishing practical and effective ET methods for the llama (*Lama glama*) would be of considerable benefit to the American llama industry; it would facilitate the importation of genetically valuable embryos from South America to improve herds in the USA and Canada. These authors have reported a 2-year study in which they used a non-surgical recovery and transfer procedure in a small llama herd; results from their ETs are in Table 1.11.

In Argentina, the feasibility of an ultrasound-guided transvaginal approach to oocyte recovery in llamas was examined by Brogliatti *et al.* (2000); 57% of follicles aspirated yielded oocytes, which were surrounded by multiple layers of compacted granulosa cells and displayed a homogeneous dark ooplasm. The same authors found the ultrastructure of llama oocytes to be similar to that of cattle except for a conspicuous accumulation of large lipid droplets in the cytoplasm. Elsewhere and earlier, Palasz *et al.* (1997) examined the ultrastructure of llama blastocysts and remarked on the presence of distinctive lipid droplets.

1.7. Human *in Vitro* Fertilization

There is obviously much common ground between procedures used in IVP/ET in farm mammals and the techniques in everyday use in human assisted reproduction. Indeed, there are those who have expressed the view that IVM may become an alternative to superovulation for all human IVF patients (Trounson, 1994). In addition, there may be a case for using the bovine model in the examination of many problems in human assisted reproduction that cannot readily be pursued for ethical or technical reasons. Wrenzycki *et al.* (2001b) observe that culture media employed with human embryos are analogous to those used in other mammals, usually the mouse, despite increasing evidence that the bovine model may well be preferable, based on timing of genome activation, intermediate metabolism and interaction with the culture medium. Certainly, there are those who contend that rodents, such as the mouse and hamster, may be less appropriate than the cow as a model for elucidating certain aspects of human infertility. There is the fact that human centrosome inheritance is paternal, unlike the maternal pattern of rodents. This has prompted some to question the reliability of the hamster oocyte penetration assay in identifying defects in human sperm centrosomes (Simerly *et al.*, 1995); the bovine model, in which the centrosome is known to be paternally inherited, may be more suitable in assessing defects. In one laboratory, workers developed the 'Minotaur assay', in which human sperm are introduced into bovine oocytes (Schatten, 1994). In Japan, Nakamura *et al.* (2001) also concluded that heterologous ICSI into bovine oocytes with human sperm can be a relevant assay for human sperm centrosomal function, which contributes to the diagnosis and treatment of some forms of male infertility.

1.7.1. Historical aspects

The first IVF baby, Louise Brown, was conceived from the transfer of a single eight-cell embryo after retrieval and subsequent fertilization *in vitro* of a single oocyte in a natural cycle (Steptoe and Edwards, 1978). By the start of the 21st century, it was estimated that about 1 million births had occurred worldwide by way of human

Table 1.11. Embryo transfer results from llama donors (from Taylor *et al.*, 2000).

Day of collection	No. collections	Total no. embryos	% Recovered	No. embryos transferred	No. (%) pregnant
7	49	27	55*	19	5 (26.3)
8	47	37	79*	27	11 (40.7)
9	3	3	100	3	2 (66.6)

*Mean values with an asterisk in the same column are significantly different at the $P < 0.10$ level.

assisted-reproduction techniques (see Hardy *et al.*, 2002). Breakthroughs contributing to the overall success of human reproductive technology include the development of protocols of ovulation induction, improved IVC methods, effective embryo cryopreservation and ICSI. However, quite apart from problems requiring assistance, natural fertility in the human is poor, with the probability of establishing a viable conception in any one menstrual cycle being about 20–25% for a healthy, fertile couple. This is in contrast to what occurs in cattle, where a conception rate of 50% in a cycle would be regarded as poor. It follows from this that much needs to be understood about factors influencing embryo mortality in the human species.

1.7.2. Establishment of pregnancy by embryo transfer

Although the advent of assisted-reproduction techniques has been of immense value in the treatment of human infertility, it is the poor viability of embryos, with consequent implantation failure, which is seen to be a major factor determining the establishment of pregnancies. In human IVF, it is well accepted that the success rate is greater when more than one embryo is transferred (Racowsky, 2002); the same appears to be true in the normally monotocous cow, where pregnancy rates can be directly related to the number of embryos transferred. In farm ruminants, much evidence has accumulated to suggest that failure in embryo–maternal signalling for pregnancy recognition is a significant source of embryo mortality; ways are now available for the pregnancy rate to be increased by agents that enhance maternal recognition of the embryo. The importance of effective communication between embryo and maternal endometrium is likely to be as great in humans as in ruminants.

1.7.3. Ovarian stimulation regimens for IVF

Although gonadotrophins were isolated from pituitary extracts more than 50 years ago, it has only been in recent decades that the structures of FSH and LH and their receptors have been described. Effective ovarian stimulation requires knowledge of the basic concepts of follicular dynamics as well as an understanding of the respective roles of FSH and LH in regulating follicular development and ovulation (Baird, 1994; McNeilly, 1994). FSH stimulates follicular development by interacting with specific receptors on granulosa cells, inducing increased division of granulosa cells as well as inducing a series of key genes involved in the maturation of the follicle and the formation of LH receptors. Such receptors are essential for completion of follicle maturation and to allow ovulation to occur in response to the preovulatory surge of LH. In contrast to FSH, LH receptors are initially confined to theca cells; LH stimulates the production of androgens which serve as a substrate for the synthesis of oestradiol by granulosa cells (Macklon and Fauser, 2000; Hillier, 2001). Although both FSH and LH use cyclic adenosine monophosphate (cAMP) as the second messenger, the effects on cell division of the two gonadotrophins are very different; relatively low amounts of cAMP stimulate cell division whereas high levels inhibit division. According to Zeleznik (2001), the process of preovulatory follicle development, including the selection of a single follicle and ovulation, may be regulated by cAMP, which, in turn, is regulated by FSH and LH.

The basic mechanisms that regulate the primate menstrual cycle are much the same as those that control the bovine oestrous cycle; there is, in fact, value in using the bovine model in a study of human ovarian follicular dynamics (Adams and Pierson, 1995; Campbell *et al.*, 1997). During each menstrual cycle, a group of follicles (a cohort) begin a well-characterized pattern of growth and development; one member only of the cohort is destined to continue development to become the preovulatory follicle. The follicle destined for ovulation, the dominant follicle, is believed to be functionally, as well as morphologically, dominant, since it inhibits the development of other competing follicles. A decreased FSH level appears to be crucial for the selection of the single dominant follicle from the recruited cohort; it escapes atresia by becoming more sensitive to FSH. Among the factors involved in this increased sensitivity to the gonadotrophin is an increase in the number of granulosa cells, the acquisition of LH receptors and an increase in FSH receptors on granulosa cells.

The fact that the normal human menstrual cycle is characterized by unifollicular ovulation whereas for human IVF the need is for multiple follicle development has led to a proliferation of ovarian stimulation methods over the years. Human menopausal gonadotrophin (HMG) preparations were used initially, essentially crude urinary extracts in which FSH alone or in conjunction with LH made up a small proportion (2–3%) of the total protein content. In the first decade of human IVF activity, ovarian stimulation was usually by way of HMG and the efficacy and safety of urinary gonadotrophins was amply demonstrated by the birth of thousands of healthy babies. The application of appropriate purification methods to urinary extracts subsequently provided products (e.g. Metrodin High Purity) that showed > 95% FSH activity with minimal inclusion of urinary proteins; this markedly reduced the systemic adverse reactions that occasionally added to the stress of IVF treatment (Eshkol, 1994). Such highly purified forms of HMG were to pave the way for the introduction of FSH and LH prepared by recombinant DNA techniques into clinical practice.

The common feature of ovarian stimulation protocols is amplification and/or prolongation of the intercycle increase in FSH, thereby encouraging maturation of several rather than one preovulatory follicle. This is achieved by increasing or prolonging the release of endogenous FSH (using clomiphene, gonadotrophin-releasing hormone (GH-RH)) or by administering exogenous gonadotrophins; the latter is the approach usually favoured, since the dose can be adjusted according to the follicular response as measured by ultrasonics. The mechanism of GH-RH action is believed to be via the down-regulation of pituitary gonadotrophins, resulting in follicular regression through removal of gonadotrophic (presumably FSH) support. Exogenous FSH is then able to stimulate uniform follicle growth because of the absence of a dominant follicle. Pretreatment with GH-RH analogues also means that a premature surge of LH can be avoided, thereby permitting greater flexibility in the timing of ovulating hormone (hCG) injection and follicle aspiration.

The advent of recombinant FSH (r-FSH) and r-LH provides the opportunity to identify more precisely the contributions that FSH and LH make to ovarian function. Recombinant FSH is likely to replace urinary forms of FSH because, as well as being more effective in inducing multiple ovulations, it is easier to produce to a constant specification and, being molecularly pure, is safer. Recombinant preparations on the market (e.g. Gonal-F; Puregon) appear to be equally suitable for use in ovarian stimulation (Hillier, 2001).

1.7.4. Recovery of human oocytes

The technique of transvaginal ultrasound-guided follicular aspiration as a method for the recovery of human oocytes was first described in 1987. The technique proved to be more rapid and less invasive than conventional laparoscopic methods used up to that time. The method also proved to be more effective than other techniques in terms of oocyte recovery rates and pregnancy rates per cycle babies born; it has been the routine procedure employed in human IVF clinics since the early 1990s. It may be noted that the first report of the technique being used in cattle was in 1988, when workers in the Netherlands performed aspirations repeatedly on the same cow over a period of several months; the procedure is now used routinely by many practitioners of cattle ET. A number of vacuum pressures have been employed in recovering human oocytes by aspiration, ranging from 40 to 200 mmHg, with most clinics using a pressure of about 100 mmHg. The possible benefits of flushing follicles have been examined by some workers, although most found no significant difference in the recovery rate of oocytes when follicles were flushed after the initial aspiration. For the collection of immature oocytes, however, there may be a much greater need to flush, because the COC is not on a pedicle as with mature oocytes in large human follicles; for that reason the COC may be more difficult to dislodge from the granulosa cell layer.

1.7.5. *In vitro* maturation of human oocytes

There are those who have expressed the view that a reliable, safe and effective method of

inducing maturation of immature oocytes *in vitro* might be the means of revolutionizing human IVF; others have expressed the view that human IVF based on the recovery and maturation of the primary oocyte could represent a significant improvement on the grounds of its economic, psychological and practical implications. A more limited but immediate application of IVM technology could be rescuing immature oocytes retrieved during IVF or in dealing with patients susceptible to polycystic ovarian syndrome (Mikkelsen and Lindenberg, 2001a,b); in the latter context, two of the earliest reports of a pregnancy after IVM of an oocyte retrieved without the use of ovulating hormone were those of Jaroudi *et al.* (1997) in Saudi Arabia and Liu *et al.* (1997) in Baltimore. There are certainly those who point to the need for techniques that will produce competent oocytes for IVM and IVF by way of minimal stimulation of recruitable follicles; this would eliminate several problems of high-order ovarian stimulation employed in conventional IVF. For such reasons, Suikkari *et al.* (2000) investigated the use of low-dose r-FSH in the late luteal phase of the cycle as a means of producing competent oocytes in women with regular or irregular cycles. In Newcastle, Herbert *et al.* (1997) found that IVM human oocytes did not elicit normal calcium (Ca^{2+}) signalling in response to sperm–oocyte fusion and suggested that this might be a factor in explaining the low fertilization and embryo development rates observed; for such reasons, sperm injection is the method usually employed to achieve fertilization in such oocytes.

At this moment, although a considerable body of evidence exists for the IVM of farm animal oocytes, especially in cattle, information on the human oocyte is much more limited (see Cha and Chian, 1998; Heikinheimo and Gibbons, 1998; Moor *et al.*, 1998; Russell, 1998; Canipari, 2000; Combelles *et al.*, 2002). Attempts by Jones and Trounson (1994) to apply IVM technology in clinical practice in Australia resulted in a disappointingly low pregnancy rate (less than 2%), which led workers in that country to acknowledge that there is a major problem of embryo developmental competence in human oocytes matured *in vitro* (Trounson *et al.*, 1998a). Authorities such as Edwards (1996) and Plachot (1999) have stressed the need for much more research to be directed towards the maturation and fertilization of human oocytes to increase the understanding of the basic biology of the human oocyte and early embryo. At the Wisconsin Primate Centre, Schramm and Bavister (1995, 1999) developed a non-human primate model (macaque monkey) to facilitate the formulation of successful strategies for improvements in the IVM of human oocytes. According to Smitz and Cortvrindt (1999), a prerequisite for improvement in human IVM must be a close interaction between reproductive clinicians and reseachers in the human and animal reproductive sciences; the field to be covered is large, including cell and tissue culture, microscopic techniques, cryobiology, fundamental endocrinology, molecular biology and molecular genetics.

In several laboratories, steps are gradually being taken towards developing successful IVM techniques. In Belgium, Soliman *et al.* (2001) found the presumed physiological concentrations of FSH and LH to be suboptimal for IVM of primary human oocytes and that a tenfold increase in these concentrations significantly enhanced both maturation and fertilization; they also found that changing the FSH : LH ratio from 1 : 7 to 1 : 2 dramatically decreased the success rate. In Denmark, Mikkelsen *et al.* (2000) found that shortening the IVM period from 36 to 28 h did not compromise subsequent embryo development but enabled insemination to be performed during the working day. Further studies in that country, this time by Mikkelsen and Lindenberg (2001b), evaluated the morphology of metaphase II oocytes obtained after IVM, using criteria previously employed with oocytes matured *in vivo*; half of the IVM oocytes were without anomalies and these gave rise to a significantly higher number of good-quality embryos.

Finally, because cattle and human oocytes are similar, there are ways in which advances in one species may be of interest to those working with the other. In that context, it is useful to take note of the work of Robert *et al.* (2001) in Canada, who have recently identified granulosa cell genes that may be used in the selection of developmentally competent bovine oocytes. In the human context, it may be possible to use gene markers in clinical practice to select the more promising oocytes for maturation.

1.7.6. Intracytoplasmic sperm injection (ICSI)

Defective sperm function remains the single most important cause of human infertility (Hull *et al.*, 1985). Although certain severe forms of male infertility have a genetic origin, others may be the result of environmental factors. During the past decade, ICSI has been applied increasingly around the world to alleviate problems of severe male infertility in human patients who either could not be assisted by conventional IVF procedures or could not be accepted for IVF because too few motile and morphologically normal sperm were present in the ejaculate of the male partner (Devroey *et al.*, 1998). There are those who believe that the development of ICSI has been the most significant assisted-reproduction advance of the 1990s. The first child born after sperm injection arrived in January 1992; by 2002, the number had grown to hundreds of thousands. It is now evident that ICSI can be carried out with fresh and frozen–thawed sperm, with epididymal sperm in patients with obstructive azoospermia and with testicular sperm in most patients with obstructive azoospermia and in some with non-obstructive azoospermia. Despite current limitations on embryo research in the EU (permissible only in Italy, Belgium and the UK) considerable progress has been made in refining the human ICSI technique as a result of the efforts of several formidable teams engaged in its clinical application.

One of the areas in which sperm injection can be useful is vasectomy reversal. Sterilization by vasectomy has been a highly effective method of controlling human fertility and simple to apply; reversing the process and restoring fertility is a different story. In Spain, Landeras *et al.* (1998) found ICSI to be an excellent option for vasectomized males with new parenthood wishes, because of the associated high pregnancy rates. The same authors note that, after achieving a pregnancy by ICSI, the couple do not require a new contraceptive method. According to De Croo *et al.* (1999), 3% of vasectomized men present for surgical reversal; results are best if reversal is done within 10 years. When vasovasostomy is unsuccessful, sperm injection in combination with the recovery of epididymal or testicular sperm is the only possibility for these men to father their own genetic children; whether a patient opts for sperm injection, however, may be more a matter of cost than convenience.

A further area in which sperm injection may be useful is in dealing with couples in which the male partner is human immunodeficiency virus (HIV)-positive. HIV-1-serodiscordant couples (seropositive male and seronegative female) cannot have children in the normal way, even when they are fertile and wish to become parents. As from the early 1990s, however, some clinics have provided for the washing of HIV-1-seropositive semen and the subsequent insemination of the female partner; hundreds of babies have been born as a result of such efforts. In Spain, Marina *et al.* (1998) reported the first pregnancy at their clinic in an HIV-1-serodiscordant couple; they suggest that sperm injection using just the one sperm could further reduce the risk of infection. An even lower risk would be possible if sperm could be safely heat-treated before injection; this is an area currently under investigation. The fact that sperm DNA is highly compacted and stabilized by protamines and disulphide bonds makes the sperm genome extremely resistant to temperature variations.

As with any new medical treatment, the early years of ICSI usage saw many questions raised, several of them yet to be fully resolved. Some questions related to the ethical and legal restrictions that apply in certain countries to the use of the technique. Unlike donor insemination (artificial insemination by donor (AID)), which many patients reject on religious, moral and/or emotional grounds, ICSI offers couples the possibility of having a child that is genetically related to both partners, even when the male is suffering from severe infertility. Sperm injection may be employed in circumstances that arouse strong ethical feelings, such as the use of ICSI in posthumous sperm retrieval. The fact that sperm injection circumvents all the sperm-selection processes that have developed in the evolution of fertilization mechanisms has been voiced as a matter of serious concern; few would disagree with the view that short-term success in the application of ICSI should in no way reduce the need for research to resolve many of the underlying problems of male infertility.

The great surge of interest in the application of ICSI in humans came in the early 1990s in the wake of reports by workers in Belgium (Palermo

et al., 1992; Van Steirteghem *et al.*, 1993, 1994). The Brussels work made it clear that fertilization rates comparable to those achieved in conventional IVF are possible with sperm injection and that this is true for epididymal and testicular sperm as well as for sperm obtained from ejaculates (see Table 1.12).

There is now a reasonable understanding of events that occur in the human oocyte after sperm injection (Dozortsev, 1996); this author set out a timetable of events, showing that sperm-associated oocyte-activating factor (SAOAF) release occurs within 30 min of injection and resumption of meiosis (early anaphase of the second meiotic division) is observed some 2–3 h after injection. Two to three hours later, the late anaphase stage is reached and sperm and oocyte chromosomes become indistinguishable from each other; the second polar body is extruded at this time. Shortly afterwards, the male and female chromatin undergoes further decondensation to develop into male and female pronuclei.

Factors influencing the technical efficiency of human ICSI are now well established. From the many studies reported to date, no clear correlation has emerged between the outcome of sperm injection and any of the normal male fertility parameters (e.g. motility, morphology, functional sperm measurements).

1.7.7. Early embryo culture

In cattle, ET is routinely performed at the 7-day-old blastocyst stage of development. In contrast, current practice in conventional human IVF patients is to transfer embryos on the second day after oocyte retrieval, when embryos are at the two- to four-cell stage. However, there are those who believe that there are advantages in culturing the human embryo to the blastocyst stage before transfer, regardless of whether fertilization has been achieved by IVF or by ICSI. Among the culture systems employed have been those based on human oviductal cells (Wiemer *et al.*, 1994), while a more practical co-culture system has used an established cell line, such as Vero cells (Sakkas *et al.*, 1994; Schillaci *et al.*, 1994). According to workers such as Gardner and Lane (1998) and Menezo *et al.* (1999), using a single culture medium from fertilization to blastocyst is obsolete: the requirement is now for sequential media, which can be used to culture the pronucleate embryo to the blastocyst stage at acceptable frequencies, providing embryos capable of establishing a high pregnancy rate and reducing the need for the transfer of multiple embryos.

In employing sequential media, fertilization can be carried out in a simple culture medium, such as Earle's salts with serum albumin; after fertilization, embryo development follows a biphasic path. Prior to genomic activation, the embryo operates on its own stores with very weak transcription activity. During the first phase, which takes the zygote to the eight-cell stage, a simple culture medium with or without amino acids but with low concentrations of glucose and phosphate is recommended. The concentrations of glucose, lactate and pyruvate in G1 and G2 were derived from measurements

Table 1.12. Outcome of embryo transfers after ICSI in relation to origin of sperm (from Van Steirteghem *et al.*, 1996).

	Ejaculated	Epididymal	Testicular
Cycles	2572	128	120
Transfers (%)	2382 (93)	117 (91)	108 (90)
Pregnancies per transfer (%)			
1 embryo	21/204 (10)	1/7 (14)	2/14 (14)
2 embryos	78/321 (24)	3/15 (20)	4/13 (32)
2 embryos (elective)	218/488 (45)	3/14 (21)	3/12 (25)
3 embryos	253/694 (36)	17/37 (46)	10/21 (48)
3 embryos (elective)	254/544 (47)	17/24 (71)	11/20 (55)
> 3 embryos	47/131 (36)	9/20 (45)	13/28 (46)
Total	871/2382 (37)	50/117 (43)	43/108 (40)
Pregnancies per cycle (%)	34	39	36

made of the concentrations of these metabolites in human reproductive tract fluid, both tubal and uterine at various times during the menstrual cycle.

The amino acid composition of each medium is based on extensive experimentation using a mouse-model system. The G1 medium is based on the concentrations of carbohydrates present in the human oviduct at the time when the cleavage-stage embryo is present; the medium also contains those amino acids which have been shown to stimulate development of the cleavage-stage embryo (i.e. the non-essential amino acids and glutamine). The chelating agent, ethylenediamine tetra-acetic acid (EDTA), is used in the G1 medium, not only to sequester any toxic divalent cations present but also to assist in minimizing the glycolytic activity of the embryo, which should reduce the risk of metabolic abnormalities. The G2 medium is based on the levels of carbohydrates present in the human uterus and contains both non-essential and essential amino acids to facilitate both blastocyst development and differentiation. Both G1 and G2 are supplemented with serum albumin.

Although there are those who believe that one of the major barriers to extending human embryo culture beyond 2–3 days has been overcome by the use of sequential media, there are others who observe that the overall proportion of newly fertilized embryos capable of implantation remains at less than 30%, whether embryo selection is made early or late (Edwards and Beard, 1999). There is the view that, in opting for blastocyst transfers, viable embryos are simply being identified at a stage when their weaker siblings have died or become arrested. It should be possible, using the polarity of the oocyte and pronuclei and new information about cleavage symmetry, to gain the same information about viability without the need for extended culture (Behr, 1999). In the meantime, however, the use of blastocyst culture may provide a non-invasive means of identifying abnormal embryos, thereby reducing the risk of an errant paternal genome being inherited by ICSI offspring (Sakkas, 1999). Looking further ahead, the next generation of embryo culture media are likely to be protein-free, using macromolecules such as hyaluronate as a physiological alternative to serum albumin; dealing with blastocysts rather than earlier-stage embryos would also permit the use of non-invasive methods of assessing embryo metabolism prior to transfer.

1.7.8. Assessing embryo quality

Various attempts have been made by workers in human IVF to devise appropriate embryo scoring systems that may be used in the quality assessment of embryos after fertilization. Studies reported by Saith *et al.* (1998) examined predictive relationships between embryo-related features and development; it was suggested that such data could help in the selection of embryos for transfer. The Oxford workers found that only four of the 53 features examined were helpful in predicting the outcome of ET; the key component of the composite embryo grading, which combined embryo grade, cell number, follicle size and follicular fluid volume, was cell number.

One of the morphological criteria used in selecting embryos for transfer is the absence of multinucleated blastomeres; such blastomeres are usually arrested cells and embryos with one or more are expected to be developmentally incompetent. Multinuclearity occurs at the start of embryo development and relatively frequently at the two-cell stage (Joris *et al.*, 1997). In a Belgian study that evaluated the impact of transferring multinucleated embryos on pregnancy rates, Pelinck *et al.* (1998) found that rates were lower than those found with mononucleated embryos, even after correction for age and embryo quality. The conclusion was that, wherever possible, embryos with multinucleated blastomeres should be avoided for transfer.

In Germany, the present law prevents embryo selection, although it does permit selection at the pronuclear stage. In that country, Ludwig *et al.* (2000b) applied a pronuclear-stage scoring system and evaluated its impact on the pregnancy rate; they concluded that such a scoring system might help in offering patients in Germany the transfer of two selected pronuclear oocytes, which would reduce the multiple pregnancy rate. The same authors noted that the practice of blastocyst transfer would have little appeal under the German embryo protection law, since it would almost inevitably reveal morphological differences among embryos that would invite selection. In another paper, Ludwig

et al. (2000a) performed a retrospective data analysis to investigate the possibility of reducing the incidence of multiple births without decreasing the overall pregnancy rate; the elective transfer of two embryos, with selection at the pronuclear stage, helped most patients to avoid high-order multiples. In Jordan, Karaki *et al.* (2002) reported that significantly fewer embryos were required for transfer at the blastocyst stage compared with day-3 embryo transfer; the authors concluded that blastocyst culture could be the means of reducing the incidence of multiple pregnancies and improving the outcome of IVF.

In France, Tesarik *et al.* (2000) found that the inclusion of criteria based on pronuclear morphology made it possible to apply a single-embryo transfer policy and optimize the selection of embryos for transfer and freezing. The morphological parameters evaluated in the French study included the number of nucleolar precursor bodies and their distribution in each pronucleus (polarized vs. non-polarized). It was found that cleaving embryos developing from zygotes with normal morphology at the pronuclear stage had a better implantation potential than those developing from zygotes showing abnormal morphology, even when the cleavage rate and morphology of embryos selected for transfer were the same. There are also those who believe that embryo evaluation based on metabolism rather than morphology and cleavage stage would be better predictors of embryo viability. In the USA, Fisch *et al.* (2001) developed the graduated embryo score (GES); this involved evaluating embryos at 25–27 h post-insemination for dissolution of the pronuclear membrane, blastomere cleavage, symmetry and degree of fragmentation and at 40–43 h for blastomere number, symmetry and percentage fragmentation. A final evaluation was carried out at 64–67 h to generate the score, which was out of a possible 200 points; embryos scoring 90–100 had 44% blastocyst formation compared with 31% scoring 70–85 and 11% scoring 30–65. The authors concluded that it should be possible to achieve the pregnancy and implantation rates associated with blastocyst transfer using cleavage-stage embryos selected on the basis of their score.

In human IVF clinics, it may be noted that most have microscopes linked to cameras and video screens; this permits comparisons in the grading of embryos between embryologists as well as comparisons in grading standards between clinics. The use of time-lapse photography allows human development to be followed in detail over many days; film cameras usually require considerably less light than video cameras and are especially useful for prolonged exposure (Hamberger *et al.*, 1998).

1.7.9. Cryopreservation of embryos and oocytes

The freezing of mammalian embryos was first shown to be possible in 1972, when David Whittingham and colleagues in London reported live births in mice after the transfer of frozen–thawed embryos. Cattle embryos were also among the first mammalian embryos to be successfully frozen and preserved in liquid nitrogen; countless thousands of normal healthy calves have been born worldwide as testimony to the value of cryopreservation as part of modern cattle ET technology. The first pregnancy from frozen–thawed human embryos was reported from Australia in 1983 by Allan Trounson. Results from the freezing of human embryos over a 10-year period have been provided by Mandelbaum *et al.* (1998); a historical review of the cryopreservation of cells and tissues relevant to human reproduction has been provided by Ludwig *et al.* (1999).

The stage of embryo development favoured for storage and the methods used in cryopreservation vary from clinic to clinic (Wood, 1997). Most reports deal with a slow, controlled cooling procedure. In terms of cryoprotectants employed, pronucleate or early-stage embryos are generally frozen in a medium containing 1, 2-propanediol; dimethylsulphoxide (DMSO) is an alternative cryoprotectant for cleavage-stage embryos and glycerol is used in the freezing of blastocysts. In Belgium, Van den Abbeel and Van Steirteghem (2000) quantified zona pellucida damage in cryopreserved human embryos; they found that sperm injection did not make embryos more susceptible to damage. They note that the introduction of plastic mini-straws as storage containers in their clinical practice had resulted in a clear improvement in the cryopreservation outcome.

The potential importance of vitrification as a method of preserving human embryos has been reviewed by Liebermann *et al.* (2002), who note that the method has been considered largely experimental as yet in human IVF clinics. They draw attention to inconsistent embryo survival rates and the wide variety of solutions and vessels used in vitrification; the authors see a need to focus attention on perfecting a single approach to vitrification.

Oocyte preservation

As noted by Whittingham (1996), the cryopreservation of the human oocyte would obviate many of the moral and ethical dilemmas associated with the storage of human embryos. It could also be valuable in facilitating the long-term storage of oocytes for patients in danger of losing ovarian function. Already, moves are afoot in several countries to provide an oocyte banking service. It has to be accepted, however, that oocyte cryopreservation is difficult, both in terms of survival after thawing and in terms of subsequent fertilization rates with IVF or ICSI (Coticchio *et al.*, 2001; Wininger and Kort, 2002). As yet, the cryopreservation of human oocytes by conventional freeze–thawing procedures has been very inefficient, with only about one baby born for every 100 oocytes thawed out. An alternative to conventional freeze–thawing is vitrification. Vitrification can only be achieved using very high, often embryotoxic, levels of cryoprotectants. However, vitrification is thought to be less harmful to oocytes than classical slow-freezing techniques (Liebermann and Tucker, 2002).

A major step forward was the work of Ali and Shelton (1993), who undertook a systematic examination of more than 6000 cryoprotectant solutions before they identified one consisting of 5.5 mol/l ethylene glycol and 1.0 mol/l sucrose; this solution was employed to good effect in mouse and sheep embryos. The same solution was used by Chen *et al.* (2000a) in Taiwan to vitrify mature human oocytes using conventional straws, with the achievement of a high morphological survival rate, fertilization and early cleavage; using Vajda's OPS method with mature mouse oocytes, Chen *et al.* (2000b) found that meiotic spindles and chromosomes were preserved better than in conventional straws. Elsewhere, some success was reported by Kuleshova *et al.* (1999); one baby was born from 11 oocytes thawed after vitrification using Vajta's OPS method.

One possible disadvantage of some vitrification methods may lie in the direct contact of the oocyte (or embryo) with liquid nitrogen contaminated with virus and other microorganisms. Although there are those who suggest that contamination may be prevented by adopting appropriate sterility precautions and cooling OPS straws in filtered liquid nitrogen (Vajta *et al.*, 1998d; Lane *et al.*, 1999a,b,c), such filtering may not be capable of dealing with viruses. In human assisted reproduction, although many articles have been written about human sperm or oocyte/embryo cryopreservation, one issue that obviously deserves close attention is that of microbiological cross-contamination in a liquid-nitrogen storage unit (Clarke, 1999; Rall, 2001); there are certainly those who argue that the use of containers that cannot be hermetically sealed may place cryopreserved material at risk of cross-contamination via liquid nitrogen. As noted by McLaughlin (2002), the advent of HIV and the serious nature of the sequelae posed new problems in the use of AI in human assisted reproduction and in the storage of semen.

1.7.10. Gender preselection

Gender preselection has considerable implications, for both animal and human reproduction. Although many attempts have been made to separate X- and Y-chromosome-bearing sperm, very few of these have been repeatable. One that is repeatable, and which has already been put to practical use on the farm, is cell sorting by way of flow cytometry. On the human front, a paper by Fugger *et al.* (1998) described the first clinical trials and births resulting from the use of the Beltsville sexing technology (MicroSort), followed by intrauterine insemination (IUI), IVF or ICSI; most (93%) of the births were of the requested female gender. Although the Virginian researchers note the concern expressed by some that somatic cells are sensitive to the Hoechst 33342 fluorescent dye, they argue that this does not appply to germ cells whose DNA is

compacted and stabilized. A further report by Fugger *et al.* (1999) draws attention to the absence of evidence of Hoechst mutagenicity in sperm and notes the birth of 32 normal and healthy babies after sperm sorting; a review by Fugger (1999) provides a detailed account of the Virginian clinical experiences in sexing. Regardless of the technical efficiency of separating human sperm by flow cytometry, sight should not be lost of the cost factor, which may well place it beyond the reach of many.

For such reasons, some continue to explore other avenues. A study by Bartsich *et al.* (2001) made use of a static density gradient to select sex-specific sperm; to avoid sperm motility as a confounding factor, the gametes were temporarily immobilized for 15 min by way of lysophosphatidylcholine before exposure to a six-layer Percoll density gradient. Fractions collected from the top layer of the gradient revealed a 62% concentration of Y-bearing sperm versus 38% of X-bearing gametes; sperm from the bottom layer showed a preponderance of X-bearing cells (58.5%). The authors found no difference in the X : Y ratio of untreated sperm and suggest that this minimally invasive technique may be developed into an effective sexing procedure for humans.

2

The Bovine Oestrous Cycle and Associated Events

2.1. Oestrus and the Oestrous Cycle

The development of effective embryo production and transfer procedures in cattle demands accurate information on all aspects of follicular development in the live animal as well as a thorough understanding of the key endocrine events associated with the cycle and ovarian function.

2.1.1. Oestrus

The bovine oestrous cycle averages 21 days in cows and 20 days in heifers, with a normal range of 18–24 days. Oestrus is the period of sexual receptivity in the cow and is characterized by her willingness to stand when mounted by a bull or companion cow; she indicates this willingness by immobility when approached and a slight arching of the back (see Fig. 2.1).

The duration of oestrus, as reported in the literature, varies considerably, probably the result of different criteria employed in defining oestrus, but 12–16 h may be taken as an average duration; ovulation usually occurs some 10–12 h after the end of the heat period. An oestrous cow will attempt to mount companion animals but, unless they are themselves in oestrus, they will move away. Cows in heat can

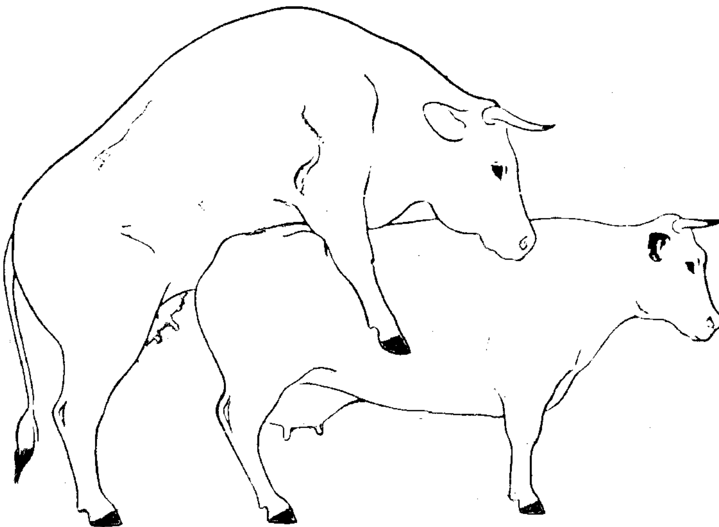

Fig. 2.1. Standing heat in the heifer and cow.

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be expected to display a variety of signs, many of which are detailed in Table 2.1.

Detecting oestrus requires keen eyesight, time, patience and familiarity with cattle behaviour (cow sense); the latter requirement may not always be in evidence with an increasing proportion of workers coming from an urban rather than a rural background.

Some workers have drawn attention to breaks or quiescent interludes in standing activity that have been observed; Stevenson *et al.* (1996) in the USA, using radiotelemetry, recorded breaks averaging 2.6 h in 67% of beef heifers. In Ireland, observations in dairy cows have shown that the onset of heat activity follows a definite pattern, with greatest activity in the early morning and late evening (Diskin and Sreenan, 2000a,b). The Irish workers concluded that careful checking for heat in the early morning and late evening would detect at least 70% of oestrous cows; three further checks during the day, at about 4–5 h intervals are required to detect 90% of animals in heat. A scoring system, based on the expression of certain behaviours, has been used by workers in the Netherlands (Lyimo *et al.*, 2000); this is set out in Table 2.2. A review of sexual behaviour and physiological events associated with oestrus in the cow has been provided by Stevenson (2001). For those dealing with zebu cattle, Galina *et al.* (1996) have provided information relevant to heat detection in that species (*Bos indicus*).

Table 2.2. Scoring system for heat detection – based on behavioural signs. The scoring system is cumulative: each time a symptom is observed the number of points is added to the total. (From Lyimo *et al.*, 2000.)

Symptoms of oestrus	Score
Mucous vaginal discharge	3
Cajoling	3
Restlessness	5
Sniffing the vagina of other cow	10
Chin resting	15
Mounted but not standing	10
Mounting (or attempt) other cows	35
Mounting head side of other cow	45
Standing heat	100

Table 2.1. Detecting the cow in heat – signs to observe. Experience shows that more than 50% of all cows come into heat during night-time when they are usually not under observation. This means that stockmen have to rely on a variety of signs and symptoms that are evident when animals are observed. The average cow comes into heat every 21–22 days and remains in heat for about 18 h, although the range is quoted as 6–36 h.

	What the cow is doing 18 h heat period		
What you see	Early	Middle	Late
Riding others	Mounts other cows	Stands to be ridden, but will still mount others	Will not stand to be ridden, but will mount others
Behaviour	Bawling, fence walking, 'spooky', butts others, trails other cows, very nervous	Complacent, friendly, still trails and licks others, does not eat, still restless	All signs of nervousness, trailing, head butting disappearing
External genitalia	Lips of vulva red and slightly swollen	Lips of vulva red and swollen, walls of vagina moist and glistening	Swelling decreased
Mucus	Very little and very watery, will note quantity when mounts others	Abundant and clear, copious in amount	Decreased in amount and very sticky and rubbery in consistency
Bloody discharge	None	Seldom	1–3 days after all signs of riding ceased
Tail and head ruffled and matted	No, but may be on cows mounted by animal coming in heat	Slightly to very observable	Most pronounced at any time

2.1.2. Expression of heat

Numerous factors may affect the detection of heat in cattle, including the way in which the animal expresses its oestrous symptoms. Detection may be a question of inclement weather, domination by other cattle or lack of interest by other cows, especially if none of them are in the vicinity at, or near, the occurrence of oestrus. Companion cows that are approaching oestrus or are in oestrus mount oestrous cows at a much higher frequency than herd-mates that have gone out of heat or those that are in the luteal phase of the cycle. It is important that cows are housed together with sufficient space to allow cow–cow interaction and for the group size to be large enough to have two animals in or near oestrus each day. If the stocking density is too high, on the other hand, the expression of oestrus is likely to be reduced, making detection difficult (Diskin and Sreenan, 2000a,b). As well as housing arrangements, there is ample evidence to show that factors such as milk yield, floor surface, foot and leg problems and the reproductive status of companion animals can all influence the expression of oestrus. Although there are those who attribute low oestrus detection rates to the farmer rather than the cow, there have also been those who have reported a low intensity of oestrous behaviour in high-yielding dairy cattle.

2.1.3. Aids to heat detection

Considerable thought has been given over the years to developing devices and methods that can be used to improve the efficiency of heat detection in cows; the variable symptoms of heat and the fact that many animals show oestrus in the hours of darkness, while others show heat of very short duration, are among the reasons for such work. Some of the techniques employed in heat detection are set out in Fig. 2.2. As observed by Senger (1994), techniques for improving heat detection need: (i) to provide for continuous surveillance of the cow; (ii) to ensure accurate and automatic identification of oestrous animals; (iii) to operate throughout the productive lifespan of the cow; (iv) to involve minimal labour costs; and (v) to be very accurate in identifying the appropriate physiological and behavioural events associated with ovulation.

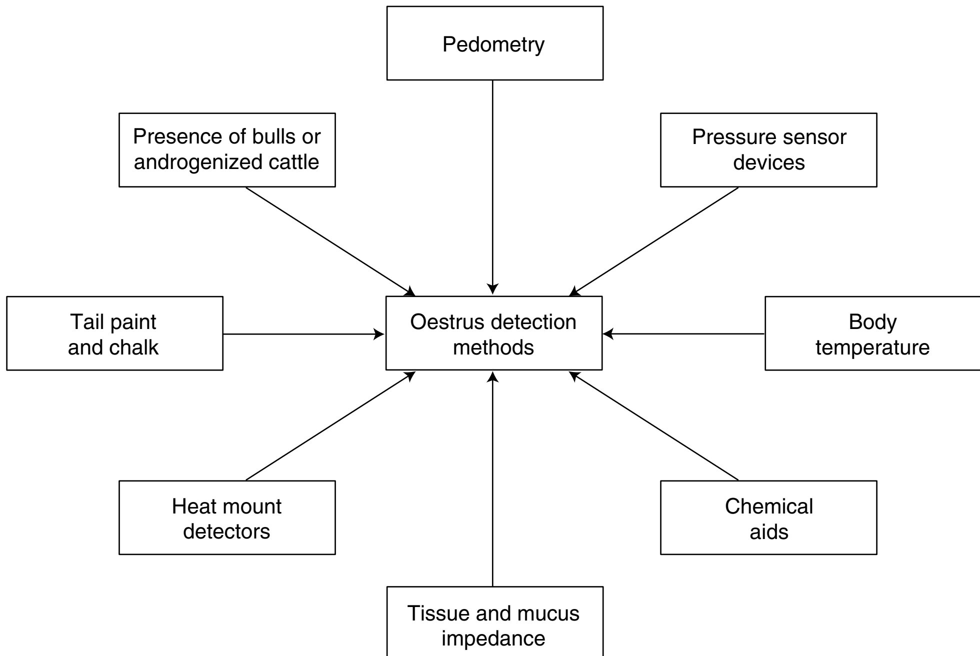


Fig. 2.2. Aids to oestrus detection in the cow.

The accuracy of detecting oestrus in cattle has been investigated using progesterone concentrations in milk in several countries; a study in Northern Ireland suggested that about 20% of cows put forward for AI may not have been in heat (White and Sheldon, 2001).

The need to develop and apply new technology for oestrus detection in dairy cattle has been emphasized in numerous reports. For dairy cows that are in and out of the milking parlour, there is the prospect of heat detection by the online measurement of progesterone. In the USA, Delwiche *et al.* (2000) reported on a biosensor that could measure milk progesterone at concentrations low enough to distinguish luteal-phase from follicular-phase animals. Such systems, using automated biosensors, may be a valuable aid in providing progesterone profiles of post-partum cows as well as cyclic animals. Research at Nottingham has shown that atypical progesterone profiles can be used to identify subfertile post-partum dairy cows (Lamming and Darwash, 1995; Lamming *et al.*, 1998).

Physical activity and vulvar impedance are regarded as being among the most promising variables for incorporation into an on-farm management system. In the age of the microchip and increasingly sophisticated electronic technology, it seems likely that miniature sensing devices implanted subdermally in the animal to detect changes in impedance, temperature or activity at oestrus may become a practical reality in the years ahead; coupled to new electronic devices for identifying cows, there is considerable scope for developments in this area. The application of electronic pressure-sensing detection methods has been reviewed by Saumande (2000) in France; it was noted that these devices do not detect all situations in which cows are in heat and some problems may be encountered with the fixation of the device on the rump. In New Zealand, Verkerk *et al.* (2001) used the Cow Trakker motion detector device attached to dairy cattle; although a useful aid to heat detection, the authors note that it must be used in conjunction with careful behavioural observations if the high proportion of false positive events is to be correctly interpreted. In the USA, Rorie *et al.* (2002) noted that pedometers are most applicable to milking cows and have greater accuracy and efficiency when combined with visual observation; intravaginal resistance measurement was held to be the least practical method because of labour and animal-handling requirements. The only real-time radiotelometric system available to them (HeatWatch, Ddx Inc.) they found required the least labour and animal handling and provided data on the time and duration of each mount. As observed by Robinson (2001), it is inevitable that future years will see greater reliance placed on automated oestrus detection and computerized recording systems.

2.1.4. Endocrine basis of oestrus

It is ovarian oestradiol acting on receptor cells in the hypothalamus that is responsible for the behavioural symptoms of oestrus. As shown in the review of Allrich (1994), the effect of oestradiol appears to be an all-or-none phenomenon; once a threshold concentration of oestradiol is reached, oestrus is induced and additional amounts of oestradiol above that threshold do not change the duration and intensity of oestrus. Prior exposure to progesterone is not required for the oestrus-inducing action of oestradiol to be achieved, other than in the early period after calving in the cow. In such post-partum cows, where a 'silent heat' (ovulation unaccompanied by oestrus) may occur, high concentrations of oestrogen during late pregnancy apparently induce a refractory state such that the brain cannot respond to the oestrus-inducing actions of oestradiol at the first ovulation after calving. In such circumstances, progesterone can sensitize receptors in the cow's brain so that the animal becomes capable of responding to subsequent oestradiol production by the preovulatory follicle; in the post-partum cow, the corpus luteum (CL) formed after the first ovulation would be the usual source of this progesterone.

Synthesis of oestradiol requires the coordinated activities of two ovarian cell types and the two gonadotrophins, follicle-stimulating hormone and luteinizing hormone (FSH and LH) (see Fig. 2.3) It is known that bovine granulosa cells are capable of producing oestradiol only when provided with an aromatizable substrate; thecal cells are the source of androgen in the follicles and androgen secretion is increased by LH, but not by FSH. In summary, a two-cell, two-gonadotrophin model for the control of

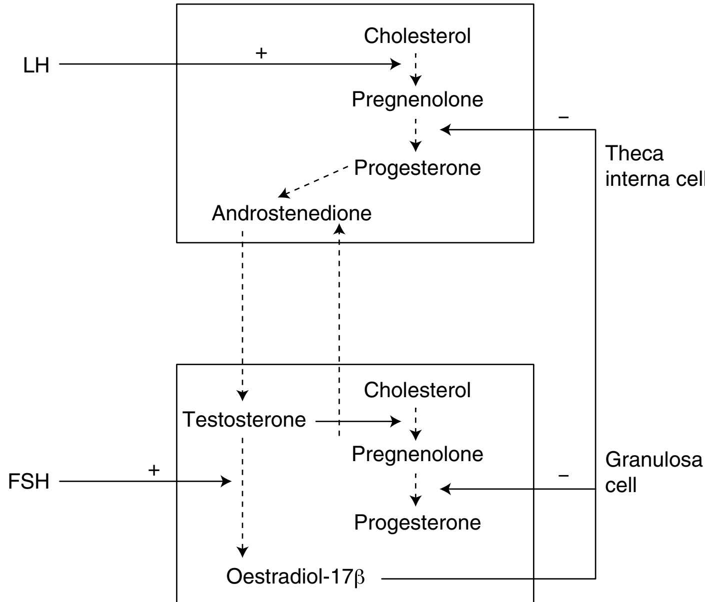


Fig. 2.3. Control of steroidogenesis in preovulatory follicles.

follicular steroidogenesis has evolved; androgens produced in thecal cells under the influence of LH are aromatized to oestradiol in the granulosa cells under the influence of FSH.

2.2. The Oestrous Cycle

The various hormonal interactions and other events that occur during the bovine oestrous cycle, involving gonadotrophin-releasing hormone (GH-RH), gonadotrophins (FSH, LH and prolactin), ovarian steroid/peptide hormones (oestradiol, progesterone, inhibin) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ of uterine origin are shown diagrammatically in Fig. 2.4. In the period prior to oestrus (pro-oestrus), gonadotrophins induce the final maturation of the preovulatory follicle, resulting in increased secretion of oestradiol; this oestrogen, in the relative absence of progesterone, acts on receptors in the brain to induce sexual receptivity and triggers the release of LH.

2.2.1. Corpus luteum and progesterone

The preovulatory LH surge brings about the rupture of the follicle destined for ovulation and the formation of the CL (yellow body); a comprehensive compilation of information on the integrative physiology of the bovine CL was provided by Hansel and others in the mid-1990s (Beal, 1996; Davis, J.S. *et al.*, 1996; Fields and Fields, 1996; Hansel and Blair, 1996; Milvae *et al.*, 1996). The CL forms after follicle rupture from cells of the granulosa and theca interna layers of the ovarian follicle (Fig. 2.5); these are believed to differentiate into the large and small luteal cells, respectively.

The large cells secrete progesterone and oxytocin and are responsive to prostaglandin E, whereas the small cells secrete progesterone and are responsive to LH. The small theca-derived cells are more numerous than the large cells, which are at least partly derived from granulosa cells; it is believed that the two hormones which principally regulate luteal function (LH and $PGF_{2\alpha}$) act on the two cell types through different second-messenger pathways. Available evidence suggests that LH has an important role in establishing a fully functional CL in the cow but is not required to maintain its function (Peters *et al.*, 1994). Morphological and ultrastructural characteristics of the bovine CL during the oestrous cycle and pregnancy have been reviewed by Fields and Fields (1996); the CL grows in 11 days to weigh ~4 g in the beef cow and is even heavier in

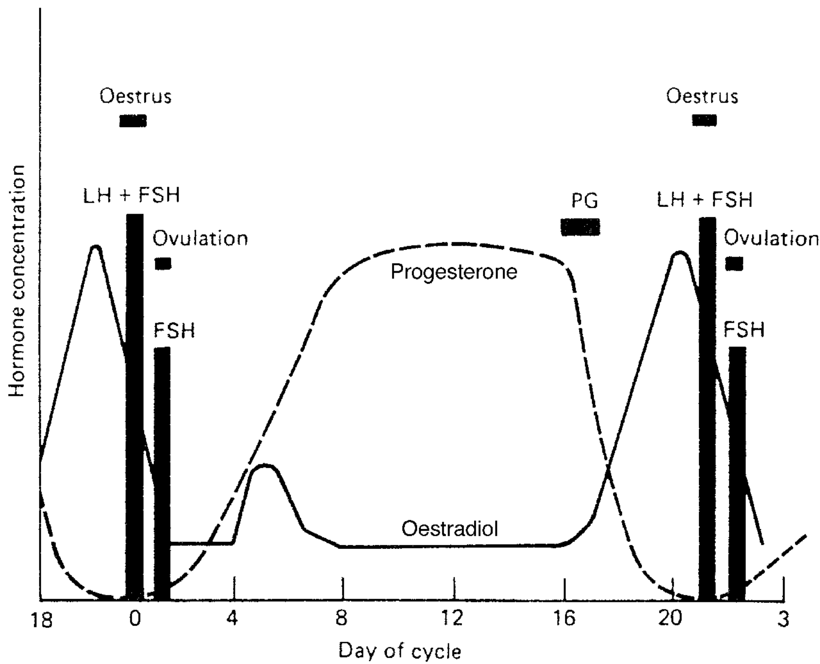


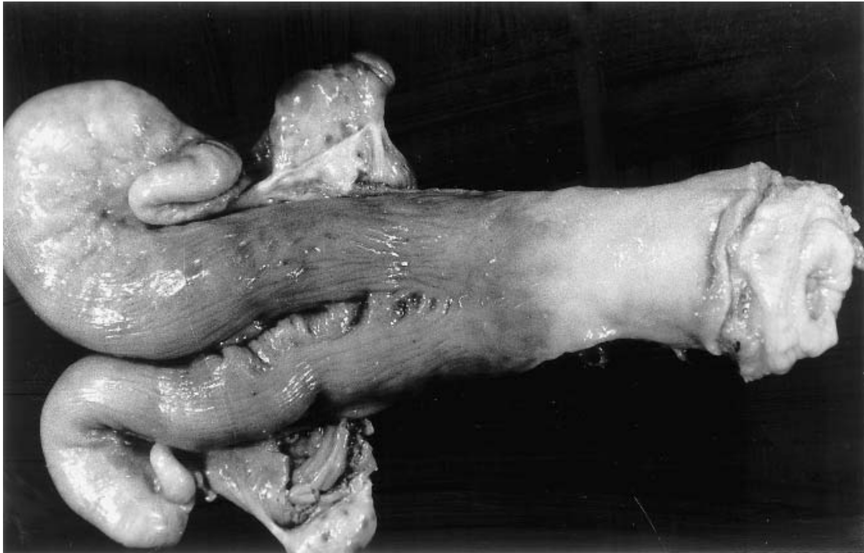
Fig. 2.4. Changing hormone concentrations during the bovine oestrous cycle (after Peters, 1985).

the dairy cow (see Table 2.3). The rapid growth of the yellow body in the first part of the cycle (i.e. from ovulation until about day 10) is illustrated in the fact that tissue mass and cell numbers - double every 72 h (Reynolds and Redmer, 1998).

The steroid hormone progesterone dominates the major part of the cow's oestrous cycle, detectable amounts being evident in the circulation 3–4 days after formation of the CL; blood concentrations of progesterone rise markedly for several days until a plateau is reached by about day 8 of the cycle. The concentration of progesterone in peripheral blood is low around the time of oestrus; the fact that it takes several days before a rise in blood level is evident may suggest that, during this transient phase, the luteal tissue does not achieve functional significance. It is only during the early part of the cycle that the developing CL of the cow is susceptible to breakdown after oxytocin treatment; it is during this same period that the yellow body is refractory to doses of $PGF_{2\alpha}$. It is also evident that the administration of progesterone during early and late metoestrus can reduce the diameter of CLs (Burke *et al.*, 1994).

Rapid regression of the cow's CL is a key event in the bovine oestrous cycle; this is responsible for the precipitous decrease in the blood concentration of progesterone at the end of the cycle, some 1–4 days prior to the onset of the heat period. Within the space of about 2 days, the progesterone level drops to a negligible value, which holds good throughout oestrus and until a fresh CL forms at the next ovulation; apoptosis is known to be responsible for this luteal regression (Zheng *et al.*, 1994), presumably triggered by hormonal changes. The time interval between progesterone reaching its minimum level and the onset of oestrus is believed to be influenced by various factors, including the presence of a dominant follicle, body condition, stress, season and probably lactation and nutrition. It is well established that $PGF_{2\alpha}$ released from the endometrium during the late luteal phase is responsible for luteolysis and the consequent dramatic fall in progesterone concentration that prepares the scene for a new ovulation. The prostaglandin release mechanism is suppressed if mating occurs and the cow becomes pregnant.

The release of $PGF_{2\alpha}$ during the cycle is stimulated by luteal oxytocin after binding to a specific receptor on the endometrial cell; oxytocin synthesis in the bovine CL is apparently achieved in exactly the same way as in the



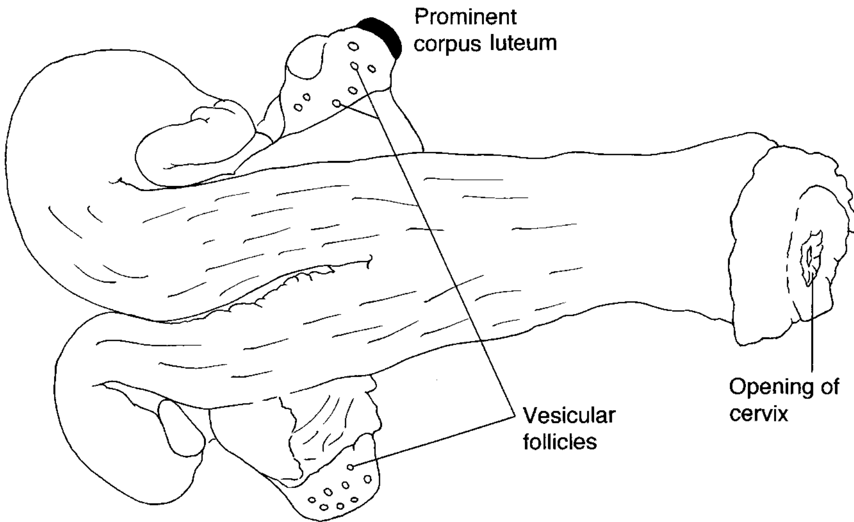


Fig. 2.5. The bovine corpus luteum.

Table 2.3. Least-square means of corpus luteum weights and progesterone levels on days 3–19 of the natural oestrous cycle[a] (from Fields and Fields, 1996).

Day (*n*)[b]	3 (15)	7 (23)	11 (16)	14 (20)	17 (19)	19 (14)
CL[c]	0.64 ± 0.34	3.56 ± 0.28	4.08 ± 0.33	5.07 ± 0.30	4.03 ± 0.30	3.23 ± 0.36
CL	0.47	3.34	4.25	4.70	3.72	1.77
P_4	1.11 ± 0.63	3.91 ± 0.65	5.08 ± 0.71	6.84 ± 0.61	5.05 ± 0.83	2.83 ± 0.68

[a]Cows were primarily of Angus and Hereford breeding from the USDA Research Station, Brooksville, Florida.
[b]Number of CLs.
[c]Weights for CLs.

hypothalamus. The formation of oxytocin receptors in the cow's endometrium is essential for the synthesis of $PGF_{2\alpha}$; the formation of these receptors is apparently dependent on oestradiol of follicular origin. The wave of follicular growth that occurs between days 12 and 15 of the oestrous cycle would appear to be the source of this oestradiol; circulating oxytocin of luteal origin binds to oxytocin receptors in the endometrium and initiates prostaglandin synthesis and release. $PGF_{2\alpha}$ acts locally on the CL to reduce progesterone production and to release more oxytocin, which stimulates the further release of prostaglandin, thereby establishing a positive feedback loop between the ovary and the uterus; this results in a decline in progesterone concentrations to the baseline value in about 24 h.

2.2.2. Follicular dynamics in the cow

A sound understanding of the processes involved in the growth and differentiation of antral follicles destined for ovulation in the cow is essential for those working in the artificial maturation of bovine oocytes; greater knowledge of factors controlling follicle waves will also help in developing improved hormonal regimens to control oestrus in the cow. In a review by Fortune (1994), the author noted that there are probably two different patterns of development of large antral follicles in mammals (see Fig. 2.6). In one pattern, as shown in humans, pigs and rats, the development of ovulatory-size follicles is suppressed except during the follicular phase of the cycle. In the other pattern, as shown by cattle, sheep and horses, development of follicles to ovulatory or near-ovulatory size is not confined to the follicular phase but occurs throughout the cycle.

Growing understanding of folliculogenesis

Factors affecting folliculogenesis and follicular dynamics in the cow and other monovular species have been addressed in numerous reviews

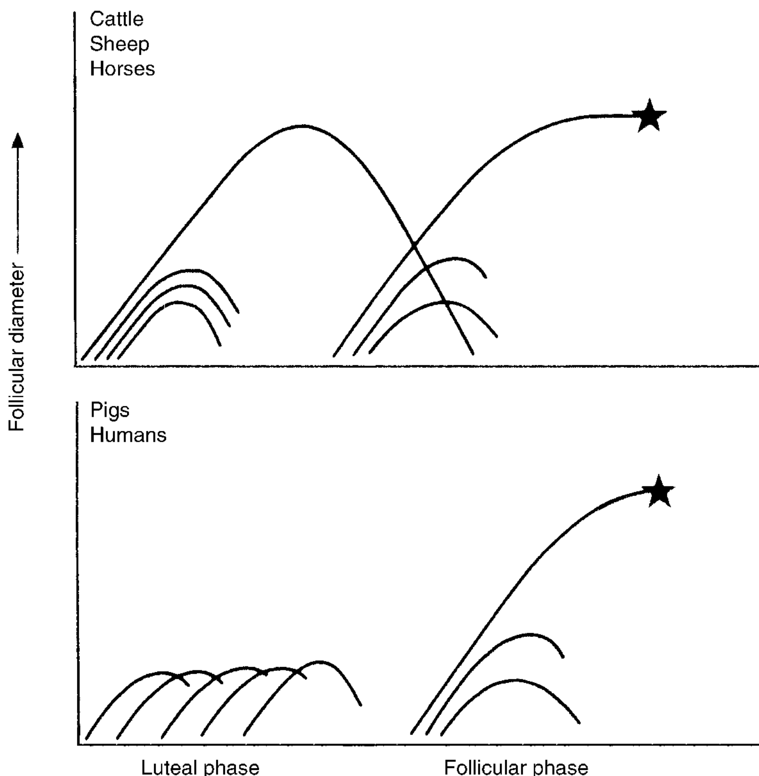


Fig. 2.6. Schematic diagrams of two patterns of follicular development.

and research reports in recent years (Adams, 1994, 1999; Mihm *et al.*, 1996, 1999, 2000; Roche, 1996; Kinoshita *et al.*, 1998, 1999; O'Rourke *et al.*, 1998; Webb and Armstrong, 1998; Austin *et al.*, 1999, 2001; Crowe, 1999; Roche *et al.*, 1999; Webb *et al.*, 1999; Bergfelt *et al.*, 2000; Duffy *et al.*, 2000; Ginther, 2000; Ginther *et al.*, 2001a,b,c; Hanzen *et al.*, 2000; Ireland *et al.*, 2000; Driancourt, 2001; Fortune *et al.*, 2001; Jimenez-Krassel *et al.*, 2001; Kulick *et al.*, 2001; Rhodes *et al.*, 2001; Rivera and Fortune, 2001; Rivera *et al.*, 2001; Zeleznik, 2001; Beg *et al.*, 2002). An understanding of normal folliculogenesis in the cow has increased as a result of all these efforts; the growth of follicles is no longer regarded as a random phenomenon but is considered to be part of a well-organized and predictable series of events. It may be noted, however, that the story of bovine ovarian follicles developing in waves goes back more than 40 years, to the time of Cupps and associates in California and Erikki Rajokoski in Finland; it is still a matter of speculation as to why the bovine ovary generates follicle waves. Driancourt (2001) suggests that such waves, at least during the luteal phase of the cycle, may be a prerequisite for luteolysis to occur. Oestradiol is involved in the luteolytic cascade in ruminants and the wave mechanism may be the means of generating the required concentration of oestradiol for luteolysis. The wave mechanism may be seen as preparing the way for the ovulation of a new follicle after luteolysis.

In terms of gaining knowledge of follicular dynamics, Fortune (2001) notes that a monotocous species such as the cow, which has fairly large preovulatory follicles, is particularly useful for studying mechanisms of follicular selection and dominance. The cow is large enough to allow: (i) sequential assessments of hormonal changes during the cycle; (ii) ultrasonographic imaging of the ovaries to trace follicular growth and regression in the live animal; and (iii) endocrine, biochemical and molecular analyses of follicular fluids and cells collected from individual follicles at all stages of the cycle There are some who note that intraovarian processes have received much less attention from researchers than endocrine changes, probably because they are less amenable to investigation (Baker and Spears, 1999).

Follicle waves occur during pregnancy, in the post-partum period and in the prepubertal animal; they can also be seen under conditions of prolonged progesterone administration. This has practical implications for those engaged in embryo production, since it is possible to aspirate the follicles of animals that are not showing regular oestrous cycles and to recover immature oocytes. Repeated scans of the ovaries of calves or heifers between 2 and 36 weeks by Evans *et al.* (1994a,b) showed that follicular development occurred in a wave-like pattern as early as 2 weeks. There is a regular emergence of follicular waves in the early months of pregnancy (Kinoshita *et al.*, 1999) and it is only in the final weeks of pregnancy that there is a complete cessation of the wave pattern (Driancourt, 2001). In the post-partum cow, emergence of the first follicle wave occurs within 2 weeks of parturition, presumably the result of FSH released as a consequence of the withdrawal of the negative feedback effect of oestrogens and progesterone before calving. It is believed that prolonged post-partum anoestrus in beef cattle is due to the failure of early dominant follicles to ovulate. In Ireland, Duffy *et al.* (2000) presented data supporting the view that LH pulse frequency is the key determinant of the fate of the dominant follicle in the early post-partum period of beef cows nursing calves.

A review by Scaramuzzi and Murray (1994) dealt with the long-term and short-term effects of nutrition on folliculogenesis. Long-term effects may involve stimulation of the earliest stages of folliculogenesis, in which follicles are activated and emerge from the primordial follicle pool; short-term nutritional effects are likely to involve stimulation of the terminal phase of follicle growth, probably by way of the paracrine and autocrine processes involved in follicle selection and atresia (see Fig. 2.7).

Elsewhere in the UK, Gutierrez *et al.* (1995) demonstrated that nutrition stimulated significant changes in the number of small follicles. In later work it was shown that short-term changes in diet may affect ovarian follicular dynamics in cattle without this involving any change in the blood levels of gonadotrophins (Gutierrez *et al.*, 1997a,b). An earlier study by Gong *et al.* (1995) showed that the early stages of follicle development in the cow (when follicles are < 4 mm diameter) are independent of gonadotrophin

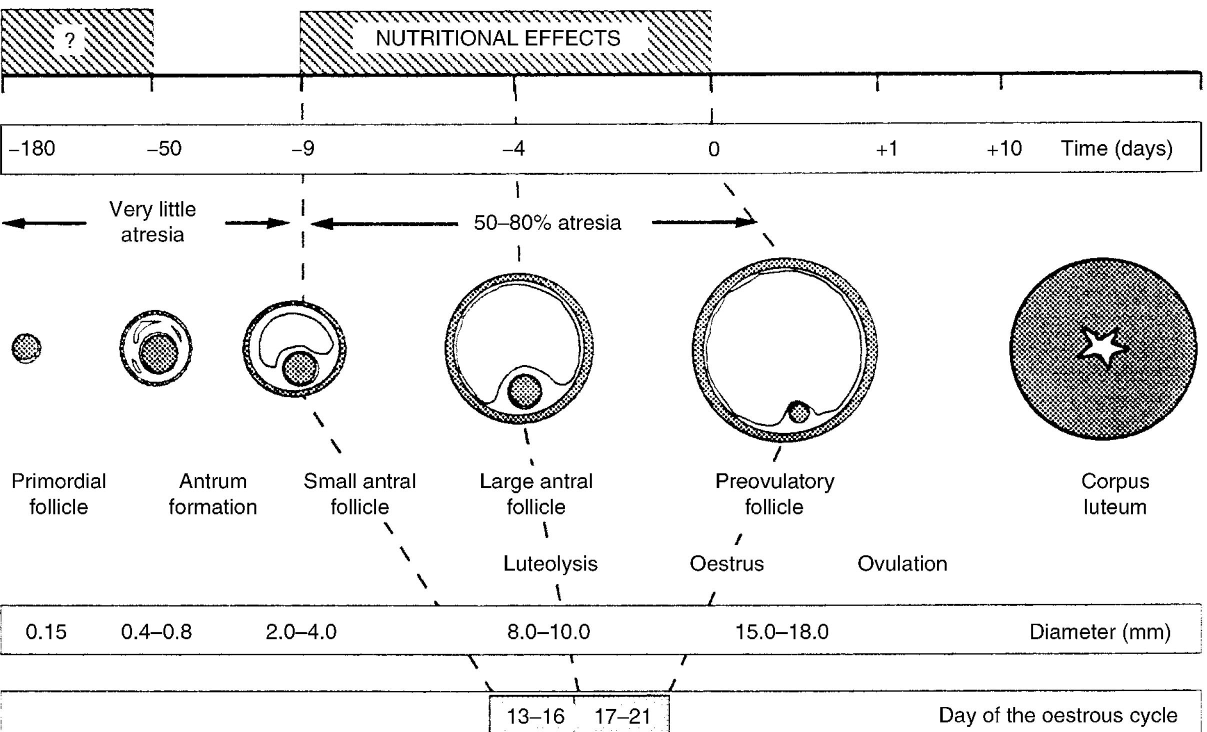


Fig. 2.7. Effects of nutrition on folliculogenesis in the cow. A follicle takes an estimated 180 days to develop from a primordial follicle (0.15 mm diameter) to a preovulatory follicle (15–18 mm diameter). Nutritional effects probably occur in follicles at the late antral stage when they are most susceptible to atresia (over the last 9 days of their development). (From Scaramuzzi and Murray, 1995.)

support. FSH is required, however, for further follicular growth (from 4 to 9 mm diameter).

Other work in the UK sought to clarify the mechanisms by which nutrition may act to influence follicle dynamics and oocyte quality (Armstrong *et al.*, 2001); dietary energy and protein can apparently affect the function of the ovarian insulin-like growth factor (IGF) system, with subsequent effects on follicular dynamics and oocyte quality. The Edinburgh workers proposed that dietary-induced changes in the ovarian IGF system increase the bioavailability of intrafollicular IGF, thereby increasing the sensitivity of follicles to FSH. In contrast to its effect on follicular growth, increased nutrient supply decreased oocyte quality, due in part to increased plasma urea concentrations. Such findings may have particular relevance to those formulating diets for high-yielding dairy cattle, where low fertility is a source of serious concern in several countries. A review by Boland *et al.* (2001b) of nutritional effects on ovarian physiology and embryo production noted that cattle with reduced energy intake have smaller dominant follicles and more three-wave cycles compared with cows on higher feed intakes. Elsewhere, results reported by Comin *et al.* (2002) suggested that acute dietary restrictions induced substantial changes at the dominant follicle level, which may be a factor in instances of poor reproductive efficiency.

There may be marked individual differences in follicular dynamics among cattle, with as few as one to as many as four waves of follicular growth occurring within an oestrous cycle; most animals would be expected to show two or three consecutive waves per cycle. These waves emerge (follicles first detected at 4 mm) approximately 0, 9 and 16 days after ovulation and are referred to as waves 1, 2 and 3, respectively; follicles of each wave pass through a common growth phase for 2 or 3 days after emergence. Each wave is stimulated by a surge of FSH, which peaks when the emerging follicles are approximately 4 mm; the concentration of the gonadotrophin falls during the common growth phase. This decrease in FSH support is believed to be caused by the combined action of inhibin and oestradiol, produced by follicles > 5 mm in diameter, which acts by way of negative feedback on the pituitary. Each follicular wave involves the recruitment of a dominant follicle, which continues to grow while apparently suppressing the growth of other follicles in the cohort. Such recruitment begins at the end of the common growth phase when deviation (growth of the largest follicle and diminished growth of subordinate follicles) begins and becomes established within a matter of hours. In the USA, Townson *et al.* (2002) examined the relationship of fertility to ovarian follicular waves before breeding in dairy cattle; fertility was greater in lactating cows inseminated after showing three waves than in those showing two. As well as the well-recognized major follicular waves of the cow's oestrous cycle, it is now evident that minor waves also occur (Ginther *et al.*, 2002a,b); these have been recorded in 50% of heifers towards the end of the cycle and appeared to be associated with FSH surges.

Zebu cattle

Most studies on follicular dynamics in cattle have involved European breeds (*Bos taurus*) and there has been a tendency to believe that the story is the same in zebu cattle (*B. indicus*); although based on limited data, a report by workers in Iran suggested that this may not be so (Niasari-Naslaji *et al.*, 1999). A study by Viana *et al.* (2000b) investigated follicular dynamics during the oestrous cycle of Gir cattle in Brazil; they recorded cycles with two (6.7%), three (60.0%), four (26.7%) and five (6.7%) follicular waves. The authors concluded that follicular dynamics in Gir cattle are characterized by a higher incidence of cycles, with three or four waves, associated with a low persistence of the dominant follicle.

2.2.3. The dominant follicle

The growth and maturation of the preovulatory bovine follicle proceed in distinct phases, including recruitment, selection and dominance; in the context of *in vitro* embryo production, it is clearly important to have a comprehensive understanding of events occurring in that follicle in devising appropriate protocols for maturing the primary oocyte. If a subluteal concentration of progesterone is maintained artificially, prolonged dominance of the dominant

follicle may ensue, and such prolongation may compromise fertility. In one study, 14% of heifers that ovulated follicles after their prolonged dominance became pregnant after breeding, in contrast to 73% of animals with normal ovulatory follicles; the cause of such decreased fertility is unclear. If the normal lifespan of an active dominant follicle in the cow (about 9 days) is extended, it may adversely affect oocyte quality (Revah and Butler, 1996). On the other hand, an endocrine imbalance may stem from prolonged follicle activity (chronic oestrogen production), which may have detrimental effects on the uterine environment.

Evidence has continued to accumulate on the activity of growth factors in the regulation of ovarian function in the cow. Spicer and Echternkamp (1995) note that IGFs are produced by granulosa, theca and luteal cells as part of an intraovarian autocrine and paracrine system. Adding to the complexity of the regulatory role of IGFs is the presence of IGF-binding proteins (IGFBPs) within the ovary; the production of these binding proteins is believed to be hormonally regulated.

There is general agreement that LH is the key hormone involved in the final growth of the dominant follicle; such sensitivity means that changes in the pattern of LH secretion can readily induce its demise. The observed time of turnover of wave 1 (day 7 to day 10) coincides with peak progesterone concentrations and a minimal frequency of LH pulses. In Ireland, Mihm *et al.* (2000) aspirated follicular fluid from individual follicles to determine if intrafollicular amounts of oestradiol, progesterone, inhibins, activin-A, follistatins and IGF proteins differed for the future dominant follicle compared with subordinate follicles during selection of the wave-1 dominant follicle; they concluded that IGFBPs and oestradiol were both reliable markers in predicting which follicle became dominant. Most research on follicular dynamics has been confined to wave 1 of the cycle, but Kulick *et al.* (2001) found that deviation mechanisms (follicle or hormone differences) were the same for waves 1 and 2.

Evidence for the role of steroids, inhibin and other peptides in the growth and regression of bovine follicles has been provided in recent years. It is clear that the attainment and loss of follicular dominance are closely related to certain crucial changes in the endocrine environment. Changes in peripheral concentrations appear to be particularly critical. It is believed that the greater development of the dominant follicle of the first follicular wave, compared with subsequent anovulatory waves, is the result of the lower progesterone levels that operate during its growth phase. There is evidence to support the view that negative feedback effects on LH pulse frequency comprise the mechanism by which changes in plasma progesterone influence follicle growth and regression. The demise of the non-ovulatory dominant follicle during the cow's oestrous cycle occurs by way of the negative feedback effects of luteal progesterone, which maintains a low LH pulse frequency and low oestradiol production. A paper by Ginther *et al.* (2001c) dealt with factors involved in follicle deviation and with evidence suggesting that the secretion of oestradiol into the circulation and the increase in oestradiol and IGF-I and decrease in IGFBP-2 concentrations in the follicular fluid at the start of follicle deviation are functions of the transient increase in LH concentrations that are associated with follicle deviation. Nicholas *et al.* (2002) found that the total number of IGFBPs was reduced in dominant follicles. In a further report, Ginther *et al.* (2002a) found evidence indicating that the conversion of a subordinant follicle to a dominant follicle involved an increase in systemic FSH, a transient increase in follicular-fluid activin A and a simultaneous increase in follicular-fluid oestradiol and restoration of an apparent growth-compatible balance of free IGF-1 and IGFBB-2 (see Fig. 2.8). Studies by Austin *et al.* (2002) have demonstrated that the early growing cohort of the first follicular wave is responsive to oestradiol and progesterone treatment, which is associated with short-term alterations in FSH and LH concentrations; they concluded that growing cohort follicles are differentially responsive to treatments that suppress LH release. This may be a factor enabling an LH-mediated survival advantage to be gained by the largest follicle in the cohort.

2.2.4. Monitoring ovarian activity

It has been recognized for many years that follicular development in the cow's ovaries is a dynamic process. Traditionally, changes in

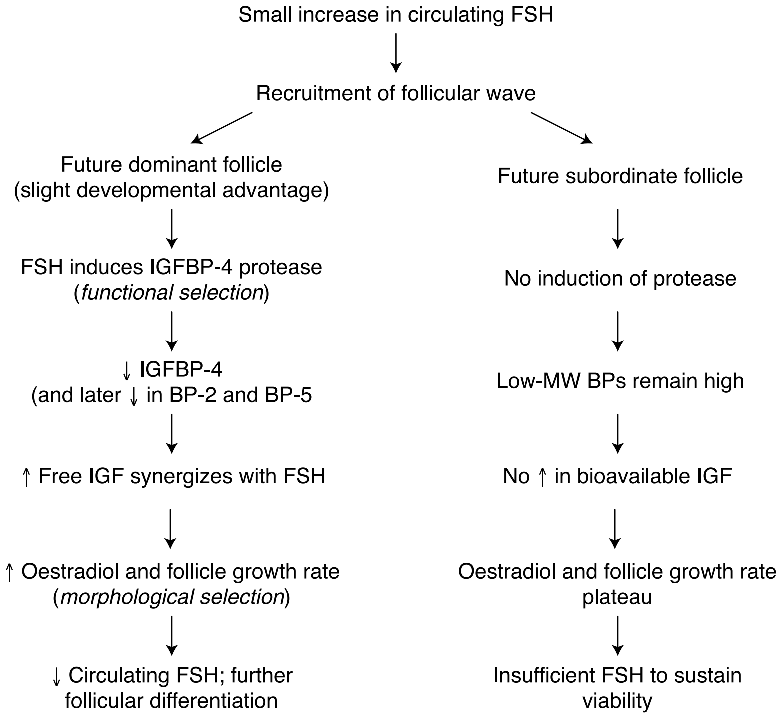


Fig. 2.8. Possible sequence of events during selection of a dominant follicle. This model suggests that a critical event of functional selection is the induction by FSH of a protease for IGFBP-4 in one follicle of a cohort and that functional selection is quickly followed by morphological selection and further differentiation of the dominant follicle. BP, binding protein; MW, molecular weight. (From Fortune *et al.*, 2001.)

antral follicle populations were determined by slicing ovaries excised at different stages of the cycle or by monitoring ovaries by way of palpation *per rectum*. The advent of real-time ultrasonics has changed all this (see Garcia *et al.*, 1999; Ribadu and Nakao, 1999; Lamb, 2002); now it is possible to visualize structures within the ovary (follicles and CLs) without disturbing normal physiological events (oestrous cycle or pregnancy).

During the 1980s, equipment became available for use on the farm which permitted a valuable new approach to the study of follicular dynamics in the cow. Ultrasonic scanning of the reproductive organs is now the basis of an extremely accurate technique for the estimation of the antral follicle population within the limits of resolution imposed by the scanning device; follicles as small as 2–3 mm diameter can be visualized, measured and sequentially monitored. Not surprisingly, such scanning has become the method of choice for monitoring follicular development in the cow. The availability of such equipment has also permitted the development of a new area of cattle embryo transfer (ET) technology, in facilitating the recovery of immature oocytes from ovarian follicles in what is now commonly known as ovum pick-up (OPU).

The ultrasound instruments employed in examining the ovaries are 'B-mode, real-time' scanners. 'B-mode' refers to brightness modality, wherein the ultrasonic imaging is a two-dimensional display of dots, the brightness of which is proportional to the amplitude of the returning echoes. 'Real-time imaging' refers to the moving display that is continuously presented on the monitor screen. Two types of real-time, B-mode ultrasound instruments are available: linear-array scanners and sector scanners. In the linear-array scanner, sound waves are emitted perpendicular to the transducer along the row of crystals. For transrectal examinations in the cow, it is important to select equipment with a durable, atraumatic probe

designed for intrarectal insertion; for the aspiration of ovarian follicles, a sector scanner equipped with a vaginal transducer is usually employed. It may also be mentioned that transrectal ultrasonography has occasionally been employed to examine the morphology of the cow's CL. In the Netherlands, Garcia and Salaheddine (2000) investigated the significance of the diameter of the luteal cavities in CLs on the pregnancy rate of bovine recipients in an ET programme; they found that, regardless of the presence and size of the luteal cavity, recipients were able to establish and maintain pregnancy after ET.

In some studies, magnetic resonance imaging (MRI) equipment has been employed in studying follicular activity. In Canada, for example, Hilton *et al.* (2000) have shown that quantitative differences in the magnetic resonance image (MRI) attributes of the bovine follicle antrum at different phases of follicular development and regression coincided with changes in the ability of the dominant follicle to produce steroid hormones and to ovulate; they concluded that the MRI attributes of the follicular antrum were indicative of physiological status and follicular health.

2.3. Endocrine Events in the Oestrous Cycle

For a follicle to reach the preovulatory stage in the cow's ovary, it has to pass through a number of developmental stages; such development requires the interaction of both systemic and locally produced factors. Although gonadotrophins (FSH and LH) have a primary role, many locally produced and extraovarian factors contribute to the final development of the single follicle destined for ovulation. The fact that the cow has two ovaries and yet controls precisely the ovulation of a single follicle in one of its ovaries points to a regulatory mechanism that operates systemically. In discussing gonadotrophins, account should be taken of evidence of the distribution of FSH and LH isoforms during the oestrous cycle (Kojima *et al.*, 1995); Irish workers have pointed to the need for sensitive assays to help in understanding the action of such isoforms during the cycle (Crowe *et al.*, 1995).

2.3.1. Gonadotrophin release

The preovulatory surge of LH, which occurs in the early hours of oestrus and about a day ahead of follicle rupture, is responsible for triggering a series of events that results in the nuclear and cytoplasmic maturation of the germinal vesicle (GV) oocyte that is present in the follicle destined for ovulation. In attempting the artificial maturation of the oocyte, it has usually been a matter of simulating certain of the critical events that occur in the live cow in the course of the final 24 h before the preovulatory follicle ruptures and releases the oocyte. The cow is a monovular species, which implies that only one oocyte usually reaches maturity at the end of each oestrous cycle. There is a belief that the endocrine and paracrine factors controlling the final maturation and ovulation in the cow are probably more complex than those in ruminants such as the sheep and goat, where multiple ovulations (two or three) are commonplace. This may be a factor explaining differences in the response of sheep and cattle to immunological and other approaches to the modification of ovulation rate.

In considering the endocrinology of the cow's oestrous cycle, it is necessary to mention that pulsatile or episodic secretion is the common mode for many of the hormones involved; in such secretion, small amounts of hormone are released at different time intervals. In terms of LH activity, high-amplitude, low-frequency pulses occur during the luteal phase of the oestrous cycle, resulting in a low average plasma LH level; during the preovulatory phase, this changes to a low-amplitude, high-frequency pattern, resulting in higher plasma concentrations of the gonadotrophin.

The secretion of LH in pulses is, in turn, a result of the pulsatile secretion of GH-RH by cells in the hypothalamus. During the luteal phase of the cycle, the frequency of LH pulses is about one every 4 h; in the preovulatory phase, and with the demise of the CL, this frequency is likely to increase to one per hour or more. Researchers seeking to measure the preovulatory LH surge must take blood samples at intervals of 5–15 min in recognition of this increase. It is this greater LH frequency that stimulates pulse frequency. Increased LH pulse frequency stimulates oestradiol secretion by the ovarian follicle and it is this oestrogen that elicits the preovulatory LH

surge, leading to ovulation and formation of the CL.

Information on factors influencing FSH secretion is more limited; it is generally accepted that GH-RH is responsible for stimulating FSH secretion, although the effects are less evident than with LH. It is believed that GH-RH plays a relatively minor role in the regulation of FSH levels; this may be reflected in the pattern of FSH secretion, which apparently is not pulsatile. The regulation of FSH release is believed to be exercised by the ovaries and involves an interaction between oestradiol and inhibin. In sheep, and perhaps in cattle, it is believed that oestradiol is involved in the control of short-term fluctuations in FSH plasma concentrations, whereas inhibin regulates the longer-term levels. Whether oestradiol and inhibin also differentially regulate the biological activity of FSH is less certain. Studies in Japan reported by Kaneko *et al.* (1995) provided strong evidence that inhibin is an important factor in the inhibitory regulation of FSH secretion during the follicular phase in the cow and that oestradiol has a synergistic effect with inhibin on FSH secretion.

2.3.2. Intraovarian events

The identification of intraovarian factors and an understanding of their role in the follicular maturation and ovulatory processes would do much to shed light on key events influencing folliculogenesis in the cow. There is, for example, a growing body of evidence to show the important role of the IGF system and other growth factors in folliculogenesis (Armstrong and Webb, 1997); this has practical implications for the employment of recombinant bovine somatotrophin (BST) in influencing the ovarian follicle population in cattle.

In terms of cytokine activity, there is evidence suggesting the existence of a complete intraovarian interleukin-1 (IL-1) system, replete with ligands, receptor and receptor antagonist. In view of the fact that IL-1 is an established mediator of inflammation and that ovulation may constitute an inflammation-like reaction, it has been suggested that IL-1 may play an intermediary role in the process of ovulation. There is support for the view that IL-1 may be the centre-piece of an intraovarian regulatory loop concerned with the promotion of the preovulatory cascade.

2.4. Synchronizing Oestrus

The hormonal manipulation of the bovine oestrous cycle is an essential part of ET technology and usually plays an important role in various aspects of embryo production, whether in dealing with cattle acting as donors of oocytes or as recipients of embryos. The aim of most synchronization programmes, although not always attained, is to induce a precise onset of oestrus (95% of animals in oestrus within a defined 12 h period) and for cows to show normal fertility after breeding, regardless of the stage of the cycle at which treatment was initiated. What synchronization system is open to the farmer will depend on the particular protocol and hormonal preparations that are licensed for use in the country in question. In the final analysis, it will come down to a question of cost and this, unfortunately for the farmer, may involve considerations (e.g. veterinary supervision of treatment) which have no bearing on the technical efficiency of the treatment.

2.4.1. Treatment regimens

The pharmacological control of oestrus was initially based on two approaches, either shortening the lifespan of the CL by inducing its regression by luteolysins, such as $PGF_{2\alpha}$ and its analogues, or extending the life of the CL or substituting for it, by the use of progesterone or a synthetic analogue (Fig. 2.9).

Over the years, the treatment protocols have been modified to include ways of influencing ovarian follicular wave patterns, either with oestradiol or with GH-RH. There are also those who point to the need for concepts of oestrus control to be extended to include resynchronization of returns to service (Macmillan and Burke, 1996). The rationale behind the various approaches to current methods of oestrus control has been dealt with in several reviews and research papers (Roche *et al.*, 1997; Rensis and Peters, 1999; Diskin *et al.*, 2001).

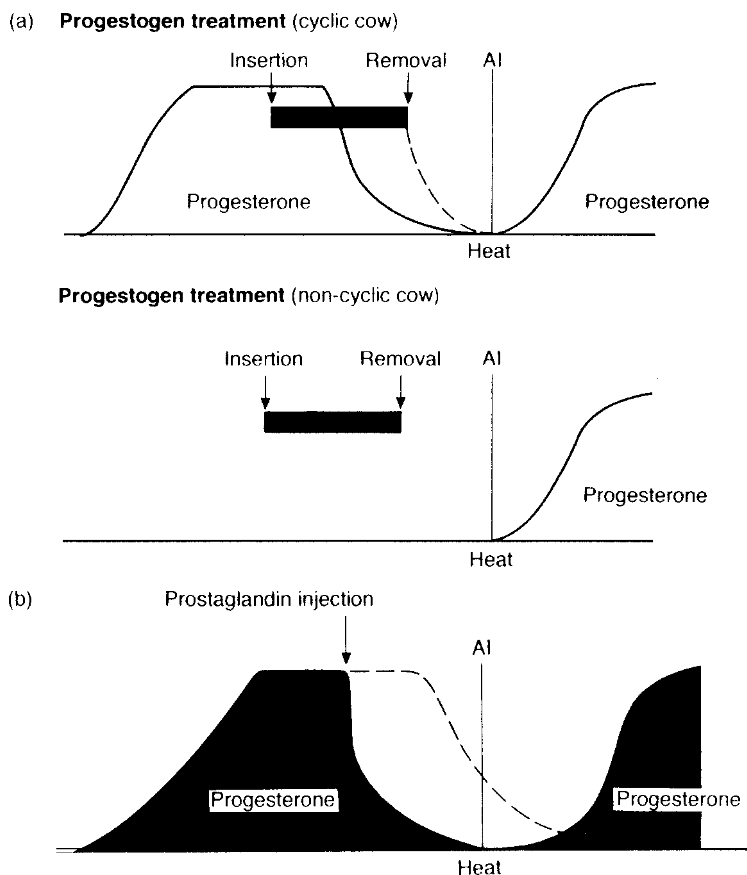


Fig. 2.9. Two approaches to oestrus control in the cow. (a) After progestogen withdrawal, cows enter the follicular phase of the oestrous cycle and become synchronized for oestrus and ovulation. (b) Prostaglandin induces regression of the cow's corpus luteum and the onset of oestrus within 2–4 days in cyclic animals.

Prostaglandins for oestrus synchronization appeared on the agricultural scene in the mid-1970s; at that time, they seemed to have most appeal and worked well with dairy heifers. The injection of $PGF_{2\alpha}$ to cows after day 5 of the cycle can be expected to induce immediate and rapid regression of the CL in most animals. However, prostaglandin is only effective in cattle between days 5 and 17 of the cycle, which leaves some 40% that show no response to the injection. A two-injection procedure, with an interval of 9–13 days between injections, should find all animals with a susceptible CL in the ovary at the time of the second prostaglandin dose; an 11-day interval is generally recommended for cyclic heifers.

In the USA, Pursley *et al.* (1995, 1997) demonstrated that a combined regimen of GH-RH on day 0, $PGF_{2\alpha}$ on day 7 and GH-RH on day 9, followed by a single fixed-time insemination can result in normal fertility (see Fig. 2.10). The first injection of GH-RH is given at a random stage of the cycle and causes either ovulation or luteinization of a dominant follicle, if present, in the majority of animals. The rationale for the second administration of GH-RH 2 days after $PGF_{2\alpha}$ is to advance the time of the LH surge and consequently advance and synchronize ovulation, so that a single insemination is sufficient to ensure normal fertility. This GH-RH – $PGF_{2\alpha}$ – GH-RH protocol, commonly known as Ovsynch, synchronizes follicular development, luteal regression and time of ovulation, thus permitting timed AI after the second GH-RH administration. In view of the fact that the retail cost of GH-RH largely determines the cost of the Ovsynch

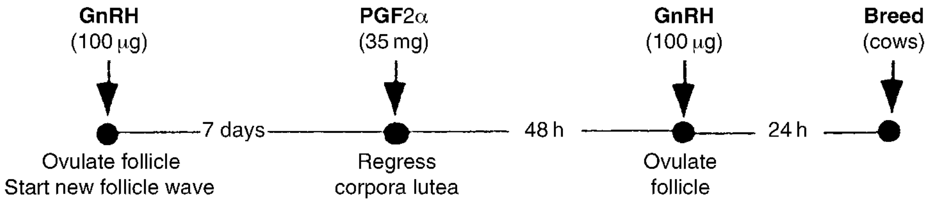


Fig. 2.10. Timing and purpose of hormone injections in synchronization protocol.

treatment, Fricke *et al.* (1998) in Wisconsin were able to show that decreasing the dosage of Gn-RH (from 100 to 50 μg) substantially reduced costs (by $6.40 per cow and $20.27 per pregnancy) without such reduction compromising the efficacy of the synchronization treatment.

The development of progesterone delivery devices, such as the progesterone-releasing intravaginal device (PRID, Sanofi), the controlled intravaginal releasing device (CIDR, InterAg) and progestogen-containing ear implants, has been an alternative route to synchronization. A recent addition to the range of products available for oestrus synchronization is a slow-release progesterone/oestradiol formulation given as an intramuscular injection of biodegradable microspheres (Whisnant and Burns, 2002). In the case of the ear implants, Synchromate B (SMB, Ceva Laboratories) uses an implant containing 6 mg of the highly potent progestogen norgestomet, combined with an intramuscular injection of 5 mg oestradiol valerate and 3 mg norgestomet; Crestar (Intervet) has the same regimen but uses implants containing only 3 mg norgestomet. Most animals (up to 85%) can be expected to show oestrus some 36–60 h after progestogen withdrawal. A feature of all these systems is the administration of oestradiol at the start of treatment, designed to shorten the lifespan of the CL, terminate the existing follicular wave and induce the emergence of a new follicle. An oestradiol-containing capsule has been the mode of oestrogen administration in conjunction with the PRID and CIDR, but Irish workers have shown that this may not always increase plasma oestrogen levels to the point where follicular activity is decisively influenced (O'Rourke *et al.*, 1998); intramuscular injection rather than intravaginal administration of oestradiol may be indicated. According to Diskin *et al.* (2001), InterAg, the New Zealand company marketing CIDRs, has modified its recommendations to provide for the injection of oestradiol benzoate (1.0 mg for heifers; 2 mg for cows) at the start of treatment.

2.5. Prenatal Development of the Bovine Ovary

The series of events that result in the female gamete (oocyte) being able to perform its ultimate function of interacting with the spermatozoon develops over a long period, extending from the stage of oogonia differentiation in the fetal ovary to the final maturation of the oocyte just before its release at ovulation. The process of differentiation and maturation of the oocyte can be arranged into a prenatal and postnatal phase, each consisting of periods of enhanced activity followed by periods of quiescence. Various aspects of oogenesis and folliculogenesis in the prenatal heifer and the postnatal cow have been dealt with in several papers and reviews (Wassarman and Albertini, 1994; Beckers *et al.*, 1996; Braw-Tal and Yossefi, 1997; Fair and Hyttel, 1997; Fair *et al.*, 1997a,b, 1998; McNatty *et al.*, 1999; Derrar *et al.*, 2000; Picton, 2001; Tanaka *et al.*, 2001). In the prenatal calf, by late gestation, all stages of oocyte and follicle development up to the antral stage are to be found in the ovary. Although there are considerable numbers of zebu cattle in Asia and other parts of the world, little is known about the morphological events that occur during the prenatal development of gonads in *B. indicus*. In Brazil, Diniz *et al.* (2002) attempted to fill that gap by describing the development of the gonad, including the appearance of oogonia, oocytes and follicles in Nellore breed embryos and fetuses; results showed such development to be similar to that found in European cattle (*B. taurus*).

It has long been recognized that very few of the GV oocytes present in the the bovine ovaries

develop to the point of becoming secondary oocytes and being ovulated. It has been estimated that there may be more than 200,000 oocytes in primordial follicles in the ovaries of the heifer calf at birth. In Japan, working with 4-year-old cattle, Miyamura *et al.* (1996) found that the numbers of primordial, primary, secondary and Graafian follicles added up to a total of 77,169 (see Table 2.4); this is some indication of the oocytes that may eventually be utilized from one animal in the laboratory production of embryos. It can be calculated that no more than about 300 oocytes are ever likely to reach the ovulatory stage during the normal reproductive lifespan of the cow.

2.5.1. Migration of primordial germ cells

Special cells, the primordial germ cells (PGCs), derived from the inner cell mass of the developing blastocyst, are believed to be responsible for initiating the processes that result in the invasion of the gonad and the differentiation of the ovary. These PGCs are the only source of adult germ cells and their early history is the same for the bull fetus as for the heifer; in the male, however, spermatogonia act as stem cells and give rise to sperm from puberty onwards, whereas the heifer calf finds itself from birth possessing a finite number of oocytes. The primordial germ cells are characteristically large, possessing a round nucleus with one or more prominent nucleoli and a few organelles, including small mitochondria, tubuli and cisternae of the endoplasmic reticulum, polyribosomes, scattered Golgi membranes and microfilaments, together with variable numbers of glycogen particles and lipid droplets, in their cytoplasm (Motta *et al.*, 1997; Gosden, 1998). The PGCs can be identified because of their large size and their high alkaline phosphatase activity, which makes them recognizable by appropriate histochemical staining techniques.

When the bovine embryo is about 15 mm in length, the PGCs migrate by amoeboid movement from the yolk-sac epithelium, through the hind-gut and dorsal mesentery to the primitive kidneys, where they eventually colonize the adjacent gonadal (germinal) ridges. During the course of their migration, the PGCs proliferate by way of a series of mitoses. With the arrival of the germ cells, the epithelium of the gonadal ridge starts to thicken and proliferate; this results in the ridge becoming visible in the bovine embryo at about 28 days of age. By 41 days, the ovary has become differentiated and, by 45 days, a well-defined cortex is evident in the developing ovary and the PGCs begin to differentiate into oogonia; the germ cells lose their ability to move, stain less intensively for alkaline phosphatase activity, become more spherical and possess fewer cytoplasmic organelles (Picton, 2001). Sometime after day 35 of prenatal life, germ cells undergo mitotic divisions before entering meiosis. According to Picton (2001), there are many mitotic cycles, extending over a period of several months until shortly before birth. As a result of germ-cell proliferation, numbers may reach as many as 2 million per animal, although only some 5% of that number may remain by the time the heifer fetus reaches birth.

2.5.2. Formation of oogonia

The PGCs that move into the cortical region of the presumptive gonad, together with supporting epithelial cells, form what are known as the cortical sex chords. The germ cells form clusters of dividing cells, which are termed oogonia. At the end of mitotic proliferation, oogonia enter meiotic prophase and differentiate into primary

Table 2.4. Number of primordial, primary, secondary and Graafian follicles in cattle ovaries (from Miyamura *et al.*, 1996).

Follicle type	Cow no. 1 (%)	Cow no. 2 (%)	Mean (%)
Primordial	82,572 (95.8)	62,990 (92.4)	72,781 (94.3)
Primary	2,530 (2.9)	4,058 (6.0)	3,294 (4.3)
Secondary	837 (1.0)	833 (1.2)	835 (1.1)
Graafian	243 (0.3)	275 (0.4)	259 (0.3)
Total	86,182 (100)	68,156 (100)	77,169 (100)

oocytes. In giving rise to oocytes, oogonia undergo the two-cell divisions of meiosis to reduce their diploid chromosome and DNA complement (2n) to the haploid state (n). After the initiation of meoisis, the germ cells (primary oocytes) progress through the leptotene, zygotene and pachytene of the first meiotic prophase before arresting at the diplotene (dictyate) stage; the oocyte grows in size as development proceeds towards the diplotene stage. The first oogonia to undergo meiotic division are located at the innermost areas of the ovarian cortex and the developmental wave of meiosis spreads outwards (Picton, 2001).

2.5.3. The primordial follicle

The initiation of meiosis in the oocytes coincides with the onset of folliculogenesis; the follicle is the functional unit of the bovine ovary and is responsible for the maturation of the oocyte and the production of steroid and peptide sex hormones and other compounds. Folliculogenesis is the process whereby a follicle grows through different stages of development from the time it emerges from the follicular store until it ovulates or becomes atresic. There are three categories of follicles: non-growing or primordial follicles, growing or preantral follicles and antral (vesicular) follicles; these follicles differ in their size, complexity and responsiveness to circulating gonadotrophins. All these categories of follicles are evident in the bovine fetal ovary; advanced stages of follicular development may be found as early as 90 days into pregnancy. Before the formation of follicles, streams of cells, arising within the medullary region of the ovary, surround and invade nests of oogonia and oocytes. During this process, dictyate oocytes become enclosed in a single layer of flattened or polyhedral pregranulosa cells derived from the cell streams, giving rise to the earliest primordial follicles. The pregranulosa cells rest on a delicate basement membrane opposite the stromal cells, some of which differentiate into a thecal layer after follicle growth begins (Gougeon, 1996).

Primordial follicles occupy the peripheral areas of the cow's ovarian cortex; they are separated from one another by compactly organized stroma consisting of fibroblasts, blood-vessels and bundles of collagen and reticular fibres. Primordial follicles constantly move from the non-growing pool to enter a growth phase (see Fig. 2.11); there is clearly an interaction between the surrounding stroma and the follicles. The regulatory factors involved in the origin, differentiation and accumulation of various organelles in the cytoplasm of the primordial oocyte remain to

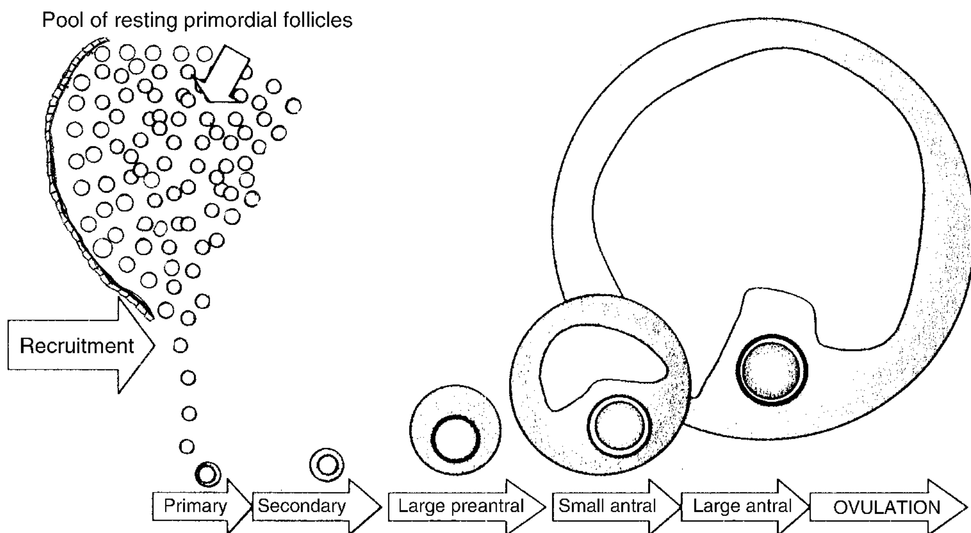


Fig. 2.11. Schematic representation of folliculogenesis in the cow.

be determined (Gosden, 1998). The limited biochemical data available on oocytes in primordial follicles suggest that there is some amount of protein synthesis during this no-growing phase; the biochemical factors responsible for the selective growth of some follicles (activation) while others remain quiescent remain unknown. Nutrients for the maintenance of primordial follicles are believed to enter the oocyte by way of a process of membrane diffusion, by pinocytosis, by active transport or through irregular gaps between adjacent follicle cells.

A classification system for the different categories of follicle has been described by Braw-Tal and Yossefi (1997). In this system, preantral follicles and the smallest antral follicles are classified as types 1–5: type 1 – primordial follicles showing one layer of flattened pregranulosa cells; type 1a – transitory follicles showing one layer of cells that are a mixture of flattened and cuboidal granulosa cells; type 2 – primary follicles showing one or two layers of cuboidal granulosa cells; type 3 – small preantral follicles, showing two to four layers of granulosa cells; type 4 – preantral follicles showing four to six layers of granulosa cells; and type 5 – follicles showing multiple layers of granulosa cells. According to Van Wezel and Rodgers (1996), 85.5% of the follicle population in cows are in the form of early primary follicles. A classification scheme based on early follicular development in the fetal sheep ovary is detailed by Picton (2001). In this, primary follicles are characterized by a single layer of cuboidal granulosa cells surrounding the oocyte; secondary follicles contain a growing oocyte surrounded by more than two layers of granulosa cells.

2.5.4. Activation of primordial follicles

The regulation of the growth of primordial and primary follicles in the cow is poorly understood; available evidence suggests that growth is gonadotrophin-independent. The regulation of follicular quiescence versus growth is considered by some to be the most intriguing question in the area of regulation of ovarian follicular development (Findlay *et al.*, 1996; Fortune *et al.*, 1998, 1999). The growth of a primordial follicle to the early antral stage involves eight doublings of the population of granulosa cells and a three- to fourfold enlargement of the oocyte (McNatty *et al.*, 1999); most, if not all, of these growth phases occur without the aid of gonadotrophins. FSH receptors are known to be present in the primary and secondary follicles of cattle (Bao and Gavernick, 1998) and certain growth-factor receptors have been detected in preantral follicles, indicating a role for some growing factors in early follicle growth. Basic fibroblast growth factor (bFGF) receptors are present in primary and secondary bovine follicles but epidermal growth factor (EGF) and IGF-I receptors are not present until after antrum formation. Workers in Japan have found evidence of an increase in serum concentrations of FSH in female bovine fetuses between day 120 and day 150 of gestation (Tanaka *et al.*, 2001); they also recorded the appearance of primordial, primary, secondary and early antral follicles at day 74, 91, 120 and 150, respectively. Such findings are taken to indicate that, in the female fetus as well as in the adult cow, the number of follicles and stages of follicular development are associated with changes in the concentration of FSH.

Studies of the bovine fetal ovaries show that quiescent primordial follicles are located in a thin avascular layer in the outer part of the ovarian cortex beneath the tunica albuginea. Growing follicles, on the other hand, are found in the corticomedullary border, a region particularly well supplied with blood-vessels (Van Wezel and Rodgers, 1996); the indications are that follicle activation and growth depend on blood-borne nutrients, hormones and growth factors. Not all primordial follicles in the corticomedullary region are activated, however, so there must be factors operating to maintain some in the resting stage. There have been suggestions that the ovarian medulla exerts an intraovarian inhibitory effect on primordial-follicle activation. This hypothesis was tested by Derrar *et al.* (2000), who failed to find evidence of such an inhibitor; they did, however, find some evidence that transforming growth factor alpha (TGF-α) may have a role in the regulation of primary follicle development.

2.5.5. Growth and development of follicles

The time taken for primordial follicles to develop to the multilayer preantral stage varies with the

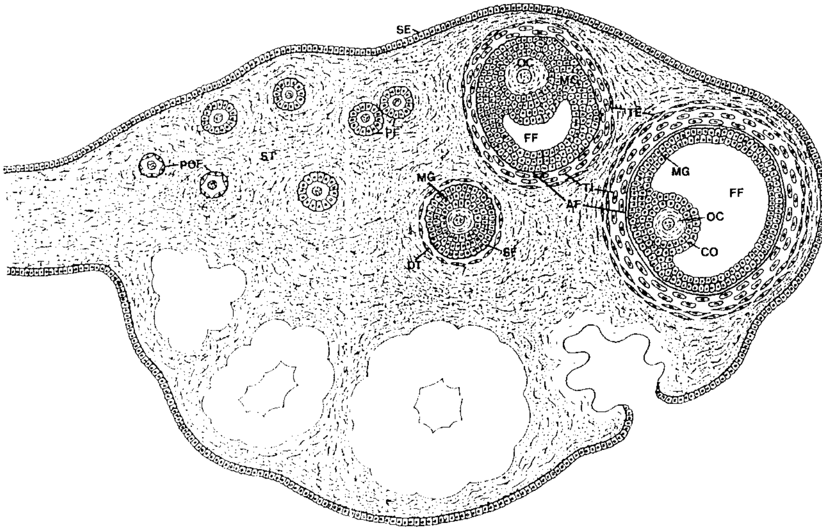


Fig. 2.12. Stages of follicle development in the bovine ovary. SE, surface epithelium; POF, primordial follicle; PF, primary follicle; SF, secondary follicle; AF, antral follicle; MG, membrana granulosa; DT, developing theca; OC, oocyte; FF, follicular fluid; TI, theca interna; TE, theca externa; CO, cumulus oophorus; ST, stromal tissue. (From Van den Hurk *et al.*, 2000.)

species; in the cow, it takes more than 30 days for follicles to grow to that stage and beyond (Telfer *et al.*, 1999); during this period, many changes are occurring in the oocyte and its associated somatic cells (see Fig. 2.12). Although apparently quiescent, oocytes in primordial follicles are not transcriptionally inert; they can demonstrate high levels of synthetic activity. With the start of follicle growth, the somatic cells have the role of nourishing and regulating further oocyte development. A third follicular cell type also emerges, the theca, but this does not become morphologically identifiable until the growing follicle is surrounded by one or more layers of granulosa cells; in due course, the thecal cells assume the appearance of typical steroid-secreting cells (Picton, 2001). As follicles grow, the granulosa cells become metabolically coupled to each other as well as to the oocyte by way of processes that pass through the developing zona pellucida to form gap junctions on the oolemma (Fair *et al.*, 1997a,b).

2.5.6. Formation of the zona pellucida

The zona pellucida (ZP) is the name given to the relatively thick extracellular coat that is formed by and surrounds the bovine primary oocyte, increasing in width as the oocyte increases in diameter. Far from being an inert structure, the ZP plays an important role in facilitating the interaction of sperm and oocyte at the time of fertilization. The ZP first appears as fibrillar material in the space between the oocyte and the cuboidal granulosa cells; as oocyte growth continues, the ZP becomes a denser and thicker meshwork of interconnected filaments completely surrounding the oocyte and largely separating it from the granulosa cells. Close contact continues to be maintained between the oocyte and surrounding granulosa cells via gap-junction complexes within the zona until the completion of oocyte maturation (Green, 1997).

In structure, the ZP is composed of three glycoproteins, termed ZP1, ZP2 and ZP3 (in some texts, ZPA, ZPB and ZPC), with molecular weights of approximately 200,000, 120,000 and 80,000, respectively (Topper *et al.*, 1997). The ZP is an open network of fibrils that permits free passage of large molecules. Of the three glycoproteins, ZP3 appears, at least in the mouse, to play a key role in the binding of the spermatozoon to the surface of the zona pellucida and in the initiation of the acrosome reaction. The ZP contains sperm receptors that mediate the sperm–oocyte interaction as a preliminary to

fertilization; after sperm penetration, the ZP also participates in the zona reaction, an important defence mechanism against polyspermy (Wassarman, 1999). It may also be mentioned that the use of ZP antigens as potential immunocontraceptive agents has become an area of much research (Fierro *et al.*, 1994), with fertility in both horses and elephants being effectively controlled by way of a porcine ZP vaccine (see Fayrer-Hosken *et al.*, 2001).

2.5.7. Development of growing follicles

The growing follicle category includes secondary follicles, distinguishable from primary follicles by virtue of possessing several layers of cubical granulosa cells and greater oocyte size. The start of follicle and oocyte growth is apparently regulated within the ovary and is believed to be a function of the size of the pool of non-growing follicles. The growth of the oocyte and its surrounding follicular cells is coordinated and the oocyte progresses through a series of well-defined morphological stages. Factors involved in the regulation of growth in preantral follicles remain largely unknown. There is general agreement that the earliest stages of follicle growth are independent of circulating gonadotrophins, although there are indications that growth is regulated by both FSH and high local concentrations of oestrogen, although the source of the steroid is in doubt. The formation of the antrum in the tertiary follicle divides the granulosa cells into two groups: cumulus and corona cells associated with the oocyte and mural granulosa cells that line the follicular wall.

2.5.8. Antral follicles

The antral follicle consists of: (i) the GV oocyte, enclosed within the ZP, containing a large nucleus and prominent nucleolus; (ii) a complex somatic component including inner layers of columnar cells, which form specialized intercellular junctions with the oolemma, several layers of granulosa cells and an outer thecal layer in communication with blood-vessels; and (iii) a large fluid-filled cavity (antrum). Within the antrum is follicular fluid, a unique body fluid containing a large number of agents derived from blood and other local secretions. The nature of the initial signals leading to the formation of the antrum is poorly understood. Throughout the entire growth phase of the oocyte and follicle, the oocyte remains arrested in the dictyate of the first meiotic prophase.

Antral follicular development in the cow has been reviewed by Monniaux *et al.* (1997). Reports dating back amost 40 years noted that antral follicles are evident in calf ovaries by day 250 of gestation; by day 270, shortly before birth, the calf ovary may show numerous antral follicles. In sheep and cattle, it takes about 40 days for an early antral follicle (diameter 0.20–0.25 mm) to reach the preovulatory stage. Antral-follicle development proceeds in two well-defined phases. In the first, up to 3–4 mm in the cow, follicles grow slowly and the follicular growth rate is closely related to the rate of proliferation of granulosa cells; this phase appears to be largely independent of gonadotrophin supply. In the second phase, which is clearly gonadotrophin-dependent, follicular growth is rapid due to the marked enlargement of the antrum; as well as that, terminal follicular development is characterized by considerable increases in the steroidogenic capacity and responsiveness of granulosa cells to FSH and LH.

There is increasing evidence that growth factors modulate folliculogenesis in the cow; presumably this holds true for the calf in prenatal life as for the animal after birth It is well established that antral follicles are present in the calf's ovaries during the late stages of its prenatal life. Studies by Carambula *et al.* (1999) in Brazil identified tertiary follicles at 210 days of gestation. As noted by Monniaux *et al.* (1997), growth factors are ubiquitous peptides, involved in the regulation of cell proliferation, differentiation and survival (see Table 2.5).

These factors are classified according to their structure and biological activity into several families: (i) the EGF family; (ii) the fibroblast growth factor (FGF) family; (iii) the platelet-derived growth factor (PDGF) family; (iv) IGF family; and (v) the TGF-β family, including inhibin, activin and the haematopoietic growth factors (cytokines). It should also be mentioned that the oocyte itself plays an important role in development beyond the primary follicle stage

(Eppig, 2001); growth differentiation factor 9 (GDF-9) is an oocyte-specific member of the TGF-β family produced by the bovine oocyte (Bodensteiner *et al.*, 1999).

The development of small antral follicles appears to be influenced by factors of the FGF, EGF and IGF families, which have a direct effect on follicle growth by enhancing granulosa-cell proliferation. In these follicles, which are poorly vascularized, paracrine regulation of growth may be particularly important; factors such as TGF-α and IGF-II, synthesized by thecal cells, may control the proliferation of nearby granulosa cells. It is possible that growth factors also act as hormones. It is known that the administration of growth hormone can stimulate the growth of small antral follicles in the cow, possibly indirectly by way of a mechanism involving IGF-I of endocrine origin.

Growth factors may play an important regulatory role in the initiation of follicular waves, in the selection of the dominant follicle and in the final development of the preovulatory follicle in the cow. Factors of the FGF, EGF and IGF families may control the growth of small antral follicles and participate with FSH in the initiation of follicular waves; factors of the IGF family are believed to be heavily involved in the selection of the dominant follicle.

Table 2.5. Action of growth factors on follicular cells (from Monniaux *et al.*, 1997).

		Granulosa	Theca
Proliferation	Stimulating action	EGF TGF-α bFGF IGF-I	TGF-α
	Inhibiting action	TGF-β	TGF-β
Differentiation	Stimulating action	IGF-I	TGF-β Inhibin
	Inhibiting action	EGF bFGF Activin A Cytokines	TGF-α Activin A

2.5.9. Follicular atresia

The growth of the bovine ovarian follicle in the fetal ovary is continuous from the primordial stage to the antral stage; in the postnatal and postpubertal animal, follicle development can progress all the way up to the preovulatory stage, culminating in ovulation. Such follicle growth can cease at any stage as follicles undergo atresia by way of a mechanism involving apoptosis (programmed cell death); sensitive and quantitative methods have been established to analyse apoptotic DNA fragmentation in ovarian cells by *in situ* DNA 3′-end-labelling (TUNEL). In practical terms, the atresic process is seen as its most striking in the fetal ovary. According to one report, the number of germ cells in the fetal calf ovaries increased to a peak value of 2,739,000 at day 110 of prenatal life, before falling dramatically to 150,000 by the time of birth. After the heifer calf is born, the process of atresia continues unabated; the same would be true in humans. It has been estimated, for example, that the infantile human ovary contains about 400,000 oocytes; in the 30 years of reproductive life about 400 oocytes may mature and be released. On this basis, only about one oocyte in a 1000 leaves the ovary by ovulation; the other 999 are lost in the process of atresia.

2.6. The Bovine Ovary in Postnatal Life

2.6.1. The prepubertal animal

Antral follicles are present in bovine ovaries at all stages from the birth of the heifer calf through to old age. In the young calf, the ovaries exhibit larger pools of antral follicles on their surface than adult ovaries, holding out the hope of recovering many oocytes at the one time. Using young calves and prepubertal heifers as donors in breeding programmes could be the means of increasing annual genetic gain by markedly decreasing the generation interval. Reports go back to the early 1950s of attempts to superovulate young calves by exogenous gonadotrophin treatment, but few follicles ovulated and *in vivo* fertilization rates were low.

In recent years, however, interest in using prepubertal heifers in breeding programmes has been revived. This is the result of applying to calves the techniques used in the production of

embryos from adult cattle (Armstrong, 2001). Oocyte collection can be achieved by laparoscopy from birth or by ultrasound-guided transvaginal OPU in animals older than about 5 months. It has also been demonstrated by some workers that it is possible to aspirate the follicles in calves twice weekly and for a prolonged period (2 months), using a protocol similar to that employed in adult animals. Such treatment has not apparently affected the growth or subsequent reproductive physiology of donors. However, on the debit side, attempts to use the oocytes from young animals have met with disappointing results, something that is equally true for oocytes for other prepubertal farm animals; the poor developmental capacity of calf oocytes has been attributed to incomplete or delayed cytoplasmic maturation

2.6.2. Antral follicle population

A summary of data setting out the number of antral follicles recorded in cattle ovaries is provided in Table 2.6; many of the reports detailed only deal with antral follicles readily visible on the surface of the ovary (> 1 mm diameter). However, it is evident that much larger numbers of follicles were recorded when all those with an antrum are included; a number of these are below the ovary and can only be observed when the ovarian cortex is exposed The size of follicles at the time of antrum formation has been given by some as 0.5 mm and by others as 0.12–0.16 mm diameter.

Data provided by Miyamura *et al.* (1996) dealing with the dimensions of follicles and oocytes in the ovaries of Japanese black beef cattle are given in Table 2.7. According to the review of Van den Hurk *et al.* (2000), bovine follicles pass from the preantral stage to the early antral stage on reaching a diameter of 115–280 μm; the formation of the terminal follicles from the early antral follicles is determined by cell proliferation and fluid formation. Some texts refer to antral follicles as tertiary follicles and to well-developed antral follicles as Graafian follicles; after the preovulatory LH surge in the early hours of oestrus in the cow, the follicle is known as the preovulatory follicle.

2.6.3. Follicle development

In the cow, follicle development takes some 12 weeks to progress from the primary to the antral stage (Katska *et al.*, 2000a,b); after that, some 40 days are required to bring the follicle to the preovulatory stage, when a diameter of 15–18 mm may be reached. Such development is only to be found in the dominant follicle, which emerges a few days earlier from a group of some 15 healthy antral follicles. In the single dominant follicle, the oocyte is surrounded by closely associated granulosa cells (corona radiata, cumulus cells), forming a compact cumulus cell–oocyte complex (COC). This complex is embedded in follicular fluid connected by a band of granulosa cells to the mural

Table 2.6. Numbers of antral follicles in the ovaries of the cow.

Year	Mean number of antral follicles	Researcher(s)
1966	47 per pair of ovaries (1 mm and above)	Erickson
1969	49 per pair of ovaries (1 mm and above)	Scanlon
1979	40 per pair of ovaries (1 mm and above)	Ireland *et al.*
1979	50 per pair of ovaries (1 mm and above)	Leibfried and First
1981	49 per pair of ovaries (1 mm and above)	Matton *et al.*
1984	52 per pair of ovaries (2 mm and above)	McNatty *et al.*
1987	42 per pair of ovaries (2 mm and above)	Pierson and Ginther
1989	38–41 per pair of ovaries (3–4 mm and above)	Prado *et al.*
1968	382 per pair of ovaries (0.4 mm and above)	Marion *et al.*
1985	142–228 per ovary (0.16 mm and above)	Dufour and Roy
1985	324 per pair of ovaries (0.14 mm and above)	Maurasse *et al.*
1987	127–490 per ovary (0.13 mm and above)	Lussier *et al.*
1989	233 per pair of ovaries (0.16 mm and above)	Le Van Ty *et al.*

Table 2.7. Size of follicles in the ovaries of Japanese black cattle (from Miyamura *et al.*, 1996).

	Oocytes		Follicles	
	Smallest	Largest	Smallest	Largest
Follicle type	(μm, mean ± SE)		(μm, mean ± SE)	
Primordial	23.52 ± 0.27	27.57 ± 0.30	37.72 ± 0.26	38.79 ± 0.32
Primary	27.85 ± 0.34	31.67 ± 0.39	41.38 ± 0.61	47.37 ± 0.67
Secondary	44.78 ± 0.70	51.00 ± 0.88	93.78 ± 3.32	115.51 ± 3.98
Graafian	87.19 ± 1.28	102.14 ± 1.46	638.83 ± 63.10	867.01 ± 84.52

SE, standard error.

granulosa. Dominant follicles that form during the luteal phase of the oestrous cycle regress, whereas those that form subsequently continue through to ovulation. Following luteal regression, the dominant follicle of the second or third wave induces the LH surge that initiates final follicular and oocyte maturation and ovulation. Follicles that do not reach ovulation eventually become atresic and are eliminated.

At the time of antrum formation, the stratum granulosum is two to seven cells thick and the theca is a thin layer of orientated stroma cells surrounding the follicle. A membrana propria is clearly visible at the primary follicle stage. In follicles of 1–2 mm diameter, the oocyte becomes surrounded by an increasingly thick layer of cumulus oophorus, several cells thick in a 2 mm follicle; the cumulus oophorus and stratum granulosum are broadly continuous. The thecal layer consists of glandular interna cells surrounded by fibrous externa cells. It takes about 1 week for the small antral follicle (2 mm diameter) to reach the preovulatory stage.

Granulosa cells

During growth, the granulosa cells of the antral follicle differentiate into two subpopulations organized as pseudostratified epithelial mural granulosa cells, in contact with the basement membrane that surrounds the follicle, and cumulus cells, enclosing the oocyte and coupled by gap junctions to both the oocyte and the surrounding mural granulosa cells (Van den Hurk *et al.*, 1994; Van Wezel *et al.*, 1999a,b). These two cell types are known to differ in their distribution of receptors (Baltar *et al.*, 2000) and in their steroidogenic characteristics. In the preovulatory follicle, they also differ markedly in their response to gonadotrophins, which stimulate cumulus cells to produce and secrete hyaluronic acid (HA) but which have no such effect on granulosa cells.

At the start of oestrus in the cow, the follicle destined for ovulation is about 10 mm in diameter; at this stage, the cumulus oophorus has increased to eight to ten cells thick and the hilus is usually a slender stalk. The stratum granulosum has increased in thickness until it exceeds 100 μm; within 24 h, follicle diameter increases to ovulation size, 15–18 mm in diameter. During the period of rapid preovulatory expansion, mitotic activity in both granulosa and theca interna cells remains constant, which is quite inadequate to maintain the thickness of these layers. The preovulatory surge of gonadotrophin, in the hours preceding ovulation, stimulates cumulus cells to undergo expansion and the mural granulosa cells to become luteinized. The development of the membrana granulosa (stratum granulosum) in antral follicles of different sizes was studied by Van Wezel *et al.* (1999a,b) in Australia, who found it to be highly structured and to change with follicular development; they proposed that proliferation occurs in the middle layers and that granulosa cells then progress basally or antrally, the latter cells undergoing terminal differentiation.

It has been estimated that the bovine tertiary follicle takes about two cycle lengths to develop from antrum formation to the preovulatory stage; the chronology of changes in follicle size and the incidence of atresia associated with each category of follicle are given in Fig. 2.13. Although atresia can begin at any stage of follicle development, the incidence tends to increase markedly as follicles grow larger. It should be noted that antral follicles must reach a certain size before they can be expected to yield a meiotically competent oocyte (i.e. one capable of resuming meiosis). This was first recognized in

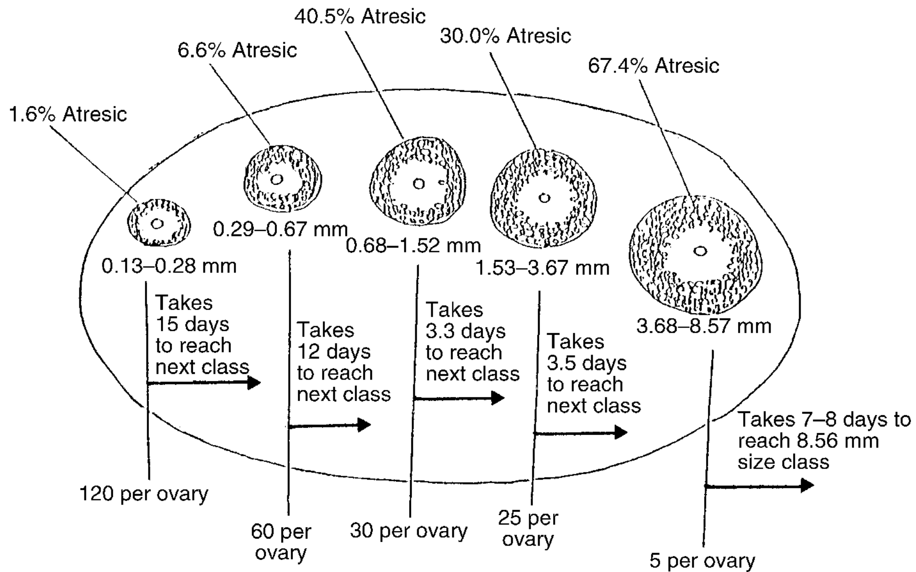


Fig. 2.13. Follicle size categories in the bovine ovary (based on data from Lussier *et al.*, 1987).

the cow by Motlik and Fulka (1986), who observed that follicles below 1–2 mm diameter are unlikely to contain meiotically competent oocytes, a fact subsequently confirmed in several other reports.

Thecal cells

In the development of the antral follicle, factors regulating the structural and functional differentiation of the thecal cells at the cellular and molecular level are poorly understood. Thecal cells are known to secrete TGF-α and TGF-β, and the cells are known to have both autocrine and paracrine functions. A study by Tajima *et al.* (2002) found evidence suggesting that theca cells regulate the fate of granulosa cells throughout the follicular maturation process by secreting factors that suppress apoptosis. The most abundant steroid products of mature theca interna cells are androgens produced from the catabolism of cholesterol; enzymatic conversion of cholesterol to androgen in the thecal cells results in the synthesis of dehydroepiandrosterone (DHEA), which is then metabolized to androstenedione.

The major source of this cholesterol in the cow is believed to be the internalization of blood-borne lipoproteins as the thecal cells of the growing antral follicle become highly vascularized. The main stimulus for the various steroidogenic activities of the thecal cells is believed to be LH; during follicle growth, thecal cells apparently respond to the gonadotrophin by activating enzyme systems that are involved in the synthesis of androgens.

Theca interna cells are believed to be a source of oestradiol in the cow, although the quantities produced, relative to androstenedione, are very small. In some species, which include cattle, thecal cells have been found to have an active aromatase enzyme system; the ability of bovine thecal cells to secrete androgens precedes the acquisition of the aromatase system by granulosa cells. Throughout the oestrous cycle, 20–60 antral follicles may produce androgens, whereas very few such follicles develop the ability to transform androgens into oestrogens. Acquisition of the aromatase system, which is regulated by FSH, may be a key factor in the selection and emergence of the dominant follicle. Using an *in vitro* culture technique, Tajima *et al.* (2002) have shown that theca cells have an important role in controlling the proliferation and apoptosis of granulosa cells; it appears that thecal cells may regulate granulosa cells by secreting factors that suppress apoptosis.

Basement membrane

The basement membrane, or lamina propria, is the acellular layer that separates the theca and granulosa cell layers. The basement membrane of the growing follicle forms a layer upon which the peripheral layer of granulosa cells rests and an outer region of bundles of collagen fibres. Rapid transfer of molecules from the theca to the outer cells of the avascular stratum granulosum is possible because of the arrangment of arterioles, capillaries and venules around the basement membrane. There appears to be no evidence of a blood–follicle barrier and the selective permeability of the basement membrane is apparently under the control of gonadotrophins and oestrogens. As the preovulatory follicle approaches ovulation, the lamina propria starts to break down, leading to invasion of the stratum granulosum by thecal cells and capillaries.

2.6.4. Oocyte growth and development

Studies by Fair (1995) marked the first attempt to describe the morphological aspects of cytoplasmic and nuclear development of the bovine oocyte throughout its entire growth period. As diameter increases, she recorded a concominant dispersal of ooplasmic organelles from the centre to the cortex of the oocyte and a number of oocyte-specific structures were generated, such as the ZP and cortical granules. An overview of the ultrastructural changes during the growth of the follicle has been ably provided in Denmark by Hyttel and colleagues (see Fair, 1995; Hyttel *et al.*, 1997; Fig. 2.14).

The resting primordial follicle encloses an ovoid to spherical oocyte; activation is characterized by a proliferation of granulosa cells and a restructuring of some into a cuboidal shape. The development of the primary follicle is

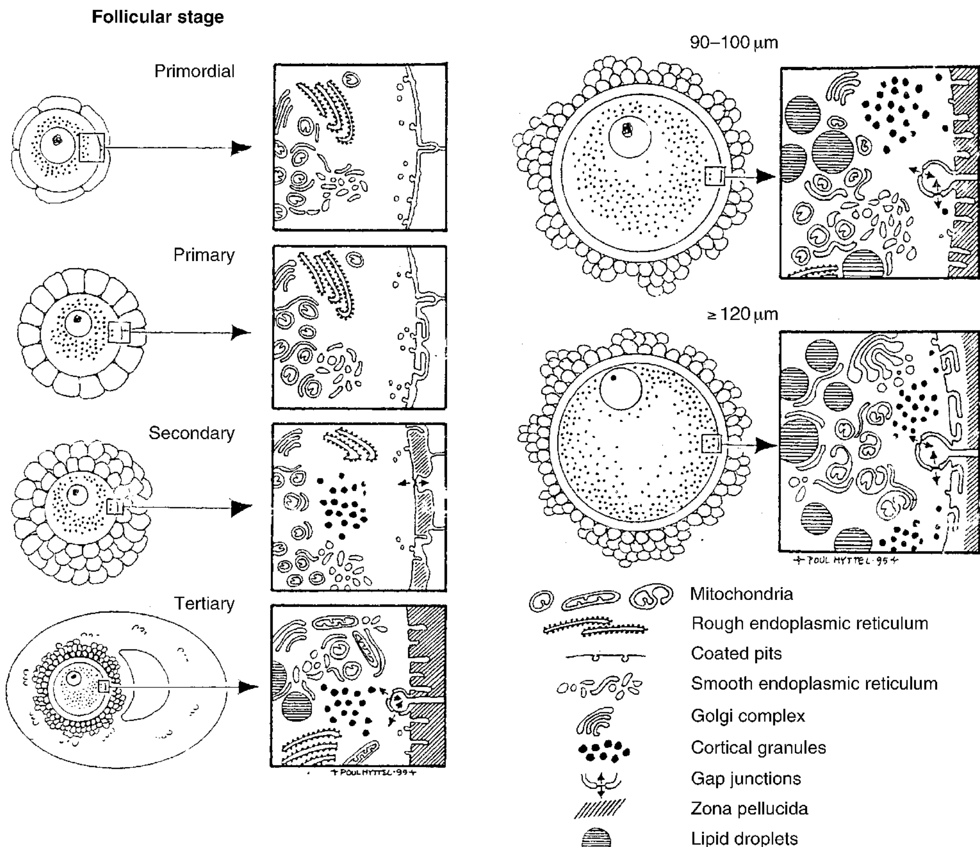


Fig. 2.14. Ultrastructural changes during follicle growth (from Fair, 1995).

characterized by the start of ZP formation and mitochondria becoming more abundant in the ooplasm, indicating the increased energy demands of the oocyte. Development of the secondary follicle is characterized by ZP and cortical granule formation. It appears that granulosa–oocyte communication may be mediated by way of receptor-mediated endocytosis in the early follicle stages and that this contact is modulated to include gap junctions with the formation of the ZP. It is possible that the granulosa–oocyte communication system serves both to maintain meiotic arrest and to stimulate oocyte growth.

Transition to the early antral stage is characterized by marked proliferation of granulosa cells, with antrum formation and complete encasement of oocytes by a ZP. The ZP is transversed by cumulus-cell process endings that terminate in indentations of the oolemma; oocyte activity increase is shown in the proliferation of various ooplasmic organelles. Elongated mitochondria become more abundant and are distributed throughout the ooplasm; lipid droplets, vesicles and Golgi complexes increase in number. In medium-sized antral follicles, oocytes are usually less than 100 μm in diameter, with their nucleus eccentrically located. Oocytes display cortical granules, mainly located in the deep cortical region; arrays of microtubules are evident in the ooplasm of most follicles. As oocyte size increases from 100 to 110 μm, formation of the perivitelline space (PvS) is observed in about 50% of them; by the time the oocyte reaches 20 μm, formation of the PvS can be expected to be complete. In the fully grown oocyte (> 120 μm), the mitochondria are either located at the periphery or dispersed all over the oocyte; many lipid droplets, vesicles and Golgi complexes are visible in the ooplasm.

Studies by Fair *et al.* (1994, 1995a,b) showed a positive correlation between bovine oocyte diameter and follicle size (see Fig. 2.15); as follicle size increases, there is a corresponding increase in oocyte diameter until the follicle has grown to 3 mm and the oocyte reaches maximum size.

The same authors examined the meiotic competence of oocytes from bovine follicles of different sizes. The evidence supports the view that the ability of bovine oocytes to mature (i.e. reach metaphase II) *in vitro* is progressively acquired (see Table 2.8). Results showed germinal vesicle breakdown (GVBD) occurring in 77% of oocytes < 100 μm diameter, but only 67% progressed to metaphase I and 21% to metaphase II. In the 100–110 μm category, 86% of all oocytes reached metaphase I but only 42% developed to metaphase II. When oocyte diameter increased beyond 110 μm, most were able to mature to metaphase II; the author concludes that bovine

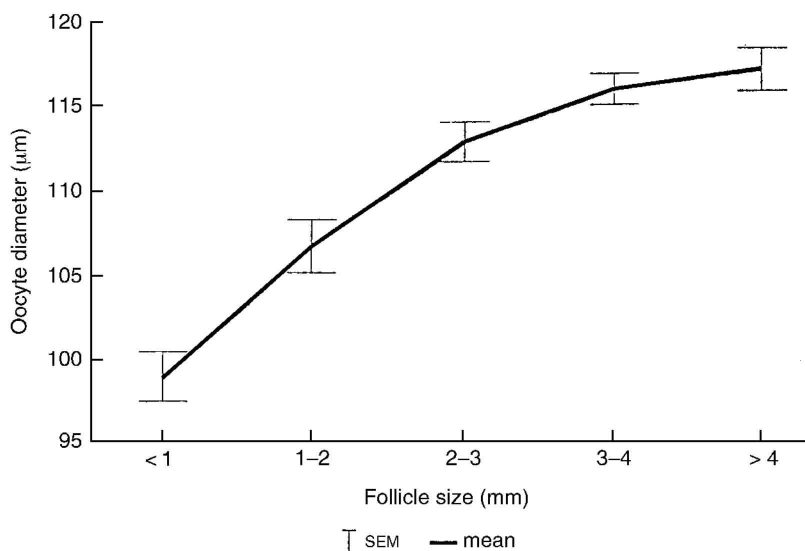


Fig. 2.15. Relationship between oocyte diameter and follicle size (from Fair, 1995). SEM, standard error of mean.

Table 2.8. Relationship between oocyte diameter and maturation rate (from Fair, 1995).

		Stage of nuclear maturation					
Oocyte size	*n*	GV	GVBD	MI	AI	TI	MII
< 100 μm	70	16 (22.8)[a]	8 (11.4)	22 (31.4)	8 (11.4)	1 (1.4)	15 (21.2)[a]
100–110 μm	104	8 (7.7)[b]	7 (6.48)	23 (22.1)	11 (10.6)	11 (10.6)	44 (42.3)[b]
110–120 μm	58	3 (5.17)[b]	1 (1.72)	6 (10.3)	1 (1.7)	3 (5.17)	44 (75.9)[c]
> 120 μm	57	1 (1.75)[b]	0	7 (12.3)	3 (5.3)	0	46 (80.7)[c]

[a, b, c] Values in the same columns with different superscripts differ significantly ($P < 0.01$).

oocytes acquire the ability to complete maturation to metaphase II at a diameter of 110 μm.

The nuclear maturational competence of oocytes from prepubertal calves (5.9–7.1 months of age) was assessed by Majerus *et al.* (1999a,b,c) in Belgium; they found few oocytes with a diameter less than 120 μm capable of completing meiosis. The Belgian workers found that full maturation competence increased as the size of oocyte became greater, a result similar to that found in adult cattle; however, even with oocytes larger than 120 μm, only 55% matured to metaphase II after 24 h in culture.Some of the reasons for the absence of meiotic competence in growing ungulate oocytes have been identified; growing pig oocytes (90 μm diameter) that are unable to resume meiosis have sufficient cell-cycle components for the G2 to M transition but lack the ability to convert these compounds to the active M-phase-promoting factor (MPF) until growth is almost completed.

Nucleus and nucleolus

The nucleus, or GV, of growing oocytes increases in diameter during the growth phase; during the period of nuclear enlargement, chromosomes remain as highly diffuse bivalents. Although rodent oocytes reach their full size before antrum formation, the bovine oocyte is still growing when the follicle reaches the antral stage. During the bovine oocyte's growth phase, gene transcripts, polypeptides and ribosomes are produced by and stored in the oocyte to sustain future early embryonic development. As shown in the studies of Fair *et al.* (1997a,b), the nucleus of a growing oocyte (i.e. an oocyte with a diameter of < 100 μm) is characterized by the presence of one to three transcriptionally active fibrillogranular nucleoli; such nucleoli are prominent structures within the nucleoplasm containing a well-defined granular component (GC) interspersed with vacuoles and numerous fibrillar centres (FC). Changes in nucleolar morphology and transcriptional activity during the growth phase have been described by Fair (1995); it is clear that the transcriptionally active fibrillogranular nucleolus found in growing oocytes is inactivated at an oocyte diameter of 110 μm, which corresponds to an antral follicle diameter of about 3 mm. It is apparent that the fully grown oocyte lacks a functional ribosome-synthesizing nucleolus throughout the two reduction divisions of meiosis and during the first three mitotic cell cycles up to the fourth embryonic cell cycle; clearly, meiosis has a marked effect on nucleolar function.

Mitochondria

Oocyte growth is accompanied not only by a substantial increase in the number of mitochondria present but also by marked changes in their ultrastructure (Fair, 1995). During the growth phase, the mitochondria become closely associated with the smooth endoplasmic reticulum. Numerous elongated and dumb-bell-shaped mitochondria are evident in growing mouse oocytes; it is believed that the dumb-bell-shaped ones are in the process of division. Mitochondria are transformed to round and ovoid highly vacuolated mitochondria that have concentrically arranged cristae as oocytes attain full size.

Golgi complex

As noted with mitochondria, the Golgi complex undergoes marked ultrastructural changes during oocyte growth, as shown by Golgi-complex activity (Fair, 1995). The sacculae become spaced further apart, vacuoles appear and lipid droplets are found in close proximity. As oocyte

growth continues, the Golgi complex exhibits increased numbers of very swollen stacked sacculae that are associated with numerous vacuoles, granules, coated vesicles and lipid droplets. Such changes are consistent with the increased participation of the Golgi complex in the processing and concentration of secretory products and cortical-granule formation during oocyte growth.

Cortical granules

Cortical granules are not found in the oocytes of primordial follicles but first appear when the oocyte is enclosed in the secondary follicle; their formation occurs at a specific stage of oocyte growth. Studies with rodent oocytes show that the first stage of cortical granule formation is the synthesis of dense material in the small Golgi complexes; deposition of this material is followed by coalescence of a few vesicles, giving rise to the formation of dense granules identical to those observed in the oocyte cortex in the mature oocyte.

Ribosomes and cytoplasmic lattices

During the growth phase of the oocyte, the number of ribosomes in the ooplasm increases while ribosome density decreases. The changes in ribosome population during oocyte growth are believed to be related to changes in rates of protein synthesis during this period. Cytoplasmic lattices are lattice-like structures (plaques, lamellae, fibrillar arrays), which are first seen in the early stages of oocyte growth and increase markedly in number throughout the growth period. The function of this extremely abundant cytoplasmic component remains unclear.

Biochemical aspects of oocyte growth

Many biochemical changes occur during oocyte growth. It is clear that the mechanisms of Ca^{2+} homoeostasis in oocytes change during growth and maturation. In mammalian cells, calcium typically carries out its signalling role in the form of very short bursts (spikes) of increased free intracytoplasmic calcium concentration (Ca^{2+}) that repeat periodically; this phenomenon is referred to as calcium oscillations, which usually take the form of waves that propagate throughout the cell. In the bovine oocyte, as in other mammalian oocytes, calcium oscillations occur during meiotic maturation and at fertilization, where they trigger oocyte activation. Workers in London have provided evidence for the mouse that the oocyte acquires the ability to respond to a penetrating spermatozoon with protracted calcium oscillations late in the maturation process (Jones and Carroll, 1994). This response is associated with an increase in the intracellular calcium store and the development of a calcium-induced calcium-release mechanism. There is an obvious need for information about this in other species, including the cow and humans.

There have been reports that the mechanisms underlying the calcium signalling events at fertilization are not present in immature human oocytes but that they develop in the oocyte in the course of its growth (Herbert *et al.*, 1994, 1997). The same authors also found that, despite nuclear maturation of human oocytes in their *in vitro* maturation (IVM) system, such oocytes did not possess a competent calcium signalling system; this may be a factor in explaining low rates of fertilization and embryonic development in human oocytes after artificial maturation.

The general pattern of protein synthesis is believed to remain constant throughout oocyte growth, although there are changes in the relative rate of synthesis of specific proteins. It is well established that the reprogramming of protein synthesis after GVBD is essential for ungulate oocytes to complete meiotic maturation and pronuclear development. Transformation of the sperm nucleus to a male pronucleus requires protein synthesis extending at least until the early metaphase II stage.

Specific proteins synthesized during oocyte growth include mitochondrial proteins, ribosomal proteins, ZP proteins, glycoproteins, histones, tubulin, actin, calmodulin, lactate dehydrogenase, creatine kinase, glucose-6-phosphate dehydrogenase (G6PD) and other proteins involved in fertilization. Structural proteins and enzymes are synthesized and stored throughout oocyte growth, but the turnover of nascent protein in growing oocytes occurs at a relatively low rate, partly accounting for the large stores inherited by unfertilized oocytes. Some of the proteins synthesized by growing

oocytes, such as those in the ZP and the cortical granules, are essential for fertilization and early embryonic development.

The RNA content of oocytes is known to increase markedly during their growth phase, with RNA accumulation exhibiting biphasic kinetics relative to oocyte volume. During the early and mid-growth stages, changes in nucleolar ultrastructure and in levels of RNA polymerase activity are consistent with high rates of transcription of ribosomal RNA. As the compact areas of the nucleolus progressively increase, the synthesis of RNA decreases.

2.6.5. Endocrine events during follicle growth and development

Gonadotrophins

FSH is known to be of crucial importance in the recruitment and selection of the dominant follicle in the cow; it is involved in stimulating aromatase activity in the granulosa cell, which converts the follicular microenvironment from androgenic to oestrogenic. In the cow's preovulatory follicle, the FSH level in follicular fluid is higher than in luteal-phase follicles; the high concentration of FSH at the time of ovulation may be associated with the assumed role of this gonadotrophin in ovulatory-related processes (such as cumulus-cell expansion and mucification).

The LH concentration in follicular fluid is thought to decrease markedly as follicle size increases; this may reflect utilization of LH by follicular cells, particularly granulosa cells, which are bathed in the fluid. In the preovulatory follicles, the levels of LH are similar to those in luteal-phase follicles, but show a peak in the follicular fluid about 4 h after the peak in the peripheral blood; this time-lag may be due to the occupation of free LH receptors in the follicular wall.

Although prolactin is believed to play a beneficial role in the process of cytoplasmic maturation in mammalian oocytes, there is no clear view as to how it exerts its effect. It is known that the level of this gonadotrophin in the peripheral blood during the luteal phase of the cow's oestrous cycle is higher than that found during preovulatory follicle development. The level of prolactin in follicular fluid does not appear to show any significant variation throughout the cycle or in the period of preovulatory development.

Oestradiol and progesterone

In non-atresic antral follicles, the oestradiol level in follicular fluid increases as the follicle grows larger and develops into the preovulatory follicle; progesterone concentration, on the other hand, shows little change and remains very low during final follicular development. After the preovulatory LH surge, the oestradiol level starts to fall, accompanied by an increase in the progesterone level; in the preovulatory follicle, oestradiol ceases to be synthesized by granulosa cells and is replaced by progesterone just prior to ovulation.

It is believed that oestradiol plays an important role in the process of follicular development and ovulation. The steroid sensitizes the granulosa cells to respond to gonadotrophins and stimulates the proliferation and differentiation of the granulosa cell; it also influences the hypothalamus–pituitary axis in producing a small increase in the LH level and a higher frequency of pulsatile release, which eventually results in the preovulatory surge of the gonadotrophin. The physiological role of progesterone in the follicle itself is not well understood; by the time oestradiol concentrations start to fall and progesterone levels to rise, events indirectly attributable to the LH surge, the meiotic maturation of the oocyte is under way. The maturation of the follicle is likely to be influenced by the change in the oestradiol/progesterone balance within the follicle.

Androstenedione and testosterone

Small antral follicles are believed to be androgenic, regardless of whether they are destined to ovulate or to become atresic. With increasing follicle size, the concentrations of androstenedione and testosterone in follicular fluid decrease gradually in both atresic and non-atresic follicles. Small follicles in the late luteal phase and follicular phases of the cycle contain appreciably more testosterone in the follicular fluid than similar-sized follicles in the early luteal phase. Preovulatory follicles contain

significantly greater concentrations of androstenedione and testosterone in the follicular fluid than luteal-phase follicles; after the preovulatory LH surge, the testosterone levels in the follicular fluid continue decreasing but the androstenedione levels increase again. Androstenedione appears to be the major form of the androgen in the follicular fluid, with a level which is more than ten times that of testosterone. It is evident that androgens synthesized by thecal cells are obligatory substrates for oestrogen biosynthesis and may play a role in follicular atresia; their role in oocyte maturation remains uncertain.

2.6.6. Follicular atresia

Atresia is the likely destiny of any antral follicle observed on the surface of the bovine ovary; the process occurs during the period when a dominant follicle is active and affects two groups of follicles. There are those that became FSH-dependent during the preceding growth phase and those that reached the FSH-dependent stage during the dominance phase but lack support for further growth; this atresia may affect some 20–24 follicles, which may comprise some 85% of the follicles in the ovary. From the practical viewpoint, it is important for those engaged in embryo production *in vitro* to know whether such atresia influences the acquisition of developmental competence by the oocyte. It is known, in fact, that follicular atresia in its early stages can promote the acquisition of developmental competence (Blondin and Sirard, 1995). It is also considered that certain events occurring during the early stages of atresia are similar to those occurring after the LH surge in the preovulatory follicle (Wise and Maurer, 1994).

Criteria employed in determining atresia include the steroid content of follicular fluid. Degenerate follicles are characterized by increased progesterone and decreased oestradiol concentrations; this is in contrast to healthy follicles, which have low progesterone and high oestradiol levels. Follicular degeneration is also a consequence of the apoptotic death of granulosa and theca interna cells. In New Zealand, Jolly *et al.* (1994a,b) found evidence suggesting that granulosa-cell apoptosis may occur in healthy follicles and/or occur early in the atresia process before either morphological or biochemical signs of degeneration or dysfunction are detectable. This was supported in Japan by Nakayama *et al.* (2000); apoptosis in granulosa cells appeared to be an initial symptom of atresia. In Australia, Van Wezel *et al.* (1999a,b) studied granulosa-cell death in healthy and atresic cattle antral follicles, using light and electron microscopy and various staining techniques; their results showed that granulosa cells within the membrana granulosa died by apoptosis. In Germany, Jewgenow *et al.* (1999a,b) found a relationship between follicular growth and atresia that varied with oestrous cycle stage and follicle diameter; follicular-stage follicles showed a higher degree of apoptosis than those at the mid-luteal stage. In Canada, Yang and Rajamahendran (2000a,b) concluded that granulosa- and theca-cell death during follicular development and atresia occurs by apoptosis; they found no evidence of apoptotic cumulus cells.

2.7. Induction of Multiple Ovulations in the Cow

For those engaged in *in vitro* fertilization (IVF) research, there may be occasions when it is necessary to obtain *in vivo* matured oocytes for comparative studies with oocytes artificially matured. For this and other reasons, information on superovulation protocols and the normality of oocytes and embryos obtained is relevant to any discussion of *in vitro* embryo production.

The treatment of cattle to induce additional ovulations (i.e. superovulation) has been the object of much research during the past 50 years and has been a major consideration in the development of commercially acceptable ET techniques. The earliest descriptions of superovulation take us back to the days of Smith and Engle in 1927, who used anterior pituitary preparations to induce a fourfold increase in ovulation rates in mice and rats. A few years further on, Cole and Hart demonstrated that the blood serum from pregnant mares would induce multiple ovulations in rats, establishing the basis for what was to become the most widely used gonadotrophin in the treatment of farm animals. Pregnant mare serum gonadotrophin (PMSG) is

a glycoprotein hormone found in the blood of the mare between days 40 and 130 of gestation and is unique among gonadotrophins in possessing both FSH and LH biological activities within the one molecule. It is now clear that PMSG is secreted by specialized trophoblastic cells that invade the mare's endometrium between days 36 and 40; for such reasons, the term equine chorionic gonadotrophin (eCG) is preferred by many. The name notwithstanding, much of the early research on cattle superovulation involved the use of serum gonadotrophin.

The principle behind the induction of multiple ovulations is simple: administer FSH at an appropriate stage of the cycle to ensure additional follicular growth and ovulation. In the conventional superovulation treatment used widely in the 1970s and 1980s, FSH administration was initiated between 8 and 12 days after oestrus, on the basis of many studies showing that there was a greater response at this time than at any other stage of the oestrous cycle; ultrasonic scanning of the ovaries had shown that the second follicle wave emerged at this time. Although the literature contains numerous reports of different regimens and hormone preparations used in superovulation, much of the variability in response can be attributed to the status of follicle wave development at the time of initiating treatment (Adams *et al.*, 1994; Callesen and Greve, 1996; Jaskowski, 1999; Webb *et al.*, 1999; Kanitz *et al.*, 2002; Mapletoft *et al.*, 2002).

2.7.1. Gonadotrophins

Factors important in the induction of multiple ovulations include: (i) the gonadotrophin preparation employed and the way in which it is administered; (ii) adjunct treatments (hormonal or otherwise) aimed at controlling follicle growth and ovulation; and (iii) donor-animal genetics and environmental influences.

Numerous comparisons have been made between different commercial gonadotrophin preparations in the past 30 years; much of the early work was concerned with comparisons between PMSG and FSH-rich anterior pituitary extracts. In more recent times, the emphasis has been on commercial preparations having a consistent and well-defined FSH and LH content, such as Folltropin (Vetrapharm, Canada), which has a low LH content, or Pluset (Laboratorios Calier), which has equal amounts of FSH and LH. Each commercial preparation is likely to have its advocates, based on their experiences with the gonadotrophin under their particular conditions of breed and reproductive management. In Germany, Kanitz *et al.* (1996) reported on dose–response relationships between FSH dose and follicular response; at low doses, response increased with increasing dose of FSH, after which the response plateaued, and then decreased when the dose was further increased. Very high doses of FSH suppressed ovulation completely. Many reports in recent years have come from South America (Benyei and Costa, 1998; Hernandez and Cahuao, 1998; Santos Filho *et al.*, 1998; Zambrano *et al.*, 1998; Ake Lopez *et al.*, 1999; Benyei and Barros, 2000, 2001; Caccia *et al.*, 2000, 2002; Nogueira *et al.*, 2000; Barros and Nogueira, 2001; Barros *et al.*, 2001; Andrade *et al.*, 2002; Coscioni *et al.*, 2002; Fernandes *et al.*, 2002a; Nigro *et al.*, 2002).

Among the areas of active research, reducing the labour involved in FSH administration (Bo *et al.*, 1994; Sugano and Shinogi, 1999; Callejas *et al.*, 2002) and using recombinant FSH preparations (Takagi *et al.*, 1999) are two that have occupied workers in various countries. Other studies have reported on repeated superovulation (Dochi *et al.*, 1998b; Oikawa *et al.*, 1998; Matoba *et al.*, 2002) and on attempts to increase the efficiency of AI in superovulated dairy cattle (Dalton *et al.*, 1999).

2.7.2. Control of follicle growth

It is clear from numerous reports that variation in the superovulatory response of donor cattle is attrributable to the animal's follicular status at the start of treatment (Guilbault *et al.*, 1996; Wolfsdorf *et al.*, 1997); the presence of a functional dominant follicle at the start of treatment is likely to result in a decreased response. Experimentally, Ede *et al.* (1999) in France punctured the largest follicle 2 days before starting superovulatory treatment, and concluded that this procedure may improve total embryo production and quality under farm conditions; similar evidence was reported by Kim, I.H. *et al.* (2000)

and Lee, D.W. *et al.* (2000) in Korea; removal of the dominant follicle 48 h prior to superovulation promoted follicular growth and ovulation in Friesian and Hanwoo cattle. In Canada, Kohran *et al.* (1998) used GH-RH to elicit the predictable emergence of a synchronous follicular wave in cattle at different stages of their oestrous cycles; this enabled them to promote a dominant follicle that could be punctured at a predictable time (4 days after GH-RH) and to initiate superovulation treatment after a further 2 days. In Argentina, Bo *et al.* (1998) treated beef heifers with oestradiol benzoate and progesterone at the time of CIDR insertion; they reported evidence of an increased yield of high-quality embryos after superovulation, initiated at the time of predicted wave emergence, 5 days after such steroid treatment.

In Canada, Mitchell *et al.* (1998) reported similar responses in dairy cattle when either oestradiol-17β and progesterone or GH-RH was given (at time of CIDR insertion) to synchronize follicle-wave emergence; in the same country, Mapletoft *et al.* (1999) achieved higher yields of transferable-quality embryos as a result of tighter synchrony of follicle-wave emergence when progestogen-treated (norgestomet) cattle received oestradiol-17β and progesterone (day 1) rather than the conventional treatment of norgestomet and oestradiol valerate (day 0). In the USA, Meyer *et al.* (2000) compared traditional superovulation regimes with those implementing the use of the CIDR with injections of oestradiol benzoate and progesterone at CIDR insertion; data from the study suggested that donors could be successfully superovulated without regard to the oestrous cycle (see Table 2.9).

Controlling ovulation

It is clear, from many reports, that there may be an inhibition or complete absence of the preovulatory LH surge in cattle undergoing superovulation treatment. Price *et al.* (1999) concluded that there is a reduction in endogenous pulsatile secretion of LH during superovulation, which may be due to the negative feedback effects of oestradiol. It is also apparent that cattle undergoing superovulation may suffer problems in the timing and character of endogenous LH release. This has led workers to devise treatments that not only assist in the recruitment of follicles but also enable many such follicles to develop to the point where they are capable of ovulating and releasing competent oocytes. Australian workers blocked the preovulatory LH surge a with a GH-RH agonist (deslorin) and induced ovulation after superovulation with an LH-rich preparation (D'Occhio *et al.*, 1998, 1999); their protocol eliminated the need to detect oestrus and enabled donor animals to be bred at a predetermined time. Elsewhere, Oussaid *et al.* (1998) examined the effect of prolonging the follicular phase in superovulated cattle with a GH-RH antagonist (antarelix); delaying the preovulatory LH surge appeared to improve oocyte developmental competence.

2.7.3. Animal and environmental effects

In France, Manciaux *et al.* (1999, 2000) investigated the influence of the paternal origin of Montbeliard donor cattle on embryo production after superovulation; evidence was found of a significant interaction between AI sire and donor paternal origin on the yield of transferable-quality embryos in this breed. In South America, Tonhati *et al.* (1999) estimated the repeatability and heritability of the superovulatory response of 2941 Friesian cattle in 473 herds; environmental factors apparently

Table 2.9. Superovulatory responses with or without CIDR-P4-OB (from Meyer *et al.*, 2000).

	Total ova		Viable embryos		Degenerate		Unfertilized	
	Brahman	Cont.	Brahman	Cont.	Brahman	Cont.	Brahman	Cont.
Control	$11.5^a \pm 0.86$	$12.4^a \pm 0.86$	$6.5^a \pm 0.57$	$5.9^a \pm 0.58$	$2.4^a \pm 0.31$	$2.5^a \pm 0.31$	$2.6^a \pm 0.50$	$3.9^a \pm 0.50$
CIDR	$9.7^b \pm 0.97$	$9.4^b \pm 0.70$	$5.8^a \pm 0.65$	$5.1^a \pm 0.47$	$1.8^a \pm 0.34$	$2.1^a \pm 0.25$	$2.0^a \pm 0.56$	$2.2^b \pm 0.40$

[a,b]Data in columns with different superscripts are different ($P < 0.05$, ± SEM, Student's *t* test).
Cont., Continental and English breeds; SEM, standard error of mean.

played the major role in determining response. The authors found little evidence that the response of individual animals was a heritable characteristic or was predictable on the basis of previous results. In Brazil, dairy (Friesian) heifers were shown to be superior to lactating cows in terms of embryo production after superovulation under high environmental temperatures (Benyei and Costa, 1998; Benyei *et al.*, 1999; Benyei and Barros, 2000); this may have been due to an inability to maintain normal body temperature under heat-stress conditions because of the metabolic demands associated with lactation.

Nutritional effects

According to Le Coustumier (1995), superovulatory response can be improved by controlling the feeding of dairy cattle; breeding is most likely to be successful if carried out either some 60–70 days or > 110 days *post-partum*; such timing helps to ensure that follicles selected for growth are selected before or after the critical period of nutrient deficit that is believed to occur in the first weeks of lactation. The same author observes that the level of nitrogen in feeds should be monitored so as to avoid high protein-energy diets, which may have a depressive effect on superovulation. Studies by McEvoy *et al.* (1996) dealt with the progesterone profiles and superovulatory responses of Simmental heifers in relation to preovulatory energy intake and progesterone priming treatment. In Ireland, Nolan *et al.* (1998b) found evidence that the dietary intake of superovulated cattle may influence the rate of development of embryos; the rate was higher in heifers on a restricted diet than in animals on an *ad libitum* diet. Other work in the same country also showed evidence of improved embryo yields after food restriction (Nolan *et al.*, 1998a) and that different nutritional levels can influence the expression of developmentally important genes in the bovine embryo (Wrenzycki *et al.*, 1999a). Further work in Ireland has shown that molecular and metabolic variations may exist in embryos derived *in vivo* and developed in donor heifers on different nutritional regimens differing in type and quantity (Wrenzycki *et al.*, 2000a); it may be possible to use molecular markers to determine conditions that best suit the production of good-quality embryos. In France, high-energy diets adversely affected both ovarian response and embryo quality (Negrao *et al.*, 1997; Humblot *et al.*, 1998a,b). Saumande *et al.* (1998), also in France, recommended a plane of nutrition giving a daily weight gain of 1 kg as optimal for the production of viable embryos in Friesian heifers; further work in that country showed that a commercial diet supplement (Nucleor Transplan, Chemoforma SA) provided for 4 weeks before superovulation may improve embryo quality in Montbéliard cows (Manciaux *et al.*, 2001).

In the UK, Gong *et al.* (1999) found that increased dietary intake (200% maintenance requirements) for 3 weeks preceding superovulation enhanced the recruitment of ovarian follicles in cross-bred heifers and led to significantly more ovulations than in control animals (18.1 vs. 10.6); the authors suggest that such treatment may provide a useful approach to superovulation in heifers. In Ireland, on the other hand, Yaakub *et al.* (1999a,b) found that *ad libitum* concentrate intake and barley-based concentrates had a negative influence on the quality of embryos; the authors conclude that the mechanisms by which such nutritional effects influence the induction of multiple ovulation remain unclear.

Although a study by Kommisrude *et al.* (2002) in Norway, seeking to assess the effects of blood urea content in donor cattle on embryo quality, found no effect of different feeding regimes, in the UK Dawuda *et al.* (2002) were able to describe circumstances in which quickly degradable urea nitrogen did compromise embryo development. It was shown that excess dietary urea had a deleterious effect on the yield and quality of embryos recovered 7 days after breeding, although the effect was only found if urea feeding commenced at the time of breeding and after the time of follicle selection. Such findings have practical relevance in explaining the reduced fertility often experienced in dairy cattle turned out to pasture in the spring. Under such circumstances, cows are subject to a rapid increase in their intake of highly degradable protein and experience elevated concentrations of blood urea and ammonia; when such animals are bred within 10 days of turnout, it is likely that they will experience a high level of embryo loss by day 7, with a consequent reduction in fertility. Of

considerable practical interest, however, is the finding recorded by Dawuda *et al.* (2002) that cows are able to adapt within 10 days to the toxic effects of excess urea, and reduced fertility should not be evident beyond that time.

2.7.4. Long-range assessments and sexed semen

In Canada, Pierson and Adams (1999) demonstrated the feasiblity of assessing the follicular status and ovarian response in donor cattle when the animals in question, the laboratory conducting the analyses and the operator carrying out the superovulation treatment were geographically separated. In commercial practice, there may be occasions when the Internet may be useful for data transfer, image analysis and clinical evaluation of animals undergoing superovulation. Another up-and-coming development may be the use of sexed semen in the breeding of superovulated donors. In Colorado, there have been encouraging results from studies in which superovulated heifers were inseminated with sexed semen (Chung *et al.*, 1998); sexing the recovered embryos showed 93% (43/52) to be of the intended sex.

2.7.5. Recombinant bovine somatotrophin (r-BST) and follicle growth

Growth hormone (somatotrophin) is secreted by the anterior pituitary gland. Its most obvious action in the growing animal is to induce linear growth of the long bones; in this, growth hormone acts on the epiphyseal growth plates of the long bones of the young animal. It is believed that most of these effects on growth are indirect and are mediated via IGF-I, which is secreted mainly by the liver in response to growth hormone. Recombinant bovine somatotrophin (r-BST) came on the commercial scene some years ago and its use by researchers has increased understanding about its involvement in ovarian function.

Growth hormone has an important role in reproductive biology. It has been suggested that growth hormone can legitimately be classified as a gonadotrophin on the basis of evidence now available. Whatever gonadotrophic action the hormone may possess, it seems likely that this will depend on the concurrent involvement of the conventional gonadotrophins (FSH/LH). For that reason, some suggest that the term 'co-gonadotrophin' may be more appropriate. The commercial availability of r-BST has led to its use in various attempts to stimulate follicular growth, usually in association with FSH. There is a considerable body of evidence suggesting the existence of an intraovarian IGF system, complete wiith ligands, receptors and binding proteins. It is clear that r-BST stimulates the synthesis of IGF-I, not only in the liver but also in several other tissues. In the ovary, for instance, IGF-I is found in high concentrations in follicular fluid, particularly in the dominant follicle. In the USA, Spicer and Chamberlain (2000) have reported data indicating that, during follicular development in cattle, changes in intrafollicular levels of IGF-I may be due to hormonally induced changes in granulosa-cell, but not thecal-cell IGF-I production. Given that r-BST can stimulate ovarian IGF-I and that this growth factor stimulates granulosa-cell function, it is not unreasonable to expect that the hormone is likely to influence superovulatory responses.

Studies in Edinburgh more than a decade ago showed that treatment with r-BST led to a twofold increase in the population of antral follicles of 2–5 mm diameter in heifers. Further work by Gong *et al.* (1995, 1996, 1997) indicated that pretreatment of heifers with r-BST significantly enhanced the superovulatory response to pituitary FSH in terms of ovulation rate and number of transferable embryos recovered per animal; it was concluded that this pretreatment would be a useful means of improving superovulatory response. Further studies in Scotland demonstrated that the effect of r-BST treatment is probably mediated through an increase in circulating levels of IGF-I or insulin or both, rather than by way of a change in the secretion of pituitary gonadotrophins or a direct effect of growth hormone on ovarian follicles. In the USA, Tripp *et al.* (2000b) treated beef heifers for 100 days with r-BST prior to follicular aspiration and treatment continued for the further 42-day period when follicles were aspirated; treatment with growth hormone yielded nearly 50% more follicles than control animals per OPU session. Recombinant growth

hormone has also been used effectively in large-scale work in Brazil to increase the number of growing follicles (Carter *et al.*, 1998; Ferraz *et al.*, 2002) and in the USA, where DeSouza *et al.* (2002) used it and oestradiol to enhance ovulatory response to FSH in beef and dairy animals.

2.7.6. Characteristics of preovulatory follicles and oocytes after superovulation

Although a report by Hafez and Ishibashi in the early 1960s suggested that the cytological characteristics of oocytes recovered from superovulated cattle were normal, subsequent reports have not always borne out that finding; it is now well recognized that superovulation in cattle can sometimes result in abnormal follicular steroidogenesis, premature maturation and ovulation of oocytes, deviant systemic hormone profiles and other changes. Nevertheless, it has also to be noted that thousands of oocytes go on to produce embryos and normal healthy calves each year. A study in Denmark by Assey *et al.* (1994a) investigated intrafollicular steroid concentrations and oocyte structure in FSH-treated and control heifers. They found that the mean oestradiol-17β and progesterone concentrations of potentially ovulatory follicles of superovulated animals were lower than those of similar follicles in control cattle. The oocytes from the FSH-treated cattle showed several deviations in comparison with controls, including reduced numbers of lipid droplets and lack of lipid–mitochondria association, enlarged rough endoplasmic-reticulum compartment, premature PvS formation and lack of nucleolar vacuolization. It is likely that such deviations would lead to impaired developmental competence of the superovulated oocytes; the only degenerate oocytes in the study were found in the superovulated heifers. A paper by Callesen *et al.* (1995) dealt with factors affecting the developmental stage of embryos recovered on day 7 from superovulated dairy cattle.

A study by Blondin *et al.* (1996) in Canada used oocytes for embryo production that were recovered from cattle superovulated with different FSH-P doses and prostaglandin; such treatment did not increase the developmental competence of the oocytes. It is known that superovulation alters the normal course of maturation in oocytes; it is believed that superovulation may induce an accelerated growth of follicles, resulting in oocytes that are not fully competent.

3

Recovering the Bovine Oocyte

Oocyte collection is usually performed by the isolation of follicles, dissection, aspiration or slicing of follicles or oviductal flushing. The recovery technique will vary according to whether pre- or postovulatory oocytes are required and whether collection is to be made from slaughtered animals or from live cattle on a single or repeated occasions. In dealing with the live animal, it may be a matter of removing ovaries by ovariectomy or by getting access to the ovaries by way of laparoscopy or ultrasound guidance (see Besenfelder *et al.*, 1999, 2001). There are also considerations for removing ovaries prior to animal slaughter, as reported by Alberio *et al.* (1998b); ovariectomy enabled them to obtain ovaries in Argentina in better hygienic conditions and with more reliable identification than those collected at the abattoir. The same authors noted that some 200,000 high-genetic-quality beef and dairy cattle are culled per year because of non-reproductive problems; utilizing their ovaries was seen to be a way of preserving genetic resources by *in vitro* embryo production.

3.1. Oocyte Recovery: Abattoir Ovaries

In the production of embryos from slaughterhouse ovaries, it is necessary to develop methods that facilitate the recovery of several good-quality oocytes per ovary; the number recovered will vary with different collection procedures. A review by Nagai (2001) focuses on the selection of ovaries likely to yield competent oocytes from animals at the abattoir.

3.1.1. Dissecting the intact follicle

Sheep and cattle

Dissection of the intact antral follicle (2–8 mm diameter) and its subsequent carefully controlled rupture was used by Cambridge workers in the 1980s as an important element of their artificial maturation technique employed in sheep. The same method was used in early cattle work in Dublin (see Fig. 3.1) as a means of recovering morphologically acceptable oocytes with the least disruption to the surrounding cumulus cells. An advantage of follicle dissection as the oocyte recovery method was held to be in identifying non-atresic follicles. Cambridge workers had described the morphological characteristics that permitted such follicles to be identified in sheep; these included features such as uniformly bright translucent appearance, evidence of extensive vascularization and possession of a regular stratum granulosum layer within the follicle. Atresic follicles, in contrast, may be expected to show a dull, grey, opaque appearance and to be poorly vascularized. It should be noted, however, that regressing follicles in the early stages of atresia may actually yield oocytes of greater developmental competence than those coming from growing follicles or those at the plateau stage.

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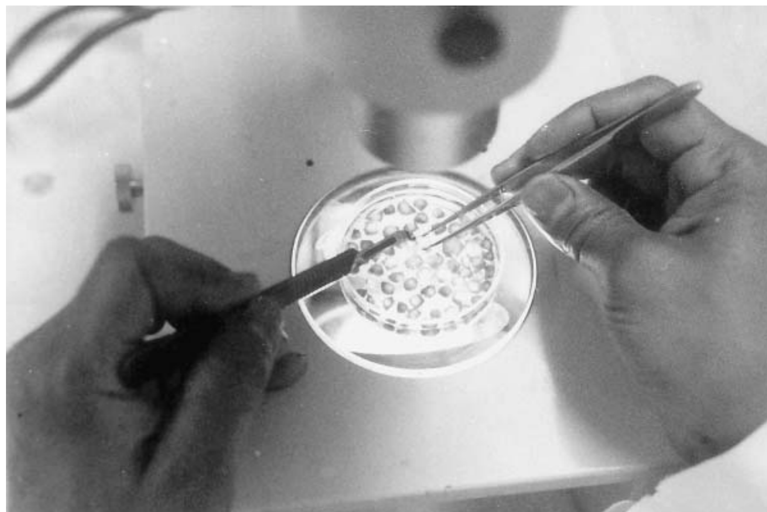

Fig. 3.1. Bovine oocyte recovery by follicle dissection. Follicle dissection was the method initially used in Ireland by Lu and associates in recovering bovine oocytes for maturation and fertilization studies. The cumulus–oocyte complex (COC) was liberated by rupturing the intact follicle in the dissection medium.

Work in Dublin comparing follicle dissection with aspiration generally supported the view that greater yields of high-quality oocytes were obtained by dissection. Part of the explanation was held to lie in the fact that aspiration resulted in greater disruption of surrounding cumulus cells; this may be due to the cumulus oophorus being firmly attached to the stratum granulosum. In New Zealand, Hagemann *et al.* (1999b) dissected follicles in abattoir ovaries and reported that those collected during phases of follicular growth (days 2 and 10) were more competent than those recovered during phases of follicular dominance (days 7 and 15); they also recorded that oocyte competence tended to increase with growing oocyte size.

Other farm animals

In India, Gogoi *et al.* (2001) compared the efficacy of aspiration and dissection techniques for the recovery of oocytes from goat ovaries; the collection of high-quality oocytes was significantly greater with dissection. The authors concluded that the higher recovery of oocytes with a good investment of cumulus cells indicated the superiority of that technique. In Italy, equine oocytes were recovered from abattoir ovaries by aspiration or after opening follicles and scraping the granulosa layer (Dell'Aquila *et al.*, 2001); it was found that the rate of abnormal fertilization was significantly higher for oocytes collected by aspiration (19% vs. 6% for dissection). These results demonstrated that the collection method may contribute to differences in results observed among laboratories working with horse oocytes.

3.1.2. Aspiration techniques: old and new

Recovery of bovine oocytes by aspiration of antral follicles, using various devices (pipette, syringe and needle, aspiration needle under vacuum pressure), has been the method most commonly employed with cattle ovaries retrieved from the slaughterhouse (see Fig. 3.2). One of the difficulties initially associated with the aspiration approach lay in the fact that oocytes might only be recovered from some 30–60% of the punctured follicles; this could contrast markedly with follicle dissection, where 100% recovery was not unusual. Quality-wise, it was common experience to find this to be lower with aspiration than with follicle dissection The advantage of follicle aspiration is in terms of speed of operation, which may be particularly important in a commercial embryo production unit; Dublin experience with aspiration as a means of acquiring oocytes in the 1980s showed

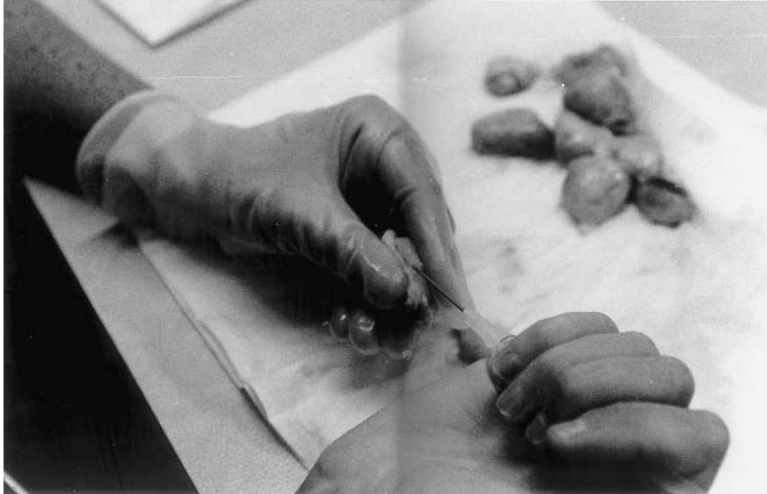

Fig. 3.2. Bovine oocyte recovery by follicle aspiration, the most widely used of the recovery procedures. In this instance, antral follicles (2–8 mm diameter) are being aspirated using a 2 ml syringe and an 18-g needle.

it to be three times faster than dissection. None the less, it is also important to take due note of quality considerations; there is little merit in speed of operations unless this results in a higher yield of developmentally competent embryos.

Bovine follicles (usually 2–8 mm diameter) have been aspirated with 18–22-g needles and 3–20 ml syringes or with 16–19-g needles attached to a vacuum pump using pressures of 75–100 mmHg. In Australia, Fry *et al.* (1997) examined the effects of aspiration pressure, needle gauge and flushing method on oocyte collection in attempts to optimize the number and quality of primary oocytes available for embryo production. With the needles tested, the easiest and most successsful aspiration of primary oocytes from ovaries was achieved using a 17-g single-lumen needle at a vacuum pressure of 55 mmHg. Increasing the vacuum pressure reduced the number of viable oocytes, presumably because cumulus cells were stripped away; there was a dramatic increase in the number of denuded oocytes found when the vacuum pressure was too high. In Belgium, Bols *et al.* (1997a,b) carried out ovum pick-up (OPU) simulation studies using short- or long-bevelled 20-g disposable needles; increased aspiration vacuum decreased the number of compact cumulus–oocyte complexes (COCs), the effect being less marked for the short- than for the long-bevelled needle.

In Japan, Hashimoto *et al.* (1999a,b) studied the effects of aspiration pressure and needle diameter on the efficiency of oocyte recovery from slaughterhouse ovaries or from live animals. For abattoir ovaries, the highest oocyte recovery rate was obtained using 18-g needles and 40 mmHg; optimal conditions for oocyte recovery from slaughtered cows are not necessarily applicable to live animals. In the Netherlands, Vos *et al.* (1999) compared two aspiration systems to recover oocytes from ovaries obtained from superovulated heifers by ovariectomy; the system using a 16-g needle and 30 mmHg of vacuum recovered a higher proportion of COCs than that using an 18-g needle and 150 mmHg. The lower vacuum system recovered COCs that were more firmly attached to the follicle wall; their higher developmental competence was reflected in a greater yield of blastocysts after processing (see Table 3.1). Similar evidence was provided by Ward *et al.* (1999) in Ireland; they recommended an aspiration vacuum pressure of < 50 mmHg to maximize the number of good-quality oocytes obtained.

3.1.3. Ovary slicing techniques

Several studies have reported on oocyte recovery techniques involving the slicing, cutting or

dissection of the bovine ovary as a means of recovering immature oocytes; slicing techniques have been either directly applied to ovaries or applied after completion of aspiration. Some workers have reported a threefold increase in oocyte numbers compared with aspiration; others have shown the method to be capable of acquiring greater oocyte numbers in sheep and goat ovaries. Dublin workers found that slicing took about three times longer per ovary than aspiration, but this was compensated for by the threefold increase in yield. In terms of workload, slicing provided the same number of oocytes per unit of time as aspiration, but with many fewer ovaries (see Table 3.2; Carolan *et al*., 1994).

In Denmark, Vajta *et al.* (1996a) reported that the number of oocytes recovered and the number of blastocysts obtained was approximately doubled by slicing ovaries in comparison with aspiration. In Italy, Vincenti *et al.* (1998) used a multiple razor-blade in slicing ovaries from Piedmontese cattle and recorded an oocyte yield of 10.7 per ovary; most of the oocytes (9.7 per ovary) were selected for processing.

In Italy, three methods for the recovery of cattle oocytes from ovaries – dissection, aspiration and slicing (in which the ovarian cortex was cut lengthwise and crosswise) – were compared by Mantovani *et al.* (1999b); comparisons of the effects of individuals performing these operations (skilled vs. unskilled) were also made. The number of COCs with more than four layers of cumulus cells and the number of oocytes classified as suitable for maturation were significantly lower for the aspiration method than for the other two methods. The number of COCs recovered in 10 min was 4.3, 3.5 and 6.6 for the dissection, aspiration and slicing methods, respectively. Operator skill had no effect on the number and quality of oocytes recovered but unskilled operators collected fewer oocytes in 10 min than skilled (3.5 vs. 5.0). The workers concluded that slicing was the best method for producing a large number of embryos. In Russia, Malenko (1999) has described a razor-blade device that enabled him to recover some 13 COCs per ovary, the majority (73%) with a dense multilayer of cumulus cells; the device enables

Table 3.1. Bovine oocyte quality and aspiration vacuum pressure (from Vos *et al*., 1999).

No. follicles aspirated	No. COCs recovered	No. COCs selected for IVP	No. blastocysts day 9	No. hatched blastocysts day 11
System 1 381	182 (47.8)[a]	128 (70.3)[b]	52 (40.6)[c]	36 (28.1)[c]
System 2 369	245[d] (66.4)[a]	208[d] (84.9)[b]	126[d] (60.6)[c]	109[d] (52.4)[c]

[a]Values in parentheses relative to follicles aspirated.
[b]Values in parentheses relative to COCs recovered.
[c]Values in parentheses relative to COCs selected.
[d]Chi-square test ($P < 0.01$).
System 1, 150 mmHg pressure; System 2, 50 mmHg pressure.

Table 3.2. Bovine oocytes obtained by slicing or aspiration (from Carolan *et al.* 1994).

	Oocyte classification*						
Treatment	A	B	C	D	E	F	Total
Aspiration	4.3 ± 0.9[a]	2.5 ± 0.6	1.7 ± 0.1[a]	3.1 ± 0.9	1.4 ± 0.5[a]	0.7 ± 0.2	13.9 ± 2.3[a]
	(30.9)	(18.0)[x]	(12.2)	(22.3)[x]	(10.1)[x]	(5.0)	(100)
Dissection	17.1 ± 1.9[b]	3.1 ± 0.4	7.5 ± 1.4[b]	5.1 ± 1.5	9.8 ± 1.8[b]	1.7 ± 0.3	44.2 ± 4.4[b]
	(38.7)	(7.0)[y]	(17.0)	(11.5)[y]	(22.2)[y]	(3.8)	(100)

*Oocyte classification: A, COCs with an even cytoplasm and four or more cumulus-cell layers; B, COCs with an even cytoplasm and less than four cumulus-cell layers; C, COCs with a granular cytoplasm; D, denuded oocytes with an even cytoplasm; E, denuded oocytes with a granular cytoplasm; F, oocytes with an expanded cytoplasm.
[a,b,x,y]Values in the same column with different superscripts differ significantly ($P < 0.05$).

oocytes to be collected without destroying their cumulus layers. In the USA, Fazio *et al.* (1999) collected oocytes by a slicing method, targeting follicles > 3 mm and < 10 mm in diameter in cyclic and pregnant beef cattle; the authors recommend that cows in good condition and/or pregnant should be selected due to increased numbers of follicles providing acceptable oocytes.

The large number of oocytes recovered by slicing is presumably due to the fact that those located in follicles under the ovarian surface (cortical location) are obtained in addition to those located in peripheral (surface-visible) follicles. When the numbers of oocytes from both peripheral and cortical follicles are combined, it is apparent that the total yield is about double that collected from one ovarian site alone (Arlotto *et al.*, 1996a); only cortical oocytes that have reached the stage of meiotic competence can be expected to mature. Apart from being time-consuming, slicing is open to criticism on the grounds of risking contamination of the COCs during the collection procedure.

Slicing and aspiration

Several reports have dealt with the slicing of ovaries after the preliminary aspiration of follicles; in terms of oocytes recovered or their quality, there appeared to be no merit in combining the two procedures.

Other farm animals

A report from India by Jain *et al.* (1995) showed that slicing was the most effective of the three recovery procedures they employed with slaughtered buffalo ovaries. It might also be noted that, in horses, ovary slicing rather than follicle aspiration has been one means of increasing the yield of oocytes per ovary; in Spain, Erice *et al.* (1998) recovered an average of 0.92 oocytes per ovary by aspiration and 1.36 by slicing.

3.1.4. Transillumination-aspiration ovary (TAO)

In Israel, Arav (2001) developed a novel technique that permits direct visualization of cortical follicles for aspiration of oocytes by transillumination of the ovarian medulla and cortex with a Plexiglas rod inserted through a small incision at the hilus; the technique, referred to as 'transillumination-aspiration ovary' (TAO), increased oocyte yield by 50% per ovary. A greater number of follicles was visible under central ovary illumination than under conventional lighting; when these follicles were aspirated with an 18-g needle, the oocytes and follicular fluid could be recovered clearly and relatively aseptically, which facilitated their screening and subsequent culture. In contrast to the slicing technique, where it is not possible to determine the size of follicles located in the cortical region, with the TAO method this difficulty does not arise; the developmental competence of oocytes was similar for both cortical and peripheral regions of the ovary. The author concluded that TAO allowed many more visible follicles to be identified in the ovary; the technique permitted 7.3 oocytes per ovary to be recovered, which was 50% greater than when using the regular aspiration method.

3.2. Abattoir Ovaries

The methods adopted in recovering oocytes for embryo production will differ according to their source. If they are to come from slaughterhouse ovaries, then due care and attention must be paid to time and temperature in handling the ovaries prior to the start of *in vitro* maturation (IVM). If the oocytes are to come from live cattle by OPU, then there is a somewhat different sequence of events leading up to the start of maturation in the laboratory.

3.2.1. Ovary storage: temperatures and time-limits

In the recovery of oocytes from slaughtered cattle at the abattoir, the question of the time interval between animal slaughter and removing the oocyte from the follicle is an important consideration; there is also the matter of defining the temperature limits that should be observed in storing ovaries before they are processed in the laboratory. In general, workers dealing with

cattle oocytes recover them within 1–2 h of animal slaughter; ovaries are usually stored at about 30°C. In Utah, Wang *et al.* (1995a,b) evaluated the developmental competence of oocytes aspirated from ovaries stored for varying intervals at two different temperatures and examined the nuclear changes resulting from storage; they concluded that storage either at 4°C or at room temperature resulted in considerable loss in the developmental capacity of bovine oocytes, even with storage times as short as 24 h. This finding was in contrast to an earlier report from Cuba indicating that ovaries could be safely preserved at 4°C for short periods (Solano *et al.*, 1994); however, the Cuban work did not go beyond the cleavage stage of development.

In Portugal, Marques *et al.* (1997a) stored ovaries from slaughtered cows at a temperature of 4 or 37°C prior to IVM; storage at 4°C resulted in a significantly lower percentage of matured oocytes and a marked decrease in the yield of good-quality embryos (2.7% vs. 27.2% for non-cooled oocytes). In Austria, Schernthaner *et al.* (1997, 1998) investigated the effect of storing ovaries for 24 h at different temperatures; they showed that it was possible to store cattle ovaries at 15–21°C before maturation for at least 12 h without adversely affecting the developmental competence of the oocytes.

3.2.2. Ovary storage to enhance oocyte quality

A review by Sirard and Blondin (1996) has noted evidence showing that the developmental competence of bovine oocytes may be enhanced by the exposure of ovaries to warm incubation conditions for a few hours before recovering the oocytes. As noted elsewhere, an earlier study by Blondin *et al.* (1995), in which whole ovaries were kept at about 30°C for 3–4 h after slaughter, had reported significantly improved oocyte developmental competence; the authors suggested that the 4 h period after slaughter might have created a specific follicular microenvironment in which changes occurred in the oocyte similar to those normally associated with the preovulatory follicle in the hours leading up to ovulation.

3.2.3. Temperature sensitivity of oocytes

Various workers have reported on the temperature sensitivity of bovine oocytes. In the USA, Aman and Parks (1994) studied the effects of abrupt cooling to 4°C on the meiotic spindle and metaphase chromosomes of IVM bovine oocytes and to determine whether such effects could be reversed by rewarming; the workers concluded that oocytes should be maintained at temperatures as close to 39°C as possible during *in vitro* procedures in order to maintain normal spindle morphology. Such councils of perfection would not seem practicable. In Canada, Martino *et al.* (1995) concluded from their studies that, although germinal vesicle (GV) cattle oocytes could be cooled to 20°C without compromising their capacity for maturation, fertilization and development *in vitro*, exposure to temperatures of 10°C or lower had adverse effects on their cleavage and development into blastocysts. Such effects were time-dependent and not obvious within the first 48 h; there was little or no effect on nuclear maturation and subsequent fertilization. In Guelph, Wu *et al.* (1998) investigated the effects of chilling GV oocytes on the formation of microtubules and meiotic spindle; compared with control oocytes held at 39°C, holding oocytes for 10 min at 31 or 24°C did not significantly alter the formation of normal spindles, but cooling oocytes to 4 or 0°C did. While cleavage of oocytes held at 24–39°C was similar (> 70%), only 42 and 37% of oocytes cooled to 4 or 0°C cleaved after fertilization. In Germany, Schwartz *et al.* (1998) reported results indicating that satisfactory embryo production could be obtained after storing COCs in tissue-culture medium 199 (TCM-199) or bovine follicular fluid at 20°C for up to 10 h.

3.2.4. Follicle size and quality

Many studies in recent years with abattoir ovaries have shown a clear relationship between follicle size and oocyte quality, with oocytes recovered from follicles > 6 mm in diameter proving superior in developmental competence to those from smaller follicles (Lonergan *et al.*, 1994a). A paper by Kubota and Yang (1998)

Table 3.3. Follicle size and oocyte development (from Kubota and Yang, 1998).

Experiment	Follicle size (mm)	No. of oocytes	No. (%) cleaved	No. (%) developed to Blastocyst (BL)	Hatched BL
1. IVF	5–8	77	59 (77)[ab]	40 (52)[a]	24 (31)[a]
	2–5	122	103 (84)[a]	56 (46)[a]	43 (35)[a]
	1–2	117	80 (68)[b]	30 (26)[b]	21 (18)[b]
	< 1	64	20 (31)[c]	7 (11)[c]	1 (2)[c]
2. Act	5–8	86	78 (91)[a]	38 (44)[a]	6 (7)[a]
	2–5	113	98 (87)[a]	43 (38)[ab]	6 (5)[a]
	1–2	74	65 (88)[a]	22 (30)[b]	4 (5)[a]
	< 1	42	20 (48)[b]	6 (14)[c]	0 (0)[a]
3. NT	2–5	90	76 (84)[a]	24 (27)[a]	–
	< 1	53	46 (87)[a]	6 (11)[b]	–

[a,b,c]Values within experiment with different superscript differ ($P < 0.05$).
Act, activation; NT, nuclear transfer.

presented data suggesting that bovine oocytes from follicles of different sizes differed in their developmental competence and that poor development of oocytes from small follicles was probably the result of cytoplasmic incompetence (see Table 3.3).

Elsewhere, in a report by Henriksen *et al.* (1998), it was recorded that oocytes from preovulatory follicles yielded twice the number of blastocysts produced by oocytes from 2–8 mm diameter follicles. Looking at the live animal, it is the dominant follicle that ovulates the oocyte after a rapid period of growth from about 4 mm to 15 mm in the space of about a week. Clearly, as the follicle grows towards the preovulatory stage, the oocyte is exposed to changes in the follicular microenvironment that influence its quality. A study by Van de Leemput *et al.* (1998) showed that preovulatory-sized follicles with a high oestradiol concentration yielded more competent oocytes than follicles showing a low level; it was not clear whether oestradiol itself was favourable to the oocyte or whether the concentration was indicative of a healthy follicle that provided the oocyte with the microenvironment favourable to its development.

Results presented by Henriksen *et al.* (1999a) dealt with the importance of the period of follicular development between approximately 8 mm and the preovulatory size; their evidence, which was in superovulated animals, suggested that oocytes might have reached their maximum competence by the time they reached that size. Atypical granulosa cells can be considered as markers for a lower-quality follicle, according to Van den Hurk *et al.* (2000); this view is based on their absence in preovulatory-sized follicles and their presence in large numbers in a high proportion of definitely atresic follicles. A study by Heleil *et al.* (2000) examined the relationship between follicle size and cumulus-cell mass morphology, oocyte chromatin quality and oocyte competence; they found that oocytes from follicles > 5 mm were much more homogeneous and were characterized by a higher percentage of immature GV stages with intact chromatin than in smaller follicles.

In Brazil, Seneda *et al.* (2000) examined the effect of follicle diameter on oocyte recovery rate, quality and competence, using oocytes obtained by OPU; in contrast to the experience of those using abattoir ovaries, they found no evidence that oocyte quality and embryo production differed between the two follicle categories (< 4 mm vs. > 4 mm) examined. They speculated that cattle destined for slaughter undergo stress and that small follicles are more susceptible to stress effects and the oocytes less competent.

3.3. Recovering Oocytes: Live Cattle

As observed by Kruip *et al.* (1994), embryo production by conventional superovulation techniques may lead to fewer than 100 embryos

over the lifetime of an individual cow, even though there may be 100,000 oocytes present in the ovaries; if more of these oocytes could be turned into embryos, this is likely to have a considerable impact on cattle-breeding programmes. *In vitro* embryo production from problem, pregnant and cyclic cows by transvaginal OPU is being employed to an increasing extent around the world, In Argentina, for example, *in vitro* embryo production is used almost exclusively to obtain young from problem cows that cannot be dealt with by conventional embryo transfer (ET) technology (Ferre *et al.*, 2002); the authors provided data showing the capability of such problem cows to produce embryos that resulted in pregnancies (see Table 3.4).

Early reports on the recovery of cattle oocytes from live cattle came from workers in North America in the early 1980s. Their efforts were directed at the recovery of *in vivo*-matured (secondary) oocytes recovered by an endoscope inserted through the skin of the right paralumbar fossa; donor animals were superovulated and endoscopy performed under local anaesthesia near the expected time of ovulation. However, as noted later, there are grounds for using a much less invasive method of recovering oocytes.

3.3.1. Advantages and alternatives

The first report of the technique of transvaginal ultrasound-guided follicular aspiration as a method of recovery of primary cattle oocytes was published by Pieterse and associates working in Utrecht in 1988. In commercial terms, OPU could be extremely useful in dealing with genetically valuable cows that respond poorly to gonadotrophin stimulation or in which embryo recovery is no longer possible (Looney *et al.*, 1994). It could also be valuable in breeding programmes that require many more births annually than is possible with conventional ET technology (Vivanco and McMillan, 1999). In the USA, Hasler (1998) reported an 8-year-old cow producing 176 embryos in a period of 3 years on a once-weekly OPU programme. Among the more unusual categories of cattle in which OPU has been practised are clones; in France, Heyman *et al.* (2000) found no evidence of differences in oocyte competence in cloned heifers in comparison with controls.

In India, where cattle slaughter is banned for religious reasons, there is obviously no opportunity for researchers to obtain oocytes from abattoir ovaries as in most other countries; for that reason, OPU has a particular value. A study by Manik *et al.* (2002) in India has demonstrated the use of the technique in recovering developmentally competent oocytes (see Table 3.5); other work at the Embryo Biotechnology Centre in India has dealt with the effect of needle gauge and aspiration vacuum, using slaughtered buffalo ovaries to develop the technique for application in the live cow (Bera *et al.*, 2002). A review by Larsson (1998), focused mainly on cattle, describes the recovery of oocytes for embryo production *in vitro* using different techniques and oocyte donors.

Not all OPU attempts involve expensive and sophisticated equipment. In Canada, Hill (1995) devised a simple method of transvaginal follicle aspiration to collect oocytes under field conditions. Under epidural anaesthesia, a needle guide was passed into the vagina to a point adjacent to the cervix; an ovary was then manipulated *per rectum* and placed against the vaginal wall over

Table 3.4. Comparisons between problem, pregnant and cyclic cows in embryo production (from Ferre *et al.*, 2002).

Groups	FR/OPU session Mean ± SD	FR/Cow Mean ± SD	Oocytes/OPU session Mean ± SD	Oocytes/Cow Mean ± SD	Embryos (%)	Pregnancy (%)
Problem	11.7 ± 10.5	7.5 ± 5.5^{b}	9.0 ± 13.4	5.7 ± 7.1^{b}	14/63 (22.2%)	4/11 (36.4%)
Pregnant	21.3 ± 10.2	12.4 ± 5.6^{a}	20.3 ± 11.8	11.8 ± 7.7^{a}	41/142 (28.9%)	12/30 (40.0%)
Cyclic	13.0 ± 7.9	7.0 ± 3.7^{b}	8.7 ± 5.6	4.7 ± 2.9^{b}	17/61 (27.9%)	5/1.3 (38.5%)

FR, follicular response; SD, standard deviation.
[a,b]Values with different superscripts in the same column differ ($P < 0.05$).

Table 3.5. Ovum pick-up in Indian cattle (from Manik *et al.*, 2002).

Animal no.	PS (*n*)	FP *n*	FP Mean	Oocytes recovered *n*	Oocytes recovered Mean	Oocytes recovered RR (%)	Grades of oocytes A + B *n* (%)	Grades of oocytes C + D *n* (%)	CR (%)
1	9	86	9.5 ± 0.9	53	5.9 ± 0.8	62	18 (33)	35 (66)	54
2	6	37	6.2 ± 0.9	21	3.5 ± 1.4	57	8 (38)	13 (62)	0
3	4	20	5.0 ± 1.5	7	1.7 ± 0.4	35	0 (0)	7 (99)	29
4	4	14	3.5 ± 0.7	11	2.7 ± 0.4	79	3 (27)	8 (72)	20
Total	23	157	6.8 ± 0.7	92	4.0 ± 0.5	59	29 (32)	63 (69)	33

PS, puncture session; FP, follicles punctured; RR, recovery rate; CR, cleavage rate.

the end of the needle guide. As the needle was passed into an ovary, an assistant applied vacuum pressure. In China, Lu, C.S. *et al.* (1999) reported on an apparatus for OPU which was based on the conventional insemination gun; trials on its efficiency yielded 12 oocytes from eight cows. In Argentina, Aller *et al.* (2000) produced IVP embryos from oocytes recovered from ovariectomized cows; the animals were ovariectomized by using an effeminator device via the vagina.

Mares

It is not only in cattle that OPU procedures have appeal. The ability to recover oocytes non-surgically from the follicles of mares would allow either *in vitro* fertilization (IVF) or *in vivo* fertilization in the recipient mare to be used as a technique for obtaining foals from older mares with poor reproductive histories. As in cattle, it is possible to recover oocytes from pregnant animals as well as cyclic mares. In the USA, Meintjes *et al.* (1994) demonstrated that oocytes could be harvested from pregnant mares during the first half of gestation and that this could be done safely without pregnancies being lost. In contrast to follicle aspiration in the cow, the oocyte recovery rate from non-ovulatory follicles is low (Dippert *et al.*, 1994; Squires and Cook, 1996; Kanitz *et al.*, 1997; Guignot *et al.*, 1999a,b); it is often necessary to flush the follicle repeatedly with medium to recover the oocyte. A study by Bezard *et al.* (1998) unsuccessfully attempted to increase the population of small follicles available for *in vivo* aspiration during early dioestrus using crude equine gonadotrophin; such results confirmed the gonadotrophin-independence of small follicles recorded by workers elsewhere.

Buffaloes and pigs

What is possible in cattle can usually be achieved, albeit less successfully, in buffaloes. In Thailand Pavasuthipaisit *et al.* (1995) and in Italy Boni *et al.* (1996) and Galli *et al.* (1998) are among researchers reporting repeated follicle aspiration by OPU in the swamp buffalo; a study by Techakumphu *et al.* (2000) demonstrated the harvesting of oocytes from buffalo calves. Although transvaginal ultrasound-guided follicular aspiration has been attempted in goats (Graff *et al.*, 1995), for most small ruminants more complex and invasive approaches, such as laparotomy or laparoscopy, are required; this makes repeat recovery sessions unacceptable because of possible side-effects.

Procedures for non-surgical transvaginal ultrasound-guided oocyte aspiration in sows have been reported (Bellow *et al.*, 2001; Caamano *et al.*, 2002). Although refinements are necessary to make the technique feasible, it seems likely that OPU technology combined with *in vitro* embryo production in pigs has the potential to be useful in research and as a useful tool to expand the breeding of superior females.

3.3.2. Laparoscopic methods of follicular aspiration

Although initial attempts to recover bovine oocytes from live cattle by laparoscopy were by way of the paralumbar fossa, workers in Germany held this method to be unsuitable for many research purposes and for animal-welfare reasons. The feasibility of collecting bovine oocytes by transvaginal laparoscopy was demonstrated by Reichenbach and colleagues in

Munich in 1993. A subsequent report from the group described an improved method for the laparoscopic examination of the internal reproductive organs of the cow through the vaginal fornix (Reichenbach *et al.*, 1994a,b); the method was used extensively to examine ovaries and to recover oocytes from follicles. The German workers suggested that the method was a useful alternative to the ultrasonically guided collection of oocytes. The authors emphasized that practical experience is essential for successful laparoscopy; they noted that proper equipment, a knowledge of the anatomy of the abdominal cavity and practice in the palpation of the cow's reproductive organs *per rectum* greatly facilitated application of their technique. A paper by Brem *et al.* (1995) dealt with *in vitro*-produced (IVP) embryos produced after repeated endoscopically guided oocyte aspiration.

Elsewhere in Germany, Becker *et al.* (1994, 1996) compared laparoscopic and ultrasonic methods. They were unable to repeat the excellent results of Reichenbach *et al.* (1994a,b); they succeeded in aspirating a comparable number of follicles but only recovered 39% of the COCs per session, in contrast to the 66–80% rates recorded by the Munich workers.

The effect of frequent aspiration sessions is a consideration, since this can result in alterations in the fornix and elsewhere; they found the probability of such events was greater with the laparoscopic method. On the other hand, laparoscopy was less traumatic for the ovaries and allowed exact positioning of the aspiration needle into the follicles; this is likely to be important in dealing with small follicles (2–3 mm diameter). In other studies in Germany, Santl *et al.* (1998a,b) compared the laparoscopic form of OPU with the ultrasonic method over two 8-week periods (15 sessions per 8-week period) in Simmental heifers; to evaluate effects of long-term OPU on the subsequent fertility of donors, all animals were bred by AI at the first natural oestrus after OPU. The authors record that the percentage of good-quality oocytes was higher after the ultrasonic method than after laparoscopy (38.7 vs. 21.0%); the ultrasonic approach was less traumatic to the vagina and they were able to attempt recovery in all animals, in contrast to laparoscopy, which was occasionally difficult or impossible to perform. A report by Havlicek *et al.* (1999) in Germany described the use of transvaginal endoscopy in collecting early-cleavage cattle embryos from the oviduct, a technique that may be useful in research.

The general conclusion is that the laparoscopic approach to oocyte collection is relatively complicated and requires special restraint and sterile surgical procedures as well as tranquillization or general anaesthesia; the invasive nature of laparoscopy limits the frequency of its use and may lead to scarring and adhesions.

3.3.3. Ultrasonic methods of follicular aspiration

Although laparoscopy has been used successfully to recover bovine primary oocytes from preovulatory and growing follicles, it is open to criticism on humane grounds as an invasive procedure. While the technique permits a clear view of the ovary's surface, laparoscopy does not allow visualization of the cohort of growing follicles just beneath the ovarian surface. Such handicaps have been factors in the development of ultrasonically guided oocyte-recovery techniques; this has the advantages of being less invasive, imposes less stress on the cow and is more easily adaptable to on-farm application than other methods.

Developments in ultrasound technology

Since the mid-1960s, hundreds of articles and books have been written about the use of ultrasound in large farm animals (see review by Lamb, 2002). The area that has probably benefited the most from the development of ultrasound technology is reproduction. It is regularly used in monitoring follicular characteristics and ovarian function in cattle. As well as its commercial application on a large scale for pregnancy diagnosis in cattle, ultrasound is now an indispensable tool for researchers; the technology enables structures such as ovaries and ovarian follicles to be visualized and measured with ease and accuracy.

Ultrasound in research and practice

From the time of its initial description by Pieterse in 1988, numerous studies have been reported on the use of the ultrasonic method for follicular

aspiration, both in adult animals (Bols *et al.*, 1994a,b, 1995, 1996a,b, 1997a,b; Scott *et al.*, 1994; Vos *et al.*, 1994; Gibbons *et al.*, 1995; Goffin *et al.*, 1995; Moyo and Dobson, 1995; Galli and Lazzari, 1996; Goodhand *et al.*, 1996; Palma *et al.*, 1996; Pollard *et al.*, 1996; Donnay et al., 1997a; Falceto *et al.*, 1997; Guyader-Joly *et al.*, 1997a,b; Bols and de Kruif, 1998; Markkula *et al.*, 1999a,c; Twagiramungu *et al.*, 1999; Watanabe *et al.*, 1999; Xu *et al.*, 1999; Hashimoto *et al.*, 2000a; Hidalgo *et al.*, 2000; Smorag *et al.*, 2000; Galli *et al.*, 2001; Seneda *et al.*, 2001) and in prepubertal cattle (Palma, 1994; Brogliatti and Adams, 1996; Damiani *et al.*, 1995, 1996a,b; Lohachit *et al.*, 1995; DeRoover *et al.*, 1996, 1997a,b, 1998, 1999, 2001; Rick *et al.*, 1996; Fry *et al.*, 1998; Bols *et al.*, 1999; Majerus *et al.*, 1999c).

Unfortunately, in comparison with oocytes from adult cattle, those from calves show impaired competence (Salamone *et al.*, 2001), so their recovery is only one step towards the production of embryos; the current belief is that the problem lies in a failure or inability to complete cytoplasmic maturation. This is not a problem unique to cattle; there is ample evidence in many mammalian species, ranging from rodents to pigs and sheep, to show that oocytes from prepubertal animals have limited potential to undergo normal embryo development and produce viable young (see Armstrong, 2001).

Most reports emphasize that careful preparation of the adult donor animal is essential to success; the cow is suitably restrained throughout the collection procedure and must have a relaxed rectal wall, so that the ovary can be manipulated without undue difficulty. As an adjunct to ET in cattle OPU is likely to have an increasingly important future. Since donors are usually not superovulated, the cost of hormones is eliminated; harvesting of oocytes can be conducted twice a week for several months. In a New Zealand study reported by Hepburn and MacMillan (1996), for example, OPU by follicle aspiration was carried out twice weekly on 34 cows and heifers over an 18-month period; the number of OPUs per animal ranged from 1 to 78. The number of oocytes recovered per session averaged 2.4 and the number of transferable embryos produced per session averaged 0.52.

As with embryos derived from abattoir ovaries, however, the success of the ultrasonic method will be largely determined by the effectiveness of the *in vitro* embryo production techniques. It is important, however, that the developmental capacity of oocytes, as well as the number recovered per OPU session, is taken carefully into account. According to Mullaart *et al.* (1999), compared with oocytes collected by aspiration from slaughterhouse ovaries, the morphological quality of COCs recovered by OPU is generally lower; they mention only 20% grade 1 oocytes for OPU compared with 49% for abattoir ovaries. As discussed elsewhere, this may well be due to inappropriate vacuum pressure and needle characteristics resulting in the partial stripping of cumulus cells. The same authors noted that follicles smaller than 2 mm were not visible, due to the limited resolution of the scanner used.

3.3.4. Developments in ovum pick-up technology

OPU is now regarded as a very flexible technique that can be applied twice a week for several weeks without having adverse side-effects on the donor animal's fertility (Gibbons *et al.*, 1994a,b,c, 1995; Bucher *et al.*, 1996; Dolman *et al.*, 1995; Goto *et al.*, 1995; Lansbergen *et al.*, 1995; Menard *et al.*, 1995; Hepburn and MacMillan, 1996; Boni *et al.*, 1997; Broadbent *et al.*, 1997a,b; Hanenberg and Van Wagtendonk-De Leeuw, 1997; Kurokin, 1998; Hashimoto *et al.*, 2000a; Park, S.J. *et al.*, 2000). In Japan, on the other hand, Imai *et al.* (2000) examined the effect of the frequency of OPU on the numbers of developing follicles in ovaries, the oocyte recovery rates and their subsequent development; they found that, under their particular conditions, OPU depended on the individual skill of the operator and that aspiration at 7-day intervals was the most effective. In Guangxi, Lu, C.S. *et al.* (1999) reported on embryo production using bovine oocytes collected by a self-made simple oocyte pick-up apparatus.

Some workers have sought to select good donor cows by follicular monitoring during a period prior to starting OPU. In Belgium, DeRoover *et al.* (1997a,b) monitored follicles three times weekly for about 6 weeks before performing OPU twice weekly for 8 weeks; after at

least five examinations, follicle numbers for OPU could be predicted with reasonable accuracy.

Where animals are collected from twice weekly, the 4-day-interval aspirations have been found to yield more oocytes per session than the 3-day-interval aspirations (Garcia and Salaheddine, 1998). It is believed that there may be no advantage in giving exogenous follicle-stimulating hormone (FSH) to animals collected from twice weekly, since they may have elevated endogenous FSH levels due to the frequency of aspiration. More good-quality oocytes may be recovered from twice-weekly collections than from once weekly; it is believed that this may be related to the oocytes being harvested from a growing, synchronous, follicular population rather than from the atresic environment that may be associated with the once-weekly collection schedule. Donor cattle can be in a wide variety of reproductive stages, varying from the prepubertal through the cyclical to the pregnant and the early post-partum animal. In terms of equipment, a scanner with a sectorial or convex probe and a suction pump are required. Decreasing collection time, minimizing the cost of operation and increasing the operator's control are regarded as important considerations in the development of commercial OPU technology (Matthews *et al.*, 1995).

One measure of the effectiveness of OPU is the recovery rate, which is the number of oocytes retrieved after the puncture of 100 follicles. The recovery rate, in turn, is affected by several factors, such as needle type, puncture frequency, hormonal pretreatment of animals, time of puncture within the oestrous cycle, aspiration vacuum and the operator's experience. During the 1990s, several research teams devoted time to improving elements of OPU technology in order to make it more practicable and economical for routine use in oocyte retrieval; one of the earliest elements examined was the aspiration needle (Bols *et al.*, 1994a,b, 1995, 1997a,b, 1998). Disposable needles were found to have several advantages over the non-disposable type; they are short and easy to manipulate; they are easy and less costly to replace, allowing a new needle to be used with each cow; they have a small dead volume, which makes them suitable for collecting follicular fluid from individual follicles; and, unlike the non-disposable needle, the disposable one did not lose its sharpness after a few aspirations. Several groups have sought to reduce the damage to oocytes arising from aspiration vacuum and needle size (Hashimoto *et al.*, 1998a,b; Vos *et al.*, 1999; Ward *et al.*, 2000b); the general consensus is that recovery rate is improved using a low aspiration vacuum and a small needle.

According to Gerber *et al.* (2000), one of the disadvantages of current OPU systems is the tendency for ultrasound probe handles to slip out of the cow's vagina unless constantly held in place. The South African workers designed a probe that could easily be used by a single operator working alone; the probe remains in place even with the cow moving or straining; some of the results they achieved with this device are given in Table 3.6). In Brazil, Brenner *et al.* (2000) employed a modified aspiration system in which a filter (Miniflush System-Minitub) was used on the aspiration line, leading to a 30% reduction in working time.

Some authors have examined the effect of OPU on the donor's oestrous cycle and follicular dynamics. In Canada, Stubbings and Walton (1995) presented evidence suggesting that a single aspiration session in mid-cycle resulted in the recruitment of a new unscheduled wave of follicles that extended the interoestrus interval by 4 days, the time required to recruit this cohort of follicles. According to these authors, if there are to be repeated aspirations over a long period of time, hormonal pretreatment (FSH) is not warranted; if it is a single recovery session,

Table 3.6. Oocyte recovery in cattle and African buffaloes using a new device (from Gerber *et al.*, 2000).

	No. aspirated follicles		No. recovered oocytes		
	Total	Per cow	Total	Per cow	% Recovered
Domestic cattle	349	8.7	178	4.5	51.0
African buffalo	117	5.6	53	2.5	45.3

gonadotrophic stimulation of the ovaries can result in a significant increase in the number of follicles available for aspiration. The characteristics of the follicle population and oocyte developmental competence were studied in oestrus-synchronized Friesian cows by Machatkova *et al.* (1996, 1999) in the Czech Republic; they demonstrated that the efficiency of OPU could be improved by utilizing the growth phase of the first follicular wave before dominant follicle selection.

In the Netherlands, Steenweg *et al.* (2000) investigated the developmental competence of COCs recovered at defined stages of a follicular wave; they recorded that the suppressive effect of a dominant follicle became apparent during its non-growing phase. In Japan, Tohei *et al.* (2001) examined the hormonal consequences of OPU and found that follicular aspiration decreased plasma concentrations of inhibin and oestradiol-17β, which resulted in a rise in plasma concentrations of FSH and luteinizing hormone (LH); the authors suggest that such increases in gonadotrophin levels stimulate the development and maturation of a new cohort of follicles within 1 week in cows. An earlier paper by Carlin *et al.* (1999) had also found that long-term OPU may modify progesterone, LH and FSH profiles and ovarian dynamics in cows.

Much attention has been given to the effect of repeated follicular puncture on subsequent ovarian function in donor animals. In Sweden, Petyim *et al.* (2000) found that dairy heifers may occasionally show cyclic activity and form corpus luteum-like structures during twice-weekly OPU; the aspiration procedure, however, apparently did not cause any major negative effects on ovarian structure and function.

Although the paternal effect in bovine embryo development is well established, there has been a lack of information about the maternal influence. For such reasons, the influence of maternal genetics on oocyte collection and quality was investigated by Chastant-Maillard *et al.* (1999a,b), Quinton *et al.* (1999) and Tamassia *et al.* (2001) in France; they found that the ability of the oocytes they obtained by OPU to support embryonic development was strongly influenced by their maternal origin. The French group pointed out that the repetition of punctures did not degrade the quality of oocytes and there was no link between quality and the quantity of oocytes recovered. They also speculated that it might eventually be possible to develop a fertility laboratory test which could be carried out on animals prior to their entry to an *in vitro* embryo production programme. In the USA, Kendrick *et al.* (1999) examined the effects of energy balance on oocytes recovered from lactating post-partum Friesian cows by OPU; they recorded that oocyte numbers increased linearly from 30 to 100 days after calving and that animals fed high-energy diets produced more good-quality oocytes than those fed low-energy diets.

There are those who have criticized the OPU technique on animal-welfare grounds; others contend that there is no problem in its application to cattle breeding. In France, workers constructed a protocol that sought to evaluate stress and ovarian lesions associated with transvaginal oocyte collection (Chastant-Maillard *et al.*, 1999a; Lauffenburger *et al.*, 1999); on the basis of the evidence obtained, they concluded that OPU is not a source of either acute or chronic stress for cattle under epidermal anaesthesia.

Commercial OPU may often be carried out on farms some distance from the laboratory in which the oocytes are to be processed. For such reasons, some workers have examined different media for use in transit that would avoid the need to equilibrate media under a special gas phase. In Australia, Fry *et al.* (2000) demonstrated that a HEPES-based medium, user-friendly for OPU on the farm, was as effective as the conventional bicarbonate-buffered medium that is equilibrated at 5% CO_2 in air.

In New Zealand, Hagemann *et al.* (1999a) suggested that the greatest improvement in OPU technology would be achieved by harvesting oocytes from the numerous small antral follicles (3–5 mm diameter) that are to be found in the ovaries. In a later report from that country, Tervit *et al.* (2002) have drawn attention to the fact that OPU-recovered oocytes are often less developmentally competent than those taken from abattoir ovaries; a major factor in this is thought to be related to the amount of cumulus cells surrounding the oocyte. It is known that these cells provide the developing oocyte with pyruvate and protect it from oxidative stress. The New Zealand workers suggest that the quality of OPU oocytes may be improved by IVM under a reduced (5%) oxygen atmosphere.

In terms of the ultrasonic equipment used in OPU, workers in Japan examined the frequency

of probes (7.5 or 5.0 MHz); Hashimoto *et al.* (1999a,b,c) evaluated the probes for their clarity of follicle image on the monitor, using slaughterhouse ovaries. The number of oocytes per donor cow obtained with the 7.5 MHz probe was significantly higher than that obtained with the 5.0 MHz probe (11.2 vs. 4.3); the difference between probes was attributed to the greater clarity of the follicle images obtained with the higher-frequency probe. In Belgium, a comparison made between a linear array and a mechanical sector transducer for OPU showed that, while both systems worked efficiently, the sector probe system appeared to yield a higher number of oocytes (Bols *et al.*, 2002).

OPU in zebu cattle

Although zebu (*Bos indicus*) breeds are known to grow and reproduce better than European cattle in the tropics, little is known about oocyte production and their developmental competence. In Brazil, Dayan *et al.* (2000) compared different zebu and European breeds in an OPU programme; no significant differences were evident between the two species in terms of numbers of oocytes, embryo production and pregnancy rates after transfers. The authors concluded that both species respond similarly and have a similar potential for assisted reproduction under tropical conditions. Other work in Brazil has evaluated two different OPU schedules in Gir donor cows (Viana *et al.*, 2000a); the outcome was more successful with oocytes collected twice weekly rather than once weekly.

3.3.5. Hormonal and nutritional pretreatments

Various groups have reported on the pretreatment of donor cattle with FSH preparations before OPU is employed to collect oocytes (Fry *et al.*, 1994; Bungartz *et al.*, 1995; Paul *et al.*, 1995; Broadbent *et al.*, 1997a,b; Lacaze *et al.*, 1997; Bottcher, 1998; Van Wagtendonk-De Leeuw and de Ruigh, 1999; Bousquet *et al.*, 2000; Guyader-Joly *et al.*, 2000; Seneda *et al.*, 2002). In the Netherlands, Mullaart *et al.* (2002) found that FSH pretreatment apparently accelerated embryo development and was associated with more normal calf birth weights than in their untreated controls. There have also been those who have reported OPU from inhibin-immunized cattle (Konishi *et al.*, 1996b,c).

Occasionally, problems arise in donor cows involved in intense superovulation and embryo collection programmes; various means, including hormonal treatments, have been employed in dealing with such animals before attempting OPU (Garcia *et al.*, 2000). In the Netherlands, Steenweg *et al.* (2000) attempted to optimize OPU in terms of oocyte recovery rate and oocyte quality; they used the technique to collect expanded and non-expanded COCs from superovulated cows in which the timing of the LH surge was controlled. The authors found that the developmental competence of COCs was unaffected by the time at which the COC was recovered relative to the preovulatory LH peak. Other work in the same country by De Ruigh *et al.* (2000) compared OPU, after FSH pretreatment, performed once every 2 weeks with a twice-weekly collection routine; the total number of oocytes recovered was higher with OPU twice weekly. In Scotland, Goodhand *et al.* (1996, 1997, 1999, 2000) used various hormonal pretreatments and recorded that FSH significantly increased the number of follicles aspirated and good-quality oocytes recovered in donors on a once-per-week OPU programme; progestogen in combination with oestradiol-17β treatment did not affect follicle number or oocyte quality. In Canada, Bordignon *et al.* (1997) showed that more oocytes could be obtained by OPU at 60 h after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from FSH-stimulated heifers treated with gonadotrophin-releasing hormone (GH-RH) at 34 h after prostaglandin; GH-RH enabled the collection of oocytes with a higher developmental competence.

In France, Oussaid *et al.* (2000), working with superovulated heifers, artificially prolonged the follicular phase by way of a GH-RH antagonist (Antarelix); they recorded no material difference in the competence of oocytes from healthy, early atresic, advanced atresic and late atresic follicles. It was concluded that the competence of the oocytes recovered from such follicles was not affected.

A study reported by McEvoy *et al.* (1997, 1998c) investigated the effects of protein and energy supply on the competence of oocytes; the

energy and rumen-degradable protein levels used in their study had no effect on the yield of healthy COCs or on the competence of oocytes from either small (2–4 mm) or medium-sized (5–9 mm) follicles. In Ireland, Lozano *et al.* (2000) were able to show that a low dietary intake improved the cleavage rate and morphological grading of oocytes in dairy cows during the early post-partum period; embryo yield tended to increase after culture of oocytes from cows offered a low dietary intake. In the same country, results reported by Papadopoulos *et al.* (2001a,b), this time in sheep, suggested that both high dietary energy and urea content influence the oocyte, with the adverse effects of urea being influenced by concomitant energy intake. In Scotland, also working with sheep, Mitchell *et al.* (2002) were able to demonstrate the effects of dietary lipid on the quality of COCs aspirated from ewes. In France, Freret *et al.* (2002) reported on studies investigating the effect of short-term reductions of energy intake on oocyte quality after superovulation in previously overfed Friesian heifers; OPU was performed 12 h after the last of five injections. The authors recorded data suggesting that, following an overfeeding period, dietary-intake restriction was capable of increasing the number of developmentally competent oocytes.

Influence of growth hormone

In some reports, recombinant bovine somatotrophin (r-BST) has been administered in addition to FSH (Pivato *et al.*, 1999); there was some evidence of an effect on the number good-quality oocytes collected. It is known that r-BST influences follicular dynamics in cattle (Lucy *et al.*, 1994; Bols *et al.*, 1998). Although the direct action of r-BST on the follicle may be small due to the limited expression of growth hormone receptors in the ovary, it is believed that the stimulatory actions of the hormone may be caused by increased concentrations of insulin-like growth factor (IGF) in the blood or in the follicular fluid. However, there have been reports on the suppression of non-surgical recovery of oocytes in heifers treated with r-BST, despite the apparent stimulatory influence of growth hormone on follicular growth (Tripp *et al.*, 1998); the reason why r-BST decreases the ratio of oocytes recovered/follicles observed remains unclear. Studies reported by Tripp *et al.* (2000a) failed to find evidence that r-BST altered the physical properties of ovarian components.

Retinol

There is evidence that the retinoid family can stimulate oocyte competence both *in vivo* and *in vitro*. For that reason, Hidalgo *et al.* (2002a) in Spain examined the effect of treatment with retinoids on donor animals and their COCs recovered by OPU. Retinol was administered 4 days before OPU sessions performed at 3–4 day intervals, with another dose given at the time of the second OPU session; there was a significant effect on the number of COCs recovered and on embryo yield. In the same laboratory, Duque *et al.* (2002) found evidence that retinoic acid, a metabolite of retinol, in their oocyte maturation medium improved the development and quality of blastocysts.

3.3.6. Oocytes from pregnant cattle

Although superovulation and embryo collection are still regarded as the most effective method for embryo production in reproductively normal cyclic cattle, OPU is the only way open to obtaining viable embryos from pregnant animals. Various authors have reported studies in which pregnant cattle have been evaluated as donors of oocytes; this is a novel means of dealing with pregnant female animals of superior genetic quality.

In Korea, Lee, B.C. *et al.* (1999) established a breeding programme using OPU in pregnant cows; aspiration was performed in pregnant animals every 10 days from day 40 until day 90 of gestation. The Korean workers recorded a significant decrease in follicle numbers at 90 days of gestation; OPU did not adversely affect the normal progress of pregnancy in the donors. Eikelmann *et al.* (2000) demonstrated that repeated OPU can be a safe and effective method for obtaining oocytes and generating viable embryos in the first trimester of gestation in heifers and in the first half of gestation in cows. With advancing pregnancy, the efficiency of the OPU technique is progressively impaired as the anatomical position of the ovaries changes and

the number of follicles available for aspiration decreases (after 3–5 months). Polish workers have also described evaluating pregnant heifers as oocyte donors (Duszewska and Reklewski, 2001); they successfully carried out twice-weekly OPU sessions over an 8-week period in heifers in the first trimester of gestation.

FSH stimulation

Trying to increase the yield of oocytes and embryos in pregnant cows by giving FSH prior to OPU has not proved successful. In Germany, Rust *et al.* (1999) compared results for FSH-treated animals (1.5–3 months pregnant) with untreated cows; the superstimulation was not effective, due to a significantly reduced recovery rate in treated animals.

3.3.7. Oocytes from post-partum cattle

Some workers have examined the possibility of obtaining oocytes from cattle in the early weeks after calving. In the USA, Perez *et al.* (2000) evaluated the use of FSH for oocyte production in early post-partum beef cows that were nursing calves; oocyte collections were performed on day 25 and again on day 35 after calving, on each occasion 18 h after the final FSH injection. They found that FSH cattle yielded a greater number of oocytes than controls on both occasions (Table 3.7).

Table 3.7. Oocytes from FSH-treated post-partum beef cattle (from Perez *et al.*, 2000).

Treatment	Follicles	Follicles aspirated	No. oocytes (%)
FSH	26.3 ± 4.3*	23.4 ± 4.3*	14.7 ± 2.4* (63%)
No FSH	4.8 ± 0.5	4.0 ± 0.4	2.6 ± 0.6 (65%)

*Means (± SEM) in a column marked with an asterisk are significantly different ($P < 0.05$).
SEM, standard error of the mean.

3.3.8. Oocytes from calves and prepubertal cattle

The interval between generations is one of the key elements in any approach to genetic improvement in cattle; the use of oocytes from young calves offers one way of reducing the generation interval, resulting in faster genetic change (Majerus *et al.*, 1998). It is known that follicular development occurs in a wave-like pattern in calves as early as 2 weeks of age (see Section 2.2.3) and that the ovaries contain antral follicles capable of yielding oocytes suitable for maturation and processing into embryos. It is also known that calf ovaries exhibit larger pools of antral follicles visible on their surface than adult ovaries; this suggests that there is ample potential for recovering numerous oocytes. In attempting OPU by the transvaginal ultrasound-guided approach, obvious limitations include lack of suitable equipment and the inability of the operator to manually immobilize the ovaries by manipulation *per rectum*. Canadian workers (Brogliatti *et al.*, 1995a,b; Brogliatti and Adams, 1996) modified a 5 MHz, convex-array ultrasound transducer designed for intravaginal use in women for calves, but were unable to immobilize the ovaries; they were able to collect oocytes from calves in the standing position (10–16 weeks old) or dorsal recumbency (6 weeks old).

Experiments reported by Armstrong *et al.* (1994) in Australia indicated that the follicular response of calf ovaries to FSH increased progressively from 3 to 9 weeks of age and that oocytes recovered from these follicles are capable of processing into embryos; administration of FSH as a single injection was much less effective than the same total dose given twice daily over a 3-day period. When a single FSH dose was allied to a low dose of pregnant mare serum gonadotrophin (PMSG), the response was equal to the multiple FSH treatment. In Massachusetts, Looney *et al.* (1995) used ultrasound-guided aspiration in prepubertal FSH-treated heifers at 30–45-day intervals between the ages of 6 and 9 months; hormonal treatment provided adequate follicles for OPU and no adverse effects on fertility were recorded. As heifer age increased, oocyte recovery rates improved, possibly due to the easier manipulation of the ovaries as the size of the animals increased.

A paper by Brown *et al.* (1996) dealt with postpubertal fertility after repeated transvaginal

oocyte collection in calves. In France, Mermillod *et al.* (1997) found evidence suggesting that oocytes from very young calves (5–15 days old) were developmentally less competent than those from 3-month-old calves; the same authors observed that the individual variation in response to FSH treatment noted in adult animals is evident in the very young calf. In studies reported in the following year, Mermillod *et al.* (1998) reported that the low developmental competence of prepubertal oocytes could be explained by a deficiency at the cytoplasmic level; although the cytoplasmic defect did not impair fertilization or first cleavage, it did have an adverse effect on development to the blastocyst stage. The authors suggested that nuclear transfer might be a valuable tool for investigating oocyte cytoplasmic maturation.

A report by Fry *et al.* (1998) demonstrated that oocytes could be recovered from 5-month-old calves by OPU, using the ultrasonic-guided transvaginal approach; they record that OPU took about 10–15 min and had no evident effects on the subsequent reproductive performance of the animals. They emphasize the need to maintain calves on an appropriate plane of nutrition so as to achieve a live weight of about 100 kg before attempting recovery; this size of animal enables the operator to manipulate the ovaries *per rectum*. Since the ovaries are very small at 5 months of age, it is necessary to stimulate the ovaries to induce the growth of follicles to a size suitable for aspiration. The Australian workers used a combination of FSH and PMSG, administered 4 days prior to OPU; the rationale was that FSH would recruit gonadotrophin-responsive follicles from the pool of small (1–2 mm diameter) follicles for growth and that the longer-acting PMSG would sustain their development to the time of OPU, 4 days later.

A study in Belgium by Majerus *et al.* (1999c) has demonstrated the possibility of recovering oocytes by repeated OPU from 7–12-month-old calves without this affecting growth or puberty; the authors believed this to be the first report of OPU being applied twice weekly for a prolonged period (2 months), using a protocol similar to that for adult animals. In Germany, Kuwer *et al.* (1999) conducted twice-weekly OPU in 6-month-old Friesian calves over a period of 6 weeks; although gonadotrophin treatment did not increase follicle numbers, the quality of the COCs was improved by gonadotrophin stimulation. In the USA, Bols *et al.* (1999) subjected 12 FSH-stimulated Friesian calves to transvaginal OPU every 2 months between 6 months and 1 year of age; although competent oocytes (capable of giving transferable embryos) were recovered at all stages, the authors concluded that further work is necessary to examine oocyte age and developmental competence.

In Australia, Kelly *et al.* (2000) examined the possibility of obtaining oocytes from FSH-treated calves at 8 and 10 weeks of age by way of midventral laparotomy under general anaesthesia; the authors recorded an average of about 26 oocytes collected at 8 weeks and 29 at 10 weeks, resulting in an average of 5.7 pregnancies per calf after embryo production.

Not all reports agree that recovering oocytes from young animals may be problem-free. A study reported from Brazil by Snel-Oliveira *et al.* (2002) evaluated the histopathological lesions in the ovaries caused by ultrasound-guided OPU in zebu prepubertal calves; these animals were stimulated by gonadotrophins and oocyte collections made on three occasions when animals were 10, 11 and 12 months old. In examinations made 3 months after the last OPU, there was evidence of pathological alterations that suggested that the function of the ovaries might have been compromised.

3.4. Live Donors: Other Considerations

There is evidence that the blastocyst yield after IVM, IVF and *in vitro* culture (IVC) of oocytes collected by OPU may be lower than for oocytes obtained from abattoir ovaries (Donnay *et al.*, 1996). It is also recognized that oocytes collected by OPU surrounded by a limited number of cumulus-cell layers may have lower developmental competence than those with many layers (Palma *et al.*, 1995a,b); part of this problem may be the loss of cumulus cells during the aspiration process. It should be kept in mind that the aspiration vacuum at the needle tip, as it punctures the follicle, will depend on the construction of the OPU device and the length and the diameter of the tubing system, as well as the diameter of the aspiration needle. In Cornell, Zhang *et al.* (1995) removed the cumulus cells

from the oocyte before maturation and recorded a dramatic decrease in the rates of oocyte maturation (4–26 vs. 93–96%). In the Netherlands, Bruynzeel *et al.* (1997) reported 22% of grade I oocytes with OPU in contrast to 72% with slaughterhouse ovaries; attempts to compensate for the possible loss of cumulus cells with cumulus monolayers have given equivocal results.

3.4.1. Recovering secondary oocytes

In human IVF, oocytes are recovered from follicles after FSH stimulation of the ovaries and at an appropriate interval (about 36 h) after the administration of an ovulating hormone (human chorionic gonadotrophin (hCG)). In this system, the majority of oocytes should have matured to metaphase II at the time of follicle aspiration; in practice, the number of immature (primary) oocytes is likely to vary with the individual, and experience has shown an incidence varying from 0 to 50%. In cattle, there have been those who take the view that oocytes aspirated from large follicles close to the time of ovulation in superovulated animals are likely to yield more blastocysts than those recovered from small (2–7 mm) follicles; in following this approach, Bousquet *et al.* (1995) in Canada characterized the progression of nuclear and cytoplasmic maturation in oocytes recovered from large (> 8 mm) follicles. Ovarian stimulation was by way of PMSG, followed by $PGF_{2\alpha}$ after 48 h; oocytes were aspirated at intervals of 36–60 h after prostaglandin. Although oocytes recovered after 60 h were further advanced in maturation and most were arrested at metaphase II, almost one-third were found at metaphase I. Results indicated that the resumption of meiosis in oocytes in large follicles was highly asynchronous. In the UK, Moyo and Dobson (1995) collected oocytes by OPU 22–24 h after an injection of GH-RH, which had been given 43 h after $PGF_{2\alpha}$ analogue; from 124 follicles punctured in five donor cows, the oocyte recovery rate was 48%; attempts to fertilize and culture the oocytes further met with little success.

In the Netherlands, Van de Leemput *et al.* (1999) aspirated *in vivo* matured oocytes from preovulatory follicles in the ovaries of superovulated heifers 22 h after a fixed-time GH-RH-induced LH surge; endogenous release of the LH surge was inhibited by a progestogen ear implant (see treatment schedule in Table 3.8). This work was part of a study comparing *in vivo* matured oocytes with those matured *in vitro*; there was a twofold increase in the developmental potential of the oocytes matured in the follicle, leading to the conclusion that IVM remained a major factor limiting laboratory embryo production.

3.4.2. Enhancing quality of primary oocytes

There have been studies that have indicated that blastocyst yield may be enhanced when oocytes in OPU programmes are matured in the presence of additional granulosa cells (Konishi *et al.*, 1995). The value of adding additional parietal granulosa cells (1–3 million ml^{-1}) to the maturation medium rather than relying on the cumulus cells attached to oocytes was demonstrated by Galli and Lazzari (1995) in Italy.

As noted in previous sections, many of the studies with OPU in live animals have been concerned with technical factors, such as needle type, aspiration vacuum pressure and collection frequency, or biological factors, such as age and hormonal pretreatment of donor animals. Clearly, such considerations may play a part in preserving the quality of a good oocyte, but they

Table 3.8. Treatment to obtain *in vivo*-matured bovine oocytes (from Van de Leemput *et al.*, 1999).

Day	Time (h)	Treatment/event
0		Oestrus
8		Removal of eventual dominant follicle
10	0	Onset of superovulation with eCG Implantation of norgestomet
12	48	Induction of luteal regression with prostaglandin
15	102	Removal of norgestomet implant Induction of LH surge with GH-RH
	104	Maximum of the preovulatory LH surge Start of *in vivo* maturation
	112	Neutralization of eCG
16	126	Ovariectomy Completion of *in vivo* maturation

do not necessarily influence the quality of oocytes that are less competent within the follicle prior to aspiration. To influence quality here, it may be a question of follicular dynamics, size of follicles aspirated, hormonal and dietary pretreatments of the donor and the reproductive status of the donor animals itself (prepubertal, cyclical, pregnant, post-partum).

Two approaches to improving embryo yield in an OPU programme have been suggested by Henriksen *et al.* (2000) in the Netherlands: (i) stimulation of donors with gonadotrophins; and (ii) *in vitro* prematuration before final maturation. As noted elsewhere, OPU performed once weekly on FSH-stimulated heifers can achieve transferable embryo yields comparable to those for twice-weekly OPU with untreated animals; there is, of course, the cost of the gonadotrophins. In taking the second approach, the hope is to improve cytoplasmic maturation while nuclear maturation is blocked by various ploys (pharmacological agents, protein synthesis inhibitors, theca-cell factors). However, as discussed in detail subsequently, although the theory seems good, there is currently no practical evidence of its success.

Increased understanding of bovine ovarian physiology in recent years has enabled effective superovulation treatment protocols to be designed for producing embryos of high quality. Such protocols usually involve the removal of medium and large follicles by aspiration *per vaginam*, which leads to an increase in FSH level (Baracaldo *et al.*, 2000); after 2 days, the emerging follicular wave is sustained by multiple injections of FSH over 2–4 days. It has also been shown that, if follicular stimulation is either decreased or stopped (resulting in FSH starvation) for 33 or 48 h after the last FSH injection, a 'coasting period' is created that simulates the *in vivo* early dominance phase. In Canada, Blondin *et al.* (2002) examined the effects on oocyte developmental competence of using four or six injections of FSH, different coasting periods (33 or 48 h) and the injection of LH 6 h before oocyte recovery; when donors were given six doses of FSH with a 48 h coasting period, *in vitro* processing of oocytes resulted in an 80% blastocyst yield, probably the highest yield yet recorded. It is believed that induction of a preovulatory-type follicular environment is necessary to trigger the bovine oocyte to complete cytoplasmic maturation before it is exposed to the conventional IVM treatment.

3.4.3. Oocyte transportation

There may be practical situations in which oocytes recovered by OPU have to be transported some distance from the farm to the embryo production laboratory. Workers in Canada sought to identify a suitable maturation environment to enable oocytes to be transported in 0.5 ml straws over long distances after being collected from abattoir ovaries (Giritharan *et al.*, 1999a,b; Giritharan and Rajamahendran, 2000); they concluded from their results that optimum temperature and CO_2 level are necessary for maturation during transport. In Brazil, Lehmkuhl *et al.* (2001) reported that follicular fluid, obtained from follicles 2–8 mm in diameter, could be used for the maintenance of immature cattle oocytes at temperatures from 22 to 38°C for 5 h. In the same country, Leivas *et al.* (2002) carried out a simulation study, using oocytes aspirated from abattoir ovaries, in which oocytes were maintained in polystyrene tubes containing 400 µl of TCM-199 + HEPES in air; they found that oocytes could be held for up to 12 h without detriment to their embryonic development. Other work reported from Brazil showed that it was possible to maintain bovine oocytes recovered by OPU in bovine follicular fluid for 6 h at 30°C before successfully maturing them *in vitro* (Alves *et al.*, 2002). Studies in the same laboratory reported by Rauber *et al.* (2002) observed a better morphological quality in embryos derived from oocytes held for 6 h in follicular fluid prior to IVM; they also recorded a higher blastocyst yield in oocytes held in bovine follicular fluid from large (> 8 mm) rather than small (3–5 mm) follicles.

3.5. Factors Affecting Oocyte Quality

A matter of great importance to those attempting to produce bovine embryos in the laboratory is the ability of the oocyte to undergo fertilization and develop into an embryo; this ability (oocyte competence) must extend all the way through to the birth of a live calf (Driancourt *et al.*, 1998).

Many factors are known to influence oocyte quality and much attention has been given to devising ways in which good-quality oocytes can be identified. Some of the features associated with the less competent oocytes may be visible immediately, while others may not be evident until fertilization of the oocyte.

3.5.1. Age of animal

Taken over the full range of mammalian species, concern is often expressed about the quality of oocytes recovered either from the very young or the very old. A progressive decline in human fertility with advancing age is a well-recognized phenomenon, but it is the young prepubertal heifer that is of most concern to those engaged in cattle *in vitro* embryo production. Although antral follicles containing fully grown oocytes are present in the ovaries of the calf at birth, attempts to use these oocytes in embryo production have usually proved disappointing (Duby *et al.*, 1996; Gandolfi *et al.*, 1996b; Presicce *et al.*, 1996, 1997; Kelly *et al.*, 1997; Khatir *et al.*, 1997c; Earl *et al.*, 1998; Steeves and Gardner, 1999a; Steeves *et al.*, 1999; Armstrong, 2001; Palma *et al.*, 2001). As with oocytes from adult cattle, the size of the calf oocyte has a marked influence on its ability to mature to metaphase II.

In France, Revel *et al.* (1995) demonstrated that fertilization and cleavage rates from 3-month-old calf oocytes did not differ significantly from those of cow oocytes but the blastocyst formation rate was much lower (9–11%) in comparison with cows (> 20%). Other studies in the same laboratory at Nouzilly showed that epidermal growth factor (EGF) in the IVM medium altered the pattern of protein neosynthesis in calf oocytes and significantly increased the blastocyst yield (Khatir *et al.*, 1996a,b,c). A study of metabolic activity and protein synthesis by Gandolfi *et al.* (1998) led them to conclude that there was a significant difference between cow and calf oocytes; they suggested that this might be related to the lower developmental capacity of oocytes from prepubertal animals.

In Italy, Lazzari *et al.* (1996) tested the effect of a protocol based on oestradiol, progesterone-releasing intravaginal sponges and PMSG on the developmental capacity of 2–3-month-old dairy calves; although an average of 4.2 embryos of normal viability was produced per calf, great variability was evident, with embryos being produced by only 50% of the calves treated. Other studies have also reported that oocytes from calves are developmentally less competent than those from adult animals (Adulyanubap *et al.*, 1998; Gandolfi *et al.*, 2000). It is known that calf oocytes may be smaller in diameter than those of adult cattle; other features that may mark them out after exposure to sperm include delayed sperm aster formation and asynchronous pronuclear formation (Duby *et al.*, 1995). In attempting to determine a possible cause for their low developmental competency, Damiani *et al.* (1996a,b, 1998) found that calf oocytes were less sensitive to calcium-releasing agonists and that a significantly higher number failed to show normal calcium oscillations after sperm penetration. In Canada, Levesque and Sirard (1994) were able to show that many constitutive proteins of adult cow cumulus cells differed from those of oocytes and several proteins were entirely absent in calf oocytes; they suggest that such proteins may be important for the initiation of a cascade of events that is required for the normal development of the bovine oocyte.

In Belgium, Majerus *et al.* (1999b) concluded that few oocytes whose diameter was less than 120 μm were able to complete meoisis; even with oocytes larger than 120 μm some proportion failed to mature to metaphase II (see Table 3.9). The same group assessed cell distribution between the inner cell mass (ICM) and the trophectoderm (TE) derived from calf and cow oocytes (Majerus *et al.*, 1999b); the lower viability of the calf oocytes could not be explained on the basis of cell numbers.

In further work, using time-lapse cinematography and other monitoring techniques, Majerus *et al.* (2000) reported that calf oocyte-derived embryos were characterized by a higher rate of developmental arrest before the nine-cell stage and by a longer lag phase preceding the major onset of embryonic genome expression; it appeared possible that such changes might be related to insufficient ‘capacitation’ of the calf oocyte during follicular growth. Reviewing a considerable body of evidence dealing with the lack of competence of the bovine oocyte before

Table 3.9. Comparison of calf vs. cow oocytes in blastocyst yield.

Year	Donor treatment	Donor age	Blastocyst %	Researcher(s)
1993	None	Calf	16	Palma *et al.*
	None	Adult	22	
1995	FSH	4 months	9	Revel *et al.*
	None	Adult	21	
1995	FSH	< 8 months	0	Looney *et al.*
	None	Adult	32	
1996	FSH	4 months	6	Damiani *et al.*
	None	Adult	33	
1997	None	3–5 months	12	Mermillod *et al.*
	None	Adult	32	

puberty, Gandolfi *et al.* (2000) noted that developmental potential was a capability of the oocyte that gradually grew from birth to puberty. In Australia, Maclellan *et al.* (2000b) examined whether Brahman heifer calves (3 months old) treated with a GH-RH agonist before and during treatment with FSH influenced the number and competence of oocytes recovered from different-sized follicles; although treatment with deslorelin tended to increase the number of larger follicles and the number of oocytes collected, there was no evident difference in their competence.

In France, Khatir *et al.* (1998a,b,c) observed differences in protein profiles between cow and calf oocytes that might explain differences in their developmental competence; there were also indications that nuclear progression during maturation is linked to changes in protein synthesis in the oocyte. A report by Massicotte *et al.* (1999) examined the ultrastructure of oocytes from 3-month-old calves treated with GH-RH and FSH; they found evidence of differences in cytoplasmic features between calf and cow oocytes, which could only be partially alleviated by FSH treatment. Among prepubertal heifers, it is known that the developmental competence of oocytes can vary greatly. Working with 14-month-old Friesian heifers, Taneja *et al.* (2000) evaluated the activities of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK); these activities were found to be lower at puberty than in adulthood.

In Spain, Pujol *et al.* (2000) evaluated the usefulness of a brilliant cresyl blue (BCB) test to select competent heifer oocytes before IVM; their study suggested that this test might be a useful tool. The BCB test determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized during oogenesis. Studies by Salamone *et al.* (2000) in Massachusetts reported that the poor developmental competence of calf oocytes is due to a lack of ooplasmic factors essential for development. Subsequent work by Salamone *et al.* (2001) showed that the activity of MPF and MAPK and the relative amount of inositol-1, 4, 5-triphosphate receptor (IP_3R) are substantially lower in artificially matured calf oocytes than in those of adult cattle; this clearly suggests that key ooplasmic components associated with fertilization, cleavage and development are compromised in calf oocytes.

A report by de Paz *et al.* (2001) in Spain examined morphometric and cytochemical differences between cow and calf oocytes, using microscopic techniques. Calf oocytes showed a greater density of microvilli on their surface and a greater number of endocytosic vesicles than those of the cow. There was a larger mitochondrial population in the oocytes from adult cattle; the cumulus cells of calf oocytes possessed a greater volume of lipid droplets. The authors describe certain other differences, as revealed by cytochemical analysis (see Fig. 3.3). As well as those describing differences between cow and calf, there are many who have attempted to improve the developmental competency of calf oocytes (Taneja *et al.*, 2000). Some have investigated the effect of adding granulosa cells taken from adult ovarian follicles and various additives during IVM (Donnay *et al.*, 2002); although adding the cells did not cause a greater number of embryos to develop, the quality of the blastocysts, as measured by cell number, was improved.

In other farm species, the results of a study in sheep by O'Brien *et al.* (1997) showed an

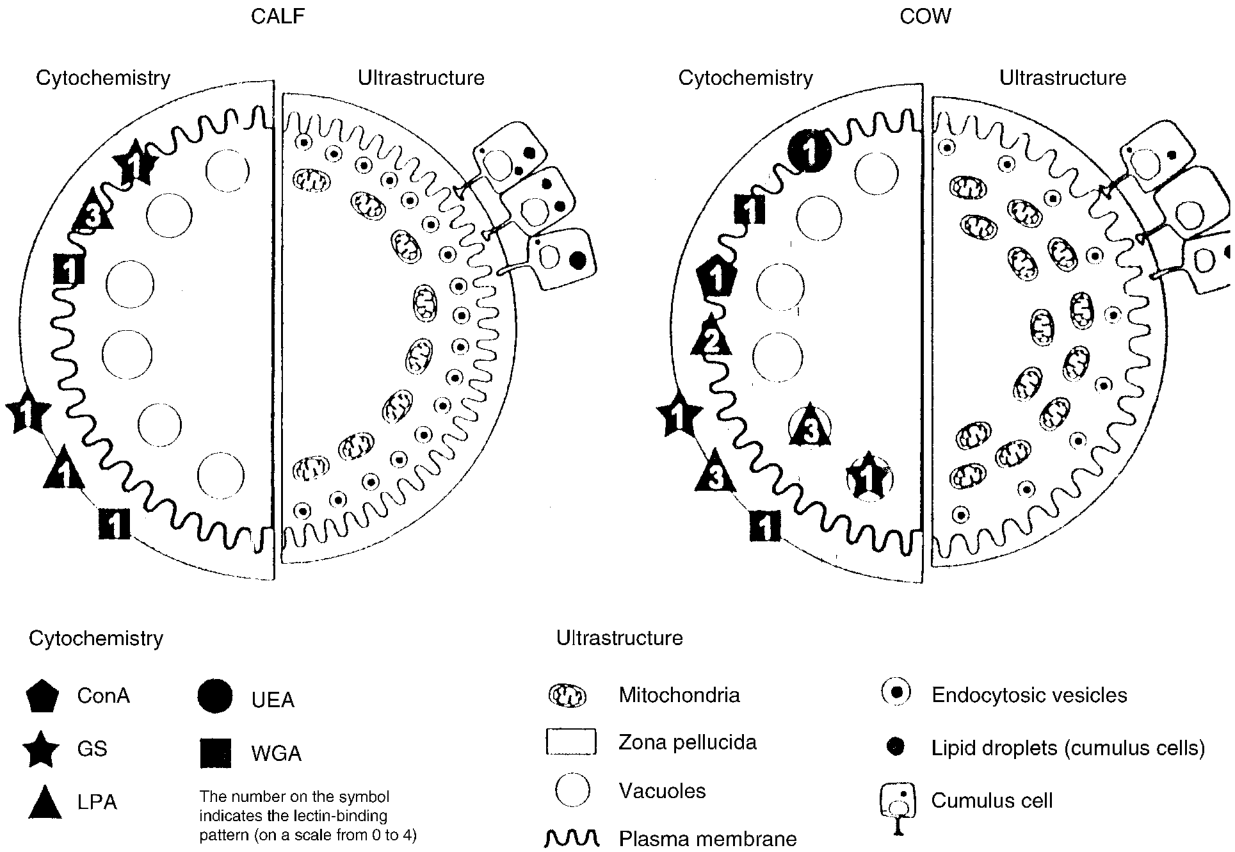

Fig. 3.3. Cytochemical and ultrastructural differences between calf and cow oocytes (from de Paz *et al.*, 2001).

increased incidence of polyspermic fertilization and a reduced *in vitro* developmental capacity of embryos derived from the oocytes of prepubertal compared with that of adult animals. In Canada, Kochhar *et al.* (2002) compared the developmental competence of IVM oocytes derived from lambs and ewes; although the cleavage rate was similar, blastocyst yield was significantly lower in lamb-derived oocytes. The aim of a study by Catt (2002) was to test whether the competence of prepubertal sheep oocytes could be altered by cytoplasmic transfer from adult oocytes; evidence suggested that the addition of 5% cytoplasm from the adult oocyte had a positive effect. It is also evident that prepubertal pig oocytes are developmentally less competent that those from postpubertal animals; the indications here, as in cattle, are that the difference lies in cytoplasmic maturation. A study by Bagg *et al.* (2002) indicates that the problem does not lie in nuclear maturation. There is evidence that oocytes from prepubertal rhesus monkeys are less competent than oocytes from adults (Zheng *et al.*, 2001); this indicates that oocyte developmental competence is affected by age in primates, as it is in cattle and other farm animals.

Oocytes from fetal ovaries

Workers in Minnesota have examined the meiotic development of fetal and adult cow oocytes (Chohan and Hunter, 1999); they dissected out follicles from the ovaries of 7.5-month-old fetuses and adult cows and matured COCs for 24 h. A significantly lower rate of maturation to metaphase II was observed for fetal oocytes from < 4 mm follicles (7.5–46.9%) compared with oocytes from 4–6 mm follicles (68.9%). Fetal oocytes from 4–6 mm follicles matured as readily to metaphase II as cow oocytes from 2–3 mm follicles.

3.5.2. Cattle category, oestrous cycle and ovarian morphology

Cattle category

The production of IVP cattle embryos in Ireland in the late 1980s was based on ovaries recovered from heifer cattle at abattoirs. Much evidence was obtained to show that the quality of oocytes recovered from heifer cattle ovaries was equal or superior to that found with cow ovaries (Gordon, 1994). In countries such as the USA, where hormones are used in the growth promotion of beef cattle, the experience may be quite different. A paper by Checura *et al.* (2002) dealt with the developmental potential of oocytes from the ovaries of cows and heifers; they found that oocytes from cull cow ovaries were markedly superior to those from fattened heifers (blastocyst yield for heifers was 16% vs. 34% for cows). The authors note that most of the beef heifers in the study had been fed melengestrol acetate at 0.5 mg day^{-1} as part of their final fattening diet. In the countries of the European Union (EU), it should be noted, there is currently a total ban on the use of growth hormones, whether for steer or for heifer cattle.

Stage of cycle

Follicle numbers and oocyte quality have been recorded during different stages of the bovine oestrous cycle and according to whether follicles are located ipsilateral or contralateral to the corpus luteum. In Canada, Boediono *et al.* (1995) tested the hypothesis that higher-quality oocytes can be obtained from ovaries in the luteal phase and from ovaries bearing the corpus luteum; their results were consistent with that hypothesis. In the Czech Republic, Machatkova *et al.* (1996) investigated the developmental competence of oocytes collected from donors at various stages of the cycle; a significantly higher proportion of oocytes developed into blastocysts when collected on days 14–16 than when collected on days 7–9, days 19–20 or day 2. A report by Hagemann *et al.* (1998a) dealt with the effect of cycle stage on the development of cattle oocytes in an IVP system. In the Netherlands, Steenweg *et al.* (2000) noted that the developmental competence of COCs appeared to be influenced by the presence of a dominant follicle (DF); they showed that the suppressive effect of the DF became apparent at the non-growing phase. A report by Machatkova *et al.* (2000) showed that utilizing the growth phase of the first follicular wave for oocyte collection improved the efficiency of blastocyst production. In New Zealand, Sisco *et al.* (2002) used suppressive subtractive hybridization to investigate the differential expression of genes in dominant and

subordinant follicles at day 1.3–2.3 of the cow's oestrous cycle; six complementary DNA (cDNA) clones were identified as being differentially expressed in the DFs.

Determining cycle stage

In the collection of ovaries from cyclic cattle at the abattoir, it may occasionally be necessary to estimate the stage of the cycle; as shown in Table 3.10, there are several features of the corpus luteum (colour,size,vasculature) that allow this to be done with reasonable accuracy.

Morphology of ovaries

In Italy, Lauria *et al.* (1996) found morphological evaluation of the ovary to be a useful method of predicting the *in vitro* developmental potential of bovine oocytes; ovaries with a DF (> 10 mm diameter) or with more than ten follicles of 2–5 mm diameter and no DF yielded higher-quality oocytes than ovaries with fewer than ten follicles and no DF, with or without a corpus luteum. A study by Varisanga *et al.* (1998) attempted to classify bovine ovaries into five categories according to their morphological features and to determine whether such features influenced the recovery and developmental competence of oocytes. Not unexpectedly, morphology was to some extent determined by stage of cycle; results supported the concept that the intraovarian environment to which oocytes are exposed can play a major role in determining their developmental competence. The Japanese workers concluded that classification of ovaries prior to follicle aspiration could be used as an indicator of the developmental competence of the collected oocytes; the presence of a DF in either or both ovaries had a negative effect on the competence of oocytes. In Taiwan, Shen and Lee (1999) reported that higher-quality oocytes could be aspirated from small antral follicles in the ovary in the absence of a DF. In New Zealand, similar findings were reported by Hagemann (1999) and Hagemann *et al.* (1999a,b), who recorded that competence was significantly greater in oocytes collected during phases of follicular growth than during phases of follicular dominance.

In Canada, Salamone *et al.* (1999) sought a better understanding of the interrelationship between the follicle and its oocyte, which would enable them to manipulate oocyte quality prior to collection. Since COCs for use in their laboratory usually came from follicles in the size range 2–6 mm, the competence of oocytes from subordinate follicles in their different stages of development (growing, static, regressing) was of particular interest. The Canadian workers demonstrated that follicular atresia was associated with changes that resemble the resumption of nuclear meiosis (oocytes with expanded cumulus-cell layers). The uncoupling of the relationship between cumulus cells and the oocyte or the decreased inhibitory effect of theca cells during atresia could explain the reinitiation of oocyte maturation. It is known that organelle rearrangement in oocytes during early follicle atresia resembles that occurring in preovulatory follicles around the time of the LH surge (Assey *et al.*, 1994a,b); for such reasons, the resumption of meiotic maturation within the early atresic follicle may be beneficial for oocyte developmental competence. It is evident that COC morphology and oocyte competence change during the

Table 3.10. Appearance of bovine corpus luteum at different stages of the cycle (based on Ireland *et al.*, 1980.)

Stages of oestrous cycle	*n*	Appearance of corpus luteum (CL)	Estimated range of days spanned by each stage of cycle
I	30	Surface of CL red, follicle recently ruptured (0.5–1.5 cm in diameter)	1–4
II	42	Point of rupture of follicle covered over, apex of bisected CL red or brown (1.6–2 cm), vasculature visible on surface of CL	5–10
III	52	Bisected CL is orange or yellow and has no red or brown colour remaining, vasculature prominent on surface of CL (2 cm)	11–17
IV	22	Vasculature absent from surface, large follicle present	18–20

various phases of subordinate follicle development; there are also significant changes in the levels of progesterone and oestradiol in follicular fluid as these changes occur.

In Germany, Jewgenow *et al.* (1999a,b) examined the developmental competence of good-quality COCs in relation to the degree of apoptosis evident in the follicle wall and other ovarian features; they concluded that the degree of apoptosis did influence the developmental competence, even of good-quality oocytes.

Cystic follicles

The oocyte quality of small antral follicles coexisting with cystic follicles (follicular cyst containing > 10 ml follicular fluid, > 3 cm in diameter) in the ovaries of Friesian dairy cattle was examined by Takagi *et al.* (1998a,b) in Japan. They reported that the numbers of oocytes obtained from an ovary, their morphology and their maturation rates were not significantly different in the presence or absence of a coexisting cystic follicle.

3.5.3. Body condition and nutritional considerations

Various grading sytems for assessing the body condition and nutritional status of cattle have been developed over the years. Some workers have looked at the developmental potential of oocytes in relation to such factors (see reviews by McEvoy, 1999; McEvoy and Robinson, 2002). A report by Lopez *et al.* (1996a,b) recorded a significantly lower proportion of good-quality oocytes in cows with a condition score of 1 (1 = emaciated; 5 = obese) than in cattle with scores of 2–3. In the USA, Fazio *et al.* (1999) examined oocyte quality in cattle after they were condition-scored (scale 1–9); the ovaries of thin cows (scores 3–4) had significantly fewer small follicles than cattle in good condition (scores 5–6) (9.9 vs. 11.6, respectively). The workers recommended that cows in good condition should be selected wherever possible. In Ireland, Yaakub *et al.* (1999a,b) examined the effect of different diets on follicle numbers and oocyte quality in superovulated beef heifers; some differences in embryo development could be related to diet. In the following year, Snijders *et al.* (2000), also in Ireland, recorded evidence suggesting greater competence in oocytes from dairy cows with a high body-condition score than those with a low score. In the UK, McEvoy *et al.* (1997) found that the developmental competence of oocytes from small follicles (< 4 mm) of non-superovulated heifers was compromised by excess dietary energy and Sinclair *et al.* (1999, 2000a) observed that oocytes from medium-sized antral follicles (> 4–8 mm diameter) appeared to be particularly sensitive to rumen-degradable nitrogen. It appeared that oocyte competence could be adversely affected by nutritional regimens that resulted in high circulating levels of ammonia in the plasma.

In the Netherlands, Kruip *et al.* (1999) examined the developmental competence of oocytes recovered from high-yielding dairy cows in the post-partum period; they found the quality of oocytes about 100 days after calving to be negatively correlated with triacylglycerol concentrations in the liver. It seemed possible that the effect of negative energy balance on oocyte quality was the result of the toxic action of non-esterified fatty acids on follicles and oocytes rather than being the effect of a low insulin level on LH pulsatility. There is evidence suggesting that different nutritional levels can affect the expression of developmentally important genes in cattle embryos (Wrenzycki *et al.*, 1999b, 2000a); investigating the relative abundance of certain gene transcripts in oocytes and embryos is likely to shed valuable new light on the reasons for their failure under different dietary regimens. Diet may influence oocyte maturation and early embryonic development by influencing the microenvironment of the reproductive tract.

The complex relationship between nutrition and reproduction in cattle has been reviewed by O'Callaghan *et al.* (1999, 2000) and Boland *et al.* (2001b). In Virginia, Walters *et al.* (2002b) sought to determine the influence of energy status on the quality of oocytes obtained from cows during early and mid-lactation; they recorded a larger number of follicles in mid-lactation cows than in those in early lactation, indicating a greater rate of follicle development for cows with a more positive energy balance. It is clear from this and other reports that the effects of nutrition are exerted very early in embryo development, possibly during the acquisition of developmental

competence by the oocyte. For optimum reproductive performance, the nutritional needs of the animal should be supplied in a physiological way, avoiding abnormal or unbalanced amounts of any one component in the diet; such nutritional balancing is all the more important in high-yielding dairy cattle. It is evident that high dietary intake or high metabolic load associated with the management of high-yielding dairy cows may compromise normal oocyte development and the establishment of pregnancy.

It is known that high dietary protein may lead to increased systemic levels of ammonia and urea in dairy cattle and may be a factor in the reduced fertility often encountered in such livestock. A study in Ireland reported by Kenny *et al.* (2002a) quantified the levels of ions and metabolites in bovine oviductal fluid and showed that increased systemic levels of ammonia and urea were unlikely to disrupt the oviductal environment to the point where embryo survival would be impaired.

3.5.4. Reproductive status of donor

There is evidence showing that pregnant cows can yield a higher number of high-quality oocytes than cyclic animals, probably due to higher progesterone levels in the circulation and constant follicular turnover.

3.5.5. Animal factors

Data from an Irish study suggest that the genetic merit of dairy cattle may affect the quality of their oocytes (Snijders *et al.*, 2000); cows of high genetic merit yielded lower-quality oocytes than their medium-genetic-merit herd mates (Table 3.11). Such findings may have relevance to the lower fertility rate found in some high-yielding dairy herds.

3.5.6. Environmental factors

There is ample evidence showing that, in tropical, subtropical and temperate regions, the fertility of dairy cattle may show a serious decline. It is also known that there may be a delayed effect of summer heat stress on oocyte quality. In practical terms, this may result in fertility remaining low during the cooler autumn months, even though the animals may no longer be under heat stress. In Israel, Roth *et al.* (1999, 2001) reported clear evidence of this effect and also showed that the removal of impaired cohorts of follicles during the autumn led to the earlier emergence of healthy follicles and higher-quality oocytes; follicles (3–7 mm diameter) were aspirated during four consecutive oestrous cycles. The workers suggest that the autumn fertility of cows in warm climates could be improved by strategies (hormonal or mechanical) that would enhance the emergence of more follicles or induce more follicular waves.

In Florida, Al-Katanani *et al.* (2002a) examined seasonal variation in oocyte competence in Friesian cows and sought to determine whether oocyte quality in summer was affected by the magnitude of heat stress; summer depression in oocyte quality was evident but cooling cows for 42 days did not alleviate the seasonal effect.

Table 3.11. Genetic merit and embryo yield in diary cattle (from Snijders *et al.*, 2000).

	High genetic merit	Medium genetic merit
Number of cows	48	46
120 days' milk yield (kg)	4096 ± 108.94^{a}	3731 ± 130.32^{b}
Body condition score at oocyte recovery	2.52 ± 0.07	3.18 ± 0.08^{b}
No. of oocytes collected	6.7 ± 0.75	7.6 ± 0.91
Cleavage rate % (no.)	$70.4\ (238/338)^{a}$	$77.4\ (278/359)^{b}$
No. of blastocysts	0.36 ± 0.19^{a}	0.85 ± 0.22^{b}
Blastocyst formation rate % (no.):		
From oocytes cultured	$6.8\ (23/338)^{a}$	$11.4\ (41/359)^{b}$
From cleaved oocytes	9.7 (23/238)	14.7 (41/278)

Values with different superscripts within rows differ^{a,b} $P < 0.05$.

Further work in the same laboratory confirmed the adverse effect of elevated summer temperature on oocyte developmental competence (Al-Katanani *et al.*, 2002b); however, while embryos produced in summer showed reduced developmental competence, there was no evidence that they were more susceptible to damage caused by heat shock after fertilization.

3.6. Assessing Oocyte Quality

There are various systems used in the grading of bovine oocytes (see Table 3.12). Most depend on a visual subjective appraisal by laboratory personnel and as such can vary with the individual and the laboratory.

3.6.1. Oocyte morphology: classification schemes

Selection of bovine oocytes for IVM on the basis of visual assessment of morphological features has been examined by several groups and there have been many reports describing classification schemes, based on the compactness and quantity of surrounding follicular cells and other morphological features that are visible by light microscopy. In terms of the importance of cumulus cells, Cetica *et al.* (1999) in Argentina studied the nuclear stage of four grades of immature oocytes selected for maturation on the basis of cumulus-cell coverage; they found that 88% of the top-grade oocytes were at the GV stage, a figure significantly higher than in the other grades. Apart from cumulus cells, it is also recognized that various constituents of follicular fluid and their concencentration may also be useful in assessing the quality of the oocyte; both progesterone and oestradiol-17β, produced by the somatic cells of the follicles, are to be found in the fluid. Atresia in follicles is generally characterized by increased progesterone and decreased oestradiol levels; conversely, healthy follicles are expected to show high oestradiol and low progesterone concentrations.

At Beltsville, Hawk and Wall (1994a,b), dealing with 1000 oocytes daily, used a limited number of quality grades in selecting oocytes for maturation on the understanding that this would avoid excessive delay in placing the oocytes into the maturation medium (see Table 3.13). The same workers also examined the relationship between morphological characteristics

Table 3.12. Criteria used in assessing bovine oocyte quality.

Category	COC
Criteria	
1	Compact multilayered cumulus investment; homogeneous ooplasm; total COC light and transparent
2	Compact multilayered cumulus investment; homogeneous ooplasm but with a coarse appearance and a darker zone at the periphery of the oocyte; total COC slightly darker and less transparent
3	Less compact cumulus investment; ooplasm irregular with dark clusters; total COC darker than 1 or 2 above
4	Expanded cumulus investment; cumulus cells scattered in dark clumps in a jelly matrix; ooplasm irregular with dark cluster; total COC dark and irregular
Criteria	
Selected	Homogeneous-appearing ooplasm and compact cumulus cells tightly adherent to the zona pellucida
Unselected	Other categories with incomplete cumulus complements and heterogeneous ooplasm
Criteria	
1	Compact multilayered cumulus investment; homogeneous ooplasm; total COC light and transparent
2	As 1 but with coarse appearance and a darker zone at the periphery of oocyte
3	Total COC darker than 1 or 2; less compact cumulus; ooplasm irregular with dark clusters
4	Expanded cumulus investment

of one-cell presumptive zygotes and the potential of the zygotes to develop to the blastocyst stage. It was found that the greatest gain in selecting oocytes and zygotes with high developmental potential was by omitting oocytes with minimal cumulus and selecting presumptive zygotes with dense, even cytoplasm.

A report by Hazeleger, N.L. *et al.* (1995) in Canada also described morphological criteria and follicular-fluid progesterone levels that may be used in the selection of COCs with a high potential for embryo development. The workers classified the oocytes into nine morphological groups (Table 3.14). The Canadian workers

Table 3.13. Beltsville criteria for bovine oocyte selection (from Hawk and Wall, 1994a).

Type of oocyte	Characteristics of cumulus	Characteristics of cytoplasm
Good quality	Compact, with full cumulus or several layers of cumulus cells. Granulosa adhering to cumulus permissible if cytoplasm clearly seen	Even, dense, finely granulated
Intermediate–marginal quality	Range of characteristics, including: thick, usually with adhering granulosa, but cytoplasm not seen clearly; compact cumulus, a few to several layers, covering all or at least half of zona pellucida	Range from even, dense, finely granulated to moderate-size granules
Rejects	Range of characteristics, including: partially expanded or fully expanded and dispersing cumulus; non-cellular framework with no cumulus cells; exceptionally small or large oocyte; discoloured cumulus (black or light brown); corona radiata with no cumulus; nude oocytes	Coarse granular or intermixed very light and very dark areas; discoloured cytoplasm (light brown or black); badly misshapen oocytes

Table 3.14. Characterizations of nine morphological groups used to classify bovine oocytes (after Hazeleger N.L. *et al.*, 1995).

Group	Classification criteria
Group 1	Compact and complete cumulus layer surrounding an oocyte with a homogeneous ooplasm consisting of fine granulation and that is medium brown in colour Oocyte > 120 μm in diameter
Group 2	Compact and complete cumulus layer The ooplasm is slightly more coarse than that of group 1, with a dark zone around the periphery, medium brown in colour Oocyte > 120 μm in diameter
Group 3	Complete cumulus layer but beginning to expand around the outer edge Ooplasm consists of dark clumps throughout, medium brown in colour Oocyte > 120 μm in diameter
Group 4	Fully, irregularly, expanded cumulus with clumped degenerated cells in a jelly matrix Ooplasm consists of dark clumps throughout Oocyte > 120 μm in diameter
Group 5	Identical to group 1, but both cumulus and ooplasm are pale in colour Oocyte > 120 μm in diameter
Group 6	Corona cells are exposed Variable cytoplasmic features Oocyte > 120 μm in diameter
Group 7	Compact and complete cumulus layer Ooplasm is uniformly black in colour Oocyte > 120 μm in diameter
Group 8	Exposed zona pellucida Variable cytoplasmic features Oocyte > 120 μm in diameter
Group 9	Similar to group 1, but oocyte is < 110 μm in diameter

found that groups 1, 2 and 3 produced the highest rates of blastocyst-stage embryos and accounted for 47% of the total oocyte population examined. Group 1 COCs did not show any apparent signs of degeneration, whereas groups 2 and 3 displayed certain characteristics associated with early atresia. The morphological appearance of these oocytes indicated a progression towards degeneration; the cumulus investment was starting to expand and the ooplasm was clumping and becoming darker in colour, possibly the result of lipid-droplet accumulation and the clustering of organelles. The high developmental potential shown by these oocytes is in keeping with what is now understood about early atresic stages (Salamone *et al.*, 1999).

Oocytes in groups 4, 6, 7 and 8 exhibited signs of a more advanced stage of degeneration, such as clumped and irregularly expanded cumulus investment (group 4), dark or clumped ooplasm (groups 4 and 7) and partial or total loss of the cumulus investment (groups 4, 6 and 8). Group 5 COCs differed only from those of group 1 in being much lighter in colour; there was a low developmental potential in this group. The smaller diameter of oocytes in group 9 may have been linked to meiotic incompetence; the Canadian group found the fertilization rate to be very poor, suggesting that these oocytes may have been incapable of undergoing normal maturation.

A study by Laurincik *et al.* (1996) used corona radiata density as a non-invasive marker of COCs selected for embryo production. The Slovakian workers showed that COCs in which the corona radiata exhibit the same density as the rest of the cumulus investment are less suitable for embryo production. In Japan, Kawasaki *et al.* (1999) examined the morphology of oocytes collected from follicles 2–6 mm in diameter and classified them into five groups: (i) oocytes with homogeneous ooplasm and surrounded by a compact and complete cumulus; (ii) oocytes with heterogeneous ooplasm and surrounded by a complete cumulus layer; (iii) oocytes surrounded by fully, irregularly expanded cumulus with clumped cells in a jelly matrix; (iv) oocytes without a cumulus layer; and (v) oocytes not categorized into the other four groups. The rates of maturation and normal fertilization of oocytes with heterogeneous ooplasm were significantly higher than those of oocytes with homogeneous ooplasm. Oocytes with heterogeneous ooplasm showed dispersion of cytoplasmic granules during maturation; those with expanded cumulus or bereft of cumulus cells had significantly lower maturation and fertilization rates.

IVM, IVF and subsequent development of cattle oocytes with homogeneous or heterogeneous ooplasm were investigated by Nagano *et al.* (1999a) in Japan; oocytes with heterologous ooplasm had higher fertilization rates due to a lower incidence of polyspermy. Examination by electron microscopy showed such oocytes to have cortical granules lined up close to the plasma membrane (vitelline membrane); some oocytes with homologous ooplasm still showed small clusters of cortical granules after maturation.

In Germany, Khurana and Niemann (2000c) examined several factors influencing the efficiency of embryo production; the morphological quality of their oocytes was based on the compactness and number of layers of cumulus cells. The German workers found that only about a quarter of total oocytes aspirated from abattoir ovaries possessed a complete, compact, multilayered cumulus mass; this would be in accord with the data of Hazeleger, N.L. *et al.* (1995) in Canada. In studying the effect of the follicular environment on the quality and developmental competence of COCs, oocytes were collected by De Wit *et al.* (2000) from non-atresic, lightly atresic, atresic and heavily atresic follicles; COCs were assigned to one of three quality groups based on the appearance of the cumulus investment. It was found that COCs placed in the second quality grade (from atresic follicles) produced more embryos than oocytes from non-atresic follicles. It was found, during the course of maturation, that the COCs from atresic follicles were a few hours ahead of those from non-atresic follicles; this may suggest the need for a longer maturation period for such oocytes.

A study by De Wit and Kruip (2001) sought to find more parameters to define the developmental competence of cattle COCs; they classified oocytes into five groups based on their morphology and level of atresia. An increasing level of atresia was associated with: (i) an increasing zona pellucida diameter; (ii) an increasing oocyte diameter; (iii) an increasing developmental competence; and (iv) an increasing percentage of oocytes exhibiting germinal vesicle breakdown

(GVBD) after 24 h culture with α-amanitin and FSH.

Oocyte diameter

The ability of the bovine oocyte to resume and complete meiosis *in vitro* is known to be related to its diameter; oocytes smaller than 110 μm are still transcriptionally active and have a reduced ability to resume meiosis. This was confirmed in the studies of Lechniak *et al.* (2002), who showed that smaller oocytes tended to follow an abnormal path of meiotic maturation, resulting in disturbances in the maturation process; the authors suggest that this may be one explanation for the reduced developmental potential of embryos derived from small oocytes. An earlier study by Lammer *et al.* (1997) reported on the effect of oocyte diameter and zona pellucida thickness on the maturation, fertilization and early cleavage of cattle oocytes.

Lipid vesicles

An investigation by Isachenko *et al.* (2001) compared the ultrastructure of lipid droplets and the effect of cooling on intracellular lipid vesicles in bovine and porcine GV oocytes; they found that lipid droplets in cattle oocytes had a homogeneous structure and that lipids were utilized without the formation of interim lipid compounds.

Oestradiol: progesterone ratio

Oocytes capable of developing into blastocysts were associated with follicular fluid that showed lower progesterone concentrations than fluid associated with less competent oocytes; such information may be useful in determining those follicles with high developmental potential. In Japan, Araki *et al.* (1998) investigated the effects of follicular-fluid oestradiol-17β concentrations of individual follicles (2–8 mm diameter) on the morphology and developmental competence of bovine COCs; oocytes aspirated from follicles with < 100 pg/ml oestradiol-17β resulted in significantly lower proportions of good-quality oocytes compared with those coming from follicles showing higher oestradiol levels.

Gene expression

There are those who believe that it would be useful to define marker genes that will enable the developmental competence of bovine oocytes to be predicted; this would allow more appropriate maturation and culture media to be developed. In North America, Calder *et al.* (2001b) reported that the differential expression of the PGE_2 receptor gene among varying COC qualities may prove to be a useful marker to predict oocyte quality.

Oocytes from zebu cattle

According to Garcia *et al.* (1998) in Brazil, data are lacking on the morphological evaluation of zebu cattle oocytes; they sought to remedy that omission by providing information on follicles and oocytes. A further report from Brazil by Dode *et al.* (2000) dealt with the effect of follicle size on the maturation of oocytes from zebu cattle; oocytes from follicles of 1–2 mm showed a significantly smaller diameter than those from larger follicles.

3.7. Oocytes from Preantral and Early Antral Follicles

A question of interest to those engaged in reproduction research is how to make much greater use of the considerable numbers of oocytes that are present in very small follicles. Despite the large numbers of oocytes found in primordial follicles, only a minute fraction of the population of oocytes grow to the point at which they are ovulated. A review by Figueiredo *et al.* (2000) in Brazil discussed the possible applications of the biotechnology they termed ‘manipulation of enclosed oocytes in preantral follicles’, with particular reference to cattle (see Fig. 3.4). Workers in the USA have described a useful biopsy method that they employed to collect primordial, primary, secondary and small (1 mm diameter) tertiary follicles (Britt *et al.*, 1999); a review by Smitz and Cortvrindt (2002) summarized current knowledge on mammalian folliculogenesis *in vivo* and dealt with the culture of preantral follicle stages, mainly in terms of work in mice and humans. In Japan, a comparison of enzymatic and mechanical methods for

Animal Isolation Growth Antrum Ovulation Embryo Birth

Goat
(*Rodrigues* et al., *1998*)

Cow
(*Figueiredo* et al., *1993*)

Sheep
(*Cecconi* et al., *1999*)

Sow
(*Hirao* et al., *1994*)

Mice
(*Eppig and Schroeder, 1989*)

Mice
(*Carroll* et al., *1990*)

Fig. 3.4. Progress in the manipulation of oocytes in preantral follicles in mice and farm animals (adapted from Figueiredo *et al.*, 2000).

the collection of bovine preantral follicles was made by Saha *et al.* (2002). Although the bovine ovary contains thousands of immature oocytes in primordial and preantral follicles (see Miyamura *et al.*, 1996), relatively few can be recovered by current procedures.

3.7.1. Birth of young in mice

There has been a keen interest, in species ranging from mice to cattle, to develop an IVC system capable of supporting follicle growth from the preantral to the antral stages, oocyte maturation, fertilization and embryonic development. Apart from the practical implications of progress in this area of research, the ability to culture preantral follicles *in vitro* would be useful for understanding the complex mechanisms in folliculogenesis at early stages of oocyte development. The production of live young from preantral follicular oocytes up to this time has been achieved only in mice, by Eppig and co-workers at Bar Harbor. They were able to report complete development of mouse oocytes, starting from the primordial follicle stage and ending with the birth of a normal pup (Eppig and O'Brien, 1996); a review by Hartshorne (1997) provided a practically oriented introduction to follicle culture *in vitro*.

3.7.2. Differences between mice and cattle follicles

Although achievements in cattle similar to those in mice are made difficult by the fibrous nature of the bovine ovary, by the larger size of the follicle, by the presence of a thick theca, which restricts the transport of nutrients and gases, and by the longer period of culture required to span the interval between the primordial and preovulatory follicle, progress has been made in the isolation and culture of primordial and preantral follicles in cattle (Figueiredo *et al.*, 1994a,b,c, 1995, 1997; Hulshof *et al.*, 1994, 1995, 1997; Perez *et al.*, 1994; Wandji *et al.*, 1994, 1996a,b,c; Ralph *et al.*, 1995b, 1996; Schotanus *et al.*, 1995, 1997; Katska and Rynska, 1996, 1998; Nuttinck *et al.*, 1996; Poehland *et al.*, 1998; Itoh *et al.*, 1999; Saha *et al.*, 1999; Telfer *et al.*, 1999; Costa *et al.*, 2001). It is generally accepted that the culture of isolated preantral bovine follicles provides a unique opportunity of examining the effect of factors influencing ovarian function.

Although it was shown, for example, more than a quarter of a century ago that the growth of one- and two-cell-layer mouse follicles was stimulated by FSH, whereas LH appeared to stimulate mitosis in the theca cells, it is only now, with the availability of recombinant FSH, that an accurate assessment of each factor is

possible. In Belgium, Cortvrindt *et al.* (1997) have shown that the long-term culture of early preantral follicle stages from mice requires FSH if full growth and meiotic competence of oocytes is to be obtained; without using LH during a 12-day culture period, oocytes were able to reach a diameter of 70–75 μm and meiotic competence. Lazzari *et al.* (1994), in a study of preantral follicles recovered from the ovaries of newborn piglets and prepubertal gilts showed that protein synthesis of follicle cells and the interactions between oocytes and their associated somatic cells change markedly during growth. The nucleolar ultrastructure of bovine primordial to early tertiary follicles has been comprehensively described by Fair *et al.* (1996a,b, 1997a,b) and Fair and Hytell (1997).

Studies reported by Van den Hurk *et al.* (1997, 1998) were designed to provide information on the ultrastructure and viability of mechanically isolated, non-cultured and cultured bovine small preantral follicles (40–100 μm diameter) and on the ultrastructure of dissected and cultured large preantral/early antral follicles (140–250 μm diameter). Various methods were employed to determine the viability of oocytes and granulosa cells. The isolation technique and culture methods employed with the small follicles proved unsatisfactory but most of the large preantral follicles showed a good ultrastructure after culture, which may have been due to the isolation/culture methods employed; the use of ultrastructural and/or viability cell markers in assessing the quality of oocytes and surrounding cells was helpful. In Japan, Saha *et al.* (2000a,b) collected preantral follicles (40–100 μm diameter) from the ovaries of slaughtered cows and used various culture media to maintain them over a 10-day period; on the basis of their results, they recommended that FSH and EGF should be used in the culture of 40–80 μm follicles. Elsewhere in the same country, Itoh and Hoshi (2000) cultured small preantral follicles (30–70 μm) for up to 30 days; they recorded an increase in the size of follicles by up to 30% in those co-cultured with fetal bovine skin fibroblasts.

In Edinburgh, McCaffery *et al.* (2000a,b) cultured preantral follicles for 6 days in a serum-free medium containing insulin and IGF-I; results showed no positive effects of the growth factor on follicle development. Other work in Scotland with preantral follicles (166 μm diameter) developed a culture system in which follicle morphology could be maintained for up to 28 days (Gutierrez *et al.*, 2000); in this system, FSH, EGF and IGF-I stimulated follicle growth and enhanced antrum formation. The authors suggest that their system may provide a valuable approach for studying the regulation of early follicular development and for the production of oocytes for processing into embryos. A paper by Thomas *et al.* (2001) dealt with the effect of ascorbic acid on the health and morphology of bovine preantral follicles during long-term culture; they demonstrated a culture system capable of supporting follicle differentiation over a 12-day culture period and showed that ascorbic acid maintained follicle health and basement-membrane remodelling during this time. In Japan, Itoh *et al.* (2002) demonstrated that the normal three-dimensional structure of the bovine preantral follicle could be maintained for up to 13 days in their serum-free culture system; they were able to use the system to identify some of the requirements and interactions of insulin and IGF-I with FSH and LH.

3.7.3. Utilizing early antral follicles

Although, in contrast to what is required in small laboratory animals, pigs and humans, a two- or even three-step culture strategy is likely to be required for the cow (Nayudu, 1994; Ralph *et al.*, 1995b; Telfer, 1996, 1998; Van den Hurk *et al.*, 1998; Telfer *et al.*, 1999), where the oocyte is relatively small and the growth rate relatively slow, the rewards of an effective culture method for bovine preantral follicles would be considerable. In the meantime, however, there are reports of measures that may be taken to make greater use of antral follicles in the cow. Attempts were made by Osaki *et al.* (1997) to fertilize bovine oocytes recovered from early antral follicles grown in culture (embedded in collagen gel) for 14 days. Studies by Yamamoto *et al.* (1999) and Miyano (2000) in Japan sought to examine whether *in vitro*-grown bovine oocytes from early antral follicles, cultured over a 14-day period in collagen gels, could be induced to develop into blastocysts in the laboratory; in due course, they were able to report the

Table 3.15. Development of *in vitro*-grown bovine oocytes (from Yamamoto *et al.* 1999).

Oocyte growth[a]	No. of oocytes (replicates)	No. (mean ± SEM) of embryos	
		Cleaved	Developed to blastocysts
In vivo	425 (4)	316 (74.6 ± 2.8)[b]	148 (35.0 ± 2.3)[b]
In vitro	135 (23)	21 (18.2 ± 5.4)[c]	6 (3.7 ± 1.7)[c]

[a] *In vitro*-grown oocytes were recovered from the collagen gels with only formation of an antral follicle-like structure after 14 days of culture, and *in vivo*-grown oocytes were collected from ovarian surface-visible follicles.
[b,c] Values with different superscripts in the same column differ significantly ($P < 0.01$).

first calf born as a result of this technology (see Table 3.15). Their results proved the feasibility of growing oocytes 90–99 μm in diameter from early antral follicles (0.5–0.7 mm diameter) to produce fertilizable oocytes with the full potential to produce live young; although much remains to be done in improving and refining the culture system, the utilization of oocytes in small-size follicles is a useful step forward in making greater use of bovine ovarian follicles.

Early antral follicles (500–700 μm diameter) were collected from abattoir ovaries by Saha *et al.* (2000a,b) and cultured in collagen gel for 14 days. Where the COCs, with their surrounding granulosa cells, were removed from the follicles before culture, the production of meiotically competent oocytes was significantly higher than in whole, intact follicles. According to a report by Katska *et al.* (2000a,b), the objective of obtaining mature oocytes from late preantral and early antral bovine follicles appears now to be more realistic, although still calling for further basic research; their results suggested that 14 days was the longest culture time compatible with oocyte growth and maintenance of nuclear configuration at the GV stage. Further studies are required to determine whether the oocytes are capable of being matured and fertilized.

3.7.4. Preantral follicles in humans and pigs

In China, a study reported by Wu, J. *et al.* (1998) investigated the development of human preantral follicles and oocyte maturation *in vitro*. This work showed that follicular aspirates obtained during oocyte retrieval in an IVF programme contained many preantral follicles that could develop into antral follicles with extrusion of oocytes in culture. A report by Wu *et al.* (2001b) in Utah, working with pigs, was the first to describe the *in vitro* development of embryos produced from oocytes that originated in the preantral follicles of a large animal species; their study demonstrated that pig antral follicles can be grown at a high frequency *in vitro* from healthy preantral follicles with an intact theca. After 4 days of culture, cumulus-enclosed oocytes were obtained and matured; 53% of the matured oocytes were fertilized and 13% of these developed to the blastocyst stage. The preantral follicles were cultured, with three follicles per well, in North Carolina State University 23 (NCSU-23) medium supplemented with FSH and porcine serum; sperm injection was as effective as IVF in achieving fertilization of the oocytes (Wu *et al.*, 2001a,c).

4

Maturing the Oocyte

4.1. Oocyte Maturation *in Vivo*

In mammalian species such as the cow, some hours before the rupture of the follicle and ovulation, the fully grown oocyte in the preovulatory follicle resumes meiosis, progressing from prophase of the first meiotic division to metaphase II (MII); this maturation process, which is accompanied by complex changes in the protein phosphorylation process (Dai *et al.*, 1998; Tomek *et al.*, 2002a,b), transforms the primary oocyte into a mature secondary oocyte. The nucleus and the cytoplasm of the oocyte undergo many changes in becoming mature, receptive to fertilization and competent to support embryonic development. In the live animal, the oocyte, which has been arrested in the dicytate stage of the first meiotic division, resumes meiosis in response to the signals associated with the preovulatory luteinizing hormone (LH) surge. *In vitro*, however, oocyte maturation can be initiated by removal of the fully grown (competent) oocyte from its follicular environment; it is this phenomenon which is exploited in *in vitro* maturation (IVM) culture systems.

4.1.1. Summary of events

The growth of the bovine oocyte in the weeks leading up to ovulation involves a series of modulations of organelles and inclusions and a period of oocyte transcription (see review by Hyttel *et al.*, 1997); this is essential for the oocyte to achieve meiotic and developmental competence. Oocyte transcription, including nucleolus function (ribosomal RNA (rRNA) synthesis), is activated in the secondary follicle and is maintained up to an oocyte diameter of about 110 μm in the 3 mm tertiary follicle. At a diameter of 100–110 μm, the oocyte gradually achieves competence to undergo meiotic maturation and sustain embryonic development. In the dominant follicle, the oocyte undergoes further ultrastructural modifications and attains full developmental competence.

Maturation events within the dominant (preovulatory) follicle are triggered by the preovulatory LH surge. In the 24 h that follow, the primary oocyte undergoes the progression of meiosis to metaphase II and various changes in the organization of its cytoplasm, which include alignment of cortical granules along the oolemma, rearrangement of mitochondria, continued development of the lipid store and the reduction of the Golgi compartment. The increased lipid store is probably an essential energy source to support the oocyte through maturation and early embryonic development. It is known that triglyceride is the major lipid component of the bovine oocyte and there is evidence that the triglyceride content can be used as a source of energy for the IVM of cattle oocytes (Ferguson and Leese, 1999a,b; Kim *et al.*, 2001). Maturation of the oocyte also sees a redistribution of the ribosomes. It is also clear that the oocyte, rightly regarded as one of the body's unique cells, is capable of a measure of control over its own destiny, as reviewed by Eppig (2001).

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During the final maturation of the oocyte, the follicle itself also undergoes a series of changes. The membrana granulosa cells stop synthesizing oestradiol and the wall of the follicle luteinizes just prior to ovulation; this luteinization coincides with a marked increase in progesterone synthesis and extensive expansion of the cumulus cells. Whether such changes in the follicle are of crucial importance to the oocyte remains unclear. During maturation, the extensions of the corona cells that penetrate the zona pellucida and connect the oocyte to outside support, which had already retracted to a more superficial position before the LH surge, are retracted still further, indicating that communication between the oocyte and its surrounding support cells decreases after the onset of maturation and is eventually lost.

As the bovine oocyte matures in response to the preovulatory LH surge, cumulus cells secrete hyaluronic acid (HA), a non-sulphated glycosaminoglycan bound to the cumulus cells by linker proteins. When the HA becomes hydrated, spaces between the cumulus cells become enlarged and the cells are embedded in a mucified matrix; the process is termed cumulus expansion or mucification. This cumulus expansion is among several important events within the follicle in the hours preceding ovulation (see Fig. 4.1).

After the oocyte is released from the ruptured follicle, the mural granulosa cells that had lined the follicle cavity, together with the theca cells, undergo differentiation and luteinization to form the corpus luteum. With the release of the secondary oocyte and its surrounding cells into the oviduct after ovulation, the disassembly of the cumulus–oocyte complex (COC) occurs within a few hours, leaving the oocyte naked; it is believed that this degradation of the matrix after ovulation is promoted by oocyte secretions that activate the expression of genes in the cumulus cells.

4.1.2. Events leading to ovulation

Much remains to be learned about events leading up to ovulation of the preovulatory follicle.

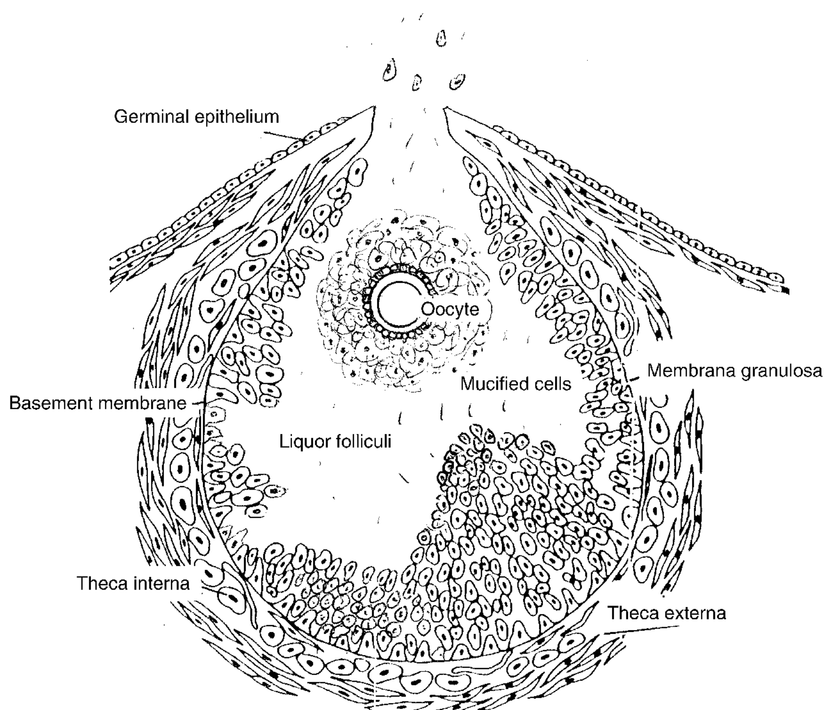

Fig. 4.1. Bovine follicle at the time of ovulation.

The need is for a greater understanding of the mechanisms responsible for the coordinated maturation of the follicle and the enclosed oocyte. During the follicular wave preceding final maturation, the dominant follicle is selected at a size of about 8 mm, which emerges around the onset of luteolysis, after which an increased LH pulse frequency maintains its development. When the dominant follicle reaches its maximum size, ultrastructural changes become apparent in the nucleus and cytoplasm of the oocyte, such as undulation of the nuclear membrane, vacuolization of the nucleolus, relocation of the cortical granules and a start of the retraction of corona-cell processes (Hyttel *et al.*, 1997).

4.1.3. Nuclear and cytoplasmic maturation

The bovine oocyte is arrested at the G2/M transition of the first meiotic division from which, after reaching full size in the follicle and after the LH surge, it undergoes its final maturation. The nuclear maturation of the bovine oocyte starts with the breakdown of the germinal vesicle (GVBD); in contrast to what occurs in rodents, such as the mouse, in the cow this process requires active protein synthesis (Tatemoto and Horiuchi, 1995: Tatemoto and Terada, 1995, 1996a,b); it appears that the new protein necessary for inducing GVBD is synthesized in the first 8 h of culture, and continuous protein synthesis after GVBD is crucial for the completion of meiotic maturation. It is also known that protein synthesis is necessary for sperm-head decondensation and for the formation of a male pronucleus (Liu *et al.*, 1998a,b,c). In the living animal, the LH surge in the early hours of oestrus is the signal for the start of maturation and GVBD, which can be observed 4–8 h after that event. GVBD is characterized by gradual chromatin condensation, the disappearance of a compact nucleolus and nuclear membrane disintegration. Many reports have appeared in recent years dealing with various aspects of meiosis in the oocytes of cattle and other domestic mammals (Levesque and Sirard, 1994, 1995, 1996; Tatemoto *et al.*, 1994; Homa, 1995; Tatemoto and Terada, 1998: Fair and Hyttel, 1999) and events in the dominant follicle as it progresses towards ovulation (Mihm *et al.*, 1999).

Increasing knowledge of factors controlling the cell cycle at the molecular level has greatly improved an understanding of the cascade of events that drives the maturation of the oocyte (see Mattioli, 1996; Wu Bin *et al.*, 1997; Anas *et al.*, 1998, 2000; Niimura and Amemiya, 2000; Abrieu *et al.*, 2001; Sirard, 2001). Oocyte maturation is known to be triggered and regulated by the coordinated action of two kinases, maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK). The MPF is a serine/threonine protein kinase composed of a regulatory subunit, cyclin B, and a catalytic subunit, p34cdc2. It is known that MPF activity appears shortly before GVBD reaches a maximal level in MI oocytes, decreases markedly during the transition from MI to MII and regains its peak level again in MII oocytes. This pattern of changes in MPF activity implies that the transition from MI to MII is correlated with a decrease in MPF activity. The importance of MPF in the maturation process is well established and studies by Gordo *et al.* (2001) have shown that MAPK activity is necessary for metaphase II arrest, the maintenance of MPF activity and spindle organization. A report by Edwards, L. *et al.* (1999) showed that significant oscillations in intracellular pH (pHi) occurred and appeared to precede the reported changes in MPF activity by about 2 h; such evidence indicates that pHi correlates with meiotic progression and MPF activity in the maturing bovine oocyte. It is clear that the oocyte itself does not possess specific receptors for either LH or follicle-stimulating hormone (FSH); much work has therefore been directed towards determining how the LH signal is communicated to the oocyte. It is known, however, that both theca and granulosa cells possess densely distributed LH receptors.

The chromosomes condense into a compact form and arrange themselves on the equatorial plate of the meiotic spindle. A study by Kim, N.H. *et al.* (2000) examined the microtubule and microfilament organization in cattle oocytes by laser-scanning confocal microscopy; microtubules and microfilaments are major cytoskeletal components and important modulators for chromosomal movement and cellular division in the bovine oocyte; the authors suggested that both microtubules and microfilaments are

closely associated with the reconstruction and proper positioning of chromatin during meiotic maturation in cattle. The oocyte undergoes its first meiotic division with the extrusion of the first polar body into the perivitelline space. The oocyte, now termed a secondary oocyte, begins the second meiotic division and proceeds until it reaches MII (see Fig. 4.2). The second meiotic division is not completed unless sperm penetration occurs or the oocyte otherwise responds to an activation stimulus. Completion of the second meiotic division is accompanied by extrusion of the second polar body into the perivitelline space. The oocyte at this stage contains the haploid number of chromosomes; the whole maturation process is completed within about 24 h.

During cytoplasmic maturation, already mentioned in an earlier context, many events occur that have relevance to the developmental competence of the bovine oocyte. In Japan, for example, Hosoc and Shioya (1995) examined the distribution of cortical granules, using lectin histochemistry to assess cytoplasmic maturation. Reviews by Homa (1995) and Carroll *et al.* (1996) have dealt with the mechanisms underlying calcium release in oocytes and discussed how release mechanisms are modified during maturation. A paper by He *et al.* (1997) dealt with calcium, calcium-release receptors and the resumption of meiosis in bovine oocytes. Work in Switzerland reported by Plaisance *et al.* (2000) has shown that IVM of the bovine oocyte is inhibited when Ca^{2+} homoeostasis is disorganized. Their results showed that GVBD in the cattle oocyte is dependent on Ca^{2+} and supports the view that the mobilization of Ca^{2+} from internal stores is a prerequisite for triggering meiotic resumption and progression to metaphase II. It is clear that bovine oocytes and cumulus cells synthesize many different proteins during the

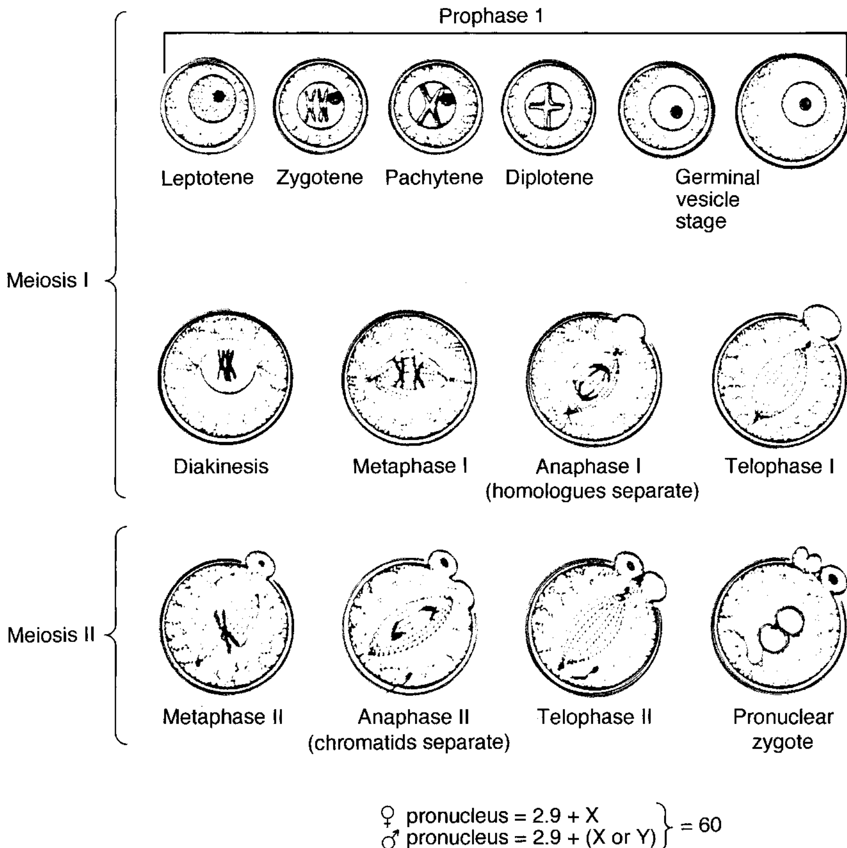

Fig. 4.2. Stages in meiosis and oocyte maturation.

maturation process (Wu *et al.*, 1996a,b). It has been suggested by Moor (1996) that useful progress in oocyte biology may be made by focusing attention on signals from associated follicular cells and the oocyte and on the mechanisms involved in the acquisition of developmental competence.

Studies reported by Fair *et al.* (2001b) investigated the localization of major nucleolar proteins from prophase I of meiosis in bovine oocytes up to the first mitotic cell division after fertilization; they found that nucleolar proteins were readily detectable by immunocytochemistry during the growth phase (prophase I) but subsequently dispersed into the ooplasm at the resumption of meiosis and reappeared at late telophase in the pronuclei following fertilization (see Fig. 4.3). The workers were the first to describe in cattle how the components of rRNA gene transcription and ribosome formation are brought from the growing oocyte stage through the processes of meiotic maturation and fertilization and delivered to the zygote; embryos that fail to progress beyond the first three cell cycles are probably lacking in these basic components.

4.1.4. Biochemical and physiological events during maturation

Maturation of the oocyte can be divided into an inductive phase and a synthetic phase (see Fig. 4.4). The inductive phase lasts for 6–8 h, culminating in GVBD. During this phase, the oocyte undergoes reprogramming by the somatic elements within the follicle. In the synthetic phase, which has a duration of 18 h, there is a total restructuring of the nuclear and cytoplasmic elements.

The inductive phase of maturation is one in which it is believed that the cumulus cells act in a particularly crucial supportive role. There is evidence that these cells may have a regulatory role in RNA synthesis; denuded oocytes show lower RNA levels than oocytes surrounded by several intact layers of cumulus at the initiation of meiosis. Data reported by Chian and Sirard (1996) indicated that the protein synthesis pattern during IVM is different between oocytes with and without cumulus cells; they also showed that FSH modulates the protein synthesis pattern of cumulus-intact oocytes. The same Canadian authors also reported that, as meiosis proceeds towards GVBD in the bovine oocyte, there is a large increase in the phosphorylation of protein. Reports by Chian *et al.* (1999a,b,c,d) have shown that protein phosphorylation and dephosphorylation play key roles in the regulation of meiotic maturation; they showed that the phosphorylated protein complexes are changed reversibly after GVBD, suggesting that such proteins may regulate transition of G2/M phases and formation of the male and female pronuclei. In Germany, Tomek *et al.* (2002b) reported a comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of messenger RNA (mRNA) during maturation of bovine oocytes *in vitro*; a detailed knowledge of the biochemical and molecular processes that occur during oocyte maturation will be useful for the improvement of IVM methods.

A study by Stojkovic *et al.* (2001) evaluated mitochondrial distribution before and after maturation; their results indicated that mitochondrial organization may be different between morphologically good and poor oocytes and that this may be responsible for their different developmental capacity after *in vitro* fertilization (IVF). In France, Vigneron *et al.* (2002) observed an accumulation of cdk1 and cyclin-B1 mRNA during the final maturation of the bovine oocyte *in vivo*; this was not seen during IVM, possibly due to inappropriate culture conditions. The same authors suggested that cdk1 and cyclin-B1 messengers may be suitable indicators of developmental competence and prove useful in improving oocyte culture conditions.

It is clear from many reports that the developmental competence of the bovine oocyte is acquired gradually (see Gandolfi, 1994, 1998a). A fully competent oocyte is able to sustain embryonic and later development all the way through to full term; this is what makes the oocyte a very special cell. The time interval between the synthesis and use of RNA and protein molecules may even be a matter of several weeks; this means that the oocyte has to possess the means of storing many molecules in a quiescent state until they reach the stage at which they are activated during maturation or early embryonic development. It is believed that in cattle maternal factors essential for sustaining development may act beyond the

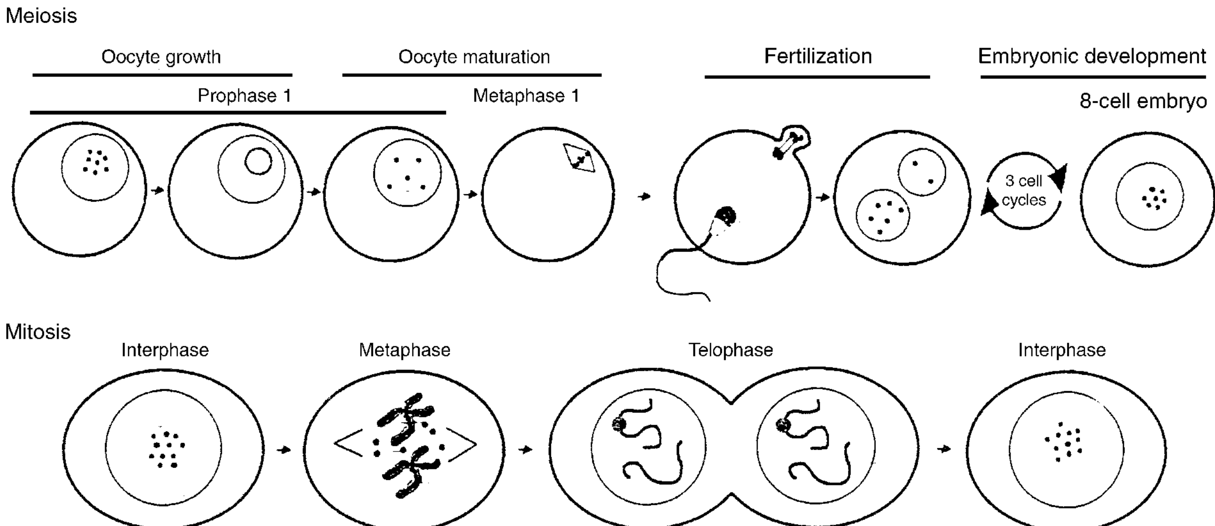

Fig. 4.3. Nucleolar proteins during bovine oocyte meiosis and mitosis. Schematic diagram comparing nucleolar protein localization during meiosis and mitosis. On completion of the growth phase during prophase I of meiosis, the nucleolus compacts and eventually fragments. At oocyte nucleus breakdown (ONBD), the nucleolar proteins disperse into the ooplasm. Following fertilization, at telophase II of meiosis the proteins target the fertilizing sperm head or male pronucleus. During the ensuing interphase, the proteins are localized to numerous foci, some of which may correspond to nucleolus precursor bodies (NPBs) in both pronuclei. Following three cell cycles, a functional nucleolus is established in each cell at interphase. At this point, the cell is similar to mitotic cells in which a functional nucleolus exists during interphase, and upon nucleus breakdown the nucleolar proteins disperse in the cytoplasm or aggregate in cytoplasmic nucleolus-derived foci or coat the chromosomes. At telophase, the nucleolar proteins target the nucleolus organiser regions (NORs) of the chromosomes, where they bind to the NPBs. A functional nucleolus is established at interphase of the next cell cycle. (From Fair *et al.*, 2001b.)

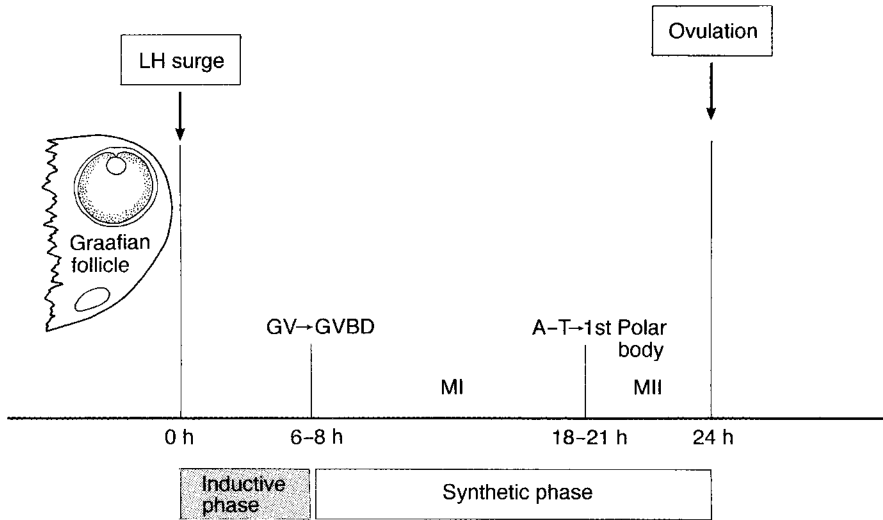

Fig. 4.4. Chronology of events during oocyte maturation in the cow. GV, germinal-vesicle oocyte; GVBD, germinal-vesicle breakdown; MI, metaphase I; A–T, anaphase–telophase; MII, metaphase II.

maternal–embryonic transition stage, which is reached between the eight- and 16-cell stage (see review by Brevini-Gandolfi and Gandolfi, 2001). The complexity of mechanisms that regulate the recruitment of maternal information has been reviewed by Duranthon and Renard (2001).

4.2. Oocyte Maturation in the Laboratory

Under the microscope, the first obvious sign of resumed meiosis is the dissolution of the nuclear membrane (GVBD). This is illustrated in Fig. 4.5. Extrusion of the first polar body (PB) and formation of the second meiotic spindle subsequently occur as the oocyte matures and the surrounding cumulus cells undergo expansion (see Fig. 4.6).

Although nuclear changes can be clearly followed in the maturing oocyte, other much less obvious changes may occur within the ooplasm. There are also crucial interactions between the oocyte and its surrounding follicular cells; a full understanding of the complex signalling mechanisms operating between granulosa cells and the oocyte is required. In evaluating the effect of the maturation process, this can be done by examining several morphological features within the oocyte and changes in the granulosa cells outside the oocyte. During IVM, extensive redistribution of intracellular organelles occurs; mitochondria migrate to occupy a perinuclear location and the cortical granules migrate outward to lie just beneath the oolemma (vitelline membrane).

4.2.1. Historical aspects

Studies on the IVM of mammalian oocytes were reported almost 70 years ago, when Pincus and his associates observed that some proportion of rabbit and human oocytes resumed meoisis spontaneously when liberated from antral follicles and cultured in a suitable medium (see Table 4.1). A detailed study of rabbit oocytes was made by Chang in the 1950s; he recorded that maturation was apparently unaffected by the addition of pituitary extracts to the culture medium, by follicle size or by the rabbit's reproductive status. In the early to mid-1950s, Bob Edwards observed that the nuclear maturation of oocytes removed from their follicle microenvironment was a common phenomenon among other mammal species, including cattle; his work at that time did much to stimulate interest in identifying the critical factors involved in bringing mammalian primary oocytes to maturity. Edwards was also one of the first to culture oocytes within the intact follicle

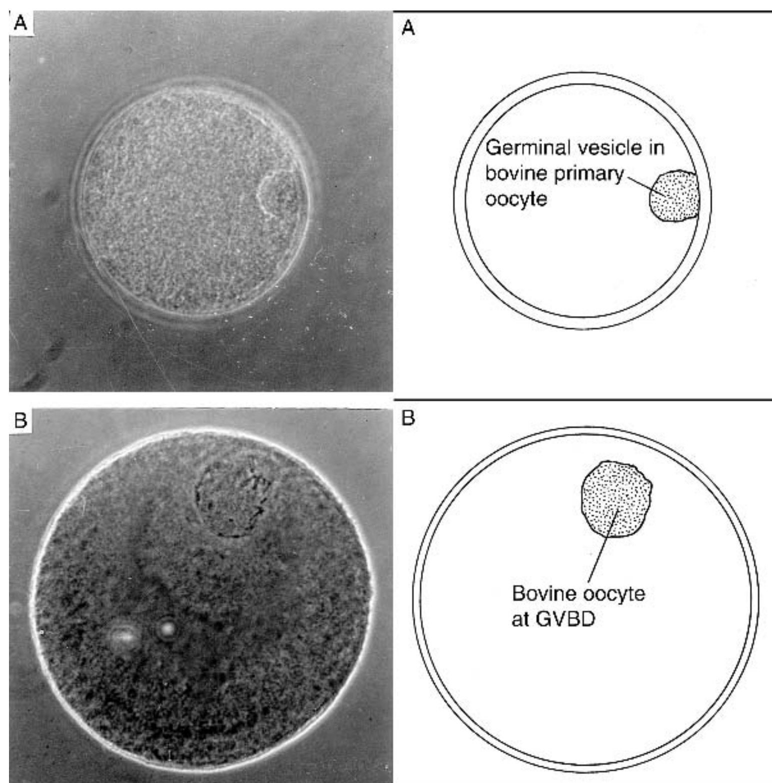

Fig. 4.5. Germinal-vesicle-stage bovine oocyte and germinal-vesicle breakdown (GVBD).

and to suggest that an inhibitory substance existed in the follicle which disappeared with the release of the oocyte.

Other work in the late 1960s and early 1970s with cattle oocytes included attempts by Sreenan and others in Ireland to determine the chronology of maturational changes in cattle and sheep oocytes. The time taken for maturation *in vitro* was about 24 h, which was the interval known to separate the preovulatory LH surge and ovulation of the mature, secondary, oocyte in the live cow and ewe.

Although it has been recognized for many years that oocytes resume meiosis spontaneously when placed in a suitable culture medium, it has also been known that, even after apparently normal nuclear maturation, those same oocytes may be quite incapable of giving rise to a normal embryo. In order to appreciate just how poor such embryonic development might be, it is possible to cite an analysis of published data (comprising some 1500 secondary oocytes from mice, rabbits, sheep and cattle) carried out by Cambridge workers during the 1970s. This showed that less than 1% of IVM oocytes were capable of normal embryonic development when transferred to recipients. It was all too evident that the concept of oocyte maturation had to be widened to cover all those events which allow the oocyte to express its developmental potential after fertilization and not merely confined to nuclear events or the ability to be fertilized.

It is now firmly established that the acquisition of developmental competence involves numerous structural and biochemical changes within the oocyte as part of cytoplasmic maturation. It is also clear that positive support must come from follicular cells during maturation if the oocyte is to express its full developmental potential.

The maturation procedure initially employed in Ireland in the late 1980s was based on the dissected follicle, with subsequent recovery of an intact COC, and employing additional cumulus/granulosa cells, trace amounts of antibiotic and oestrous cow serum

(OCS) in commercially available tissue-culture medium (TCM-199). Maturation was by way of a non-static (flux) culture system operating at 39°C in a 5% carbon dioxide atmosphere at maximum humidity.

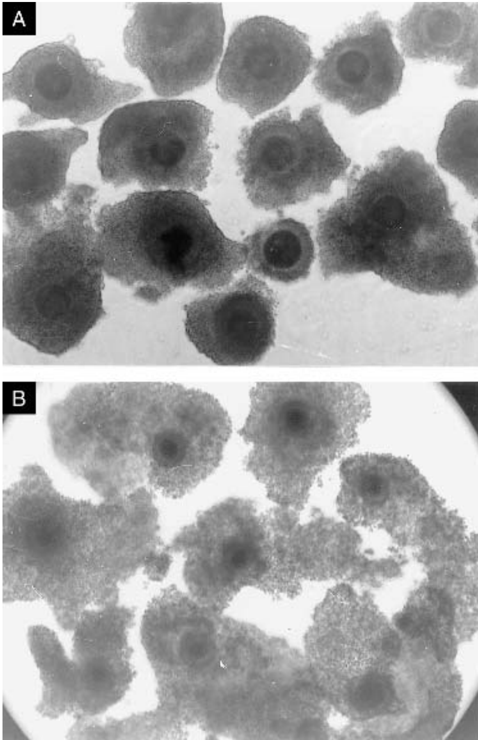

Fig. 4.6. Before and after *in vitro* maturation of the bovine oocyte. (A) Before maturation: oocytes before initiation of culture; note the tight cumulus layers (100×). (B) After maturation: oocytes after 24 h of *in vitro* culture; note the expanded cumulus (100×).

4.2.2. Current understanding of *in vitro* maturation in cattle

Not all the oocytes recovered from the cow's ovary have the ability to develop into a viable embryo. Despite the efforts of workers over a period of almost 20 years to simulate in the laboratory the events that normally take place in the microenvironment of the follicle, the current yield of blastocysts at the end of the embryo production process is no more than 30–40%; these figures are borne out in the experiences of laboratories far and wide. Studies of ovarian events in the cow and of the hormonal and other factors influencing oocyte developmental competence show that such competence is acquired under specific follicular conditions.

It is clear that the cells surrounding the oocyte are crucial in ensuring its complete maturation under culture conditions in the laboratory; selection of the COC with the required morphological features is an essential first step. There is much evidence relating COC quality to the yield of viable blastocysts. There is also plenty of evidence showing that follicular differentiation strongly influences the competence of the oocyte; the size of the follicle is certainly correlated with the ability of the oocyte to give rise to a viable embryo. The maturation of the oocyte in its own follicle is now understood to be a more gradual process than was realized 20 years ago and certain crucial steps in that maturation process actually occur before the LH surge in the dominant follicle that has been selected for ovulation. Translated into practical measures that may be employed in the artificial maturation of cattle oocytes, this may mean that oocytes should not be aspirated from abattoir ovaries immediately after animal slaughter, but should

Table 4.1. Towards an understanding of mammalian oocyte maturation.

Year	Event	Researcher(s)
1935	Observation of spontaneous resumption of meiosis in rabbit oocytes	Pincus and Enzmann
1939	Observation of resumption of meoisis in human follicular oocytes	Pincus and Saunders
1955	Detailed study of maturation of rabbit oocytes	Chang
1962–1965	Nuclear maturation *in vitro* achieved in several mammalian species	Edwards
1968	Chronology of nuclear maturation in cattle oocytes	Sreenan
1977	Appreciation of importance of cytoplasmic and nuclear maturation	Thibault *et al.*
1978	Less than 1% of ruminant oocytes attained developmental competence after artificial maturation	Moor and Warnes
1984	Crucial support of follicle cells in maturation of sheep oocytes	Staigmiller and Moor

be held for several hours in the post-mortem ovary before recovery (Blondin *et al.*, 1997a,b). During this very short time window, the follicular microenvironment can exert a decisive influence on the competence of the oocyte.

Other ways in which advantage may be taken of the follicle's influence on oocyte quality are in permitting an appropriate 'coasting' period after FSH stimulation of the cow before oocyte recovery is attempted. When a follicular wave is induced by 3 consecutive days of FSH stimulation in the absence of a dominant follicle, the period of withholding FSH (i.e. 'coasting') before oocyte recovery has an effect on blastocyst yield. According to Sirard *et al.* (1999a), optimal results may be obtained when 48 h without FSH elapses before oocyte recovery. The belief is that the follicle needs to reach a stage of stasis in its growth to signal to the oocyte the final changes required before ovulation. Reviewing oocyte quality and embryo production, Sirard and Blondin (1998) note that, during rapid follicular growth, bovine oocytes do not acquire full developmental competence. For that reason, the plateau phase at the end of the follicle growth phase, resulting in dominance or atresia, may be important for oocytes to acquire competence. Subtractive hybridization was employed to identify mRNA associated with the maturation of bovine oocytes by Robert *et al.* (2000) in Quebec; knowledge of genes and their accumulated mRNA is essential for a better understanding of the mechanisms involved in oocyte maturation and the survival of the *in vitro*-produced embryos.

In Canada, Robert *et al.* (2001) have put foward the view that mature preovulatory follicles (with functional LH receptors) possess information that is vital for the oocyte to complete its own cytoplasmic maturation. The assumption is that the granulosa cells surrounding a competent oocyte are different from those surrounding an incompetent oocyte; the Canadian workers were able to identify certain genes expressed by granulosa cells associated with competent oocytes. Research-wise, the identification of these marker genes may lead to a fuller understanding of the mechanisms involved in oocyte maturation and early embryonic development. In the laboratory, the same marker genes may be useful in defining more accurately the media most suitable for ensuring complete maturation of the oocyte *in vitro*.

It is known that there is a major relocation of mitochondria during oocyte maturation in cattle that appears to correlate with the degree of developmental competence acquired by the oocyte. A review by Bavister and Squirrell (2000) notes that mitochondria in germinal vessicle (GV) oocytes are mainly distributed in the cortex but are relocated during maturation to give two distinct patterns of mitochondrial distribution in the MII oocyte. When oocytes were matured in IVM medium containing glucose and amino acids, the mitochondria became located mainly in the centre of the oocyte; this pattern was associated with good-quality oocytes (competent to form blastocysts). In GV oocytes matured with glucose and lactate, a combination that led to poor embryo development after fertilization, mitochondria remained in the cortex of the oocyte. It appeared that the distribution pattern and location of mitochondria were related to the developmental competence of the oocyte.

In terms of the energy sources used by the bovine oocyte during maturation, Ferguson and Leese (1998, 1999a,b) examined the potential metabolic role of triglyceride; evidence suggested that bovine oocytes are as active metabolically as blastocysts and may rely on triglycerides as an energy source during maturation.

4.3. *In Vitro* Maturation (IVM) Culture Systems

Despite much work during the past decade aimed at improving *in vitro* embryo production in cattle, it is clear that the ability to mature and fertilize the oocyte and to culture the fertilized oocyte is capable of much further improvement (Enright *et al.*, 1999, 2000a,b; Ward *et al.*, 2000a,b, 2001a,b, 2002; Rizos *et al.*, 2000, 2001a,b,c,d, 2002a,b; Lonergan *et al.*, 2001a,b; Korfiatis *et al.*, 2002). In the normal course of events, the ability of the oocyte to develop into a viable embryo and eventually into a normal, healthy calf is acquired gradually during its progressive differentiation throughout folliculogenesis, a process that culminates in a state of developmental competence, which some have termed 'oocyte capacitation', a state that is finally attained in the cow's preovulatory follicle in the hours immediately preceding ovulation. It

is clear, however, after more than a decade of research that many bovine oocytes fail to become developmentally competent after conventional IVM, despite the use of a wide range of culture techniques.

4.3.1. Culturing intact follicles

In the early days of IVM of bovine oocytes, some workers attempted to do this by culturing the intact follicle; their interest may have been in oocyte maturation or in studying endocrine and other events within the follicle. Studies in the USA in the early 1970s reported studies with small antral follicles (1–2 mm diameter) cultured for 60 h on stainless-steel grills immersed in hormone-supplemented medium; no evidence of oocyte maturation was obtained. Around the same time, Cambridge workers demonstrated that 40–50% of sheep oocytes matured within intact follicles *in vitro* subsequently gave rise to normal embryos and young after fertilization; this was in marked contrast to their consistent failure at that time to mature oocytes liberated from follicles. The Cambridge group also used intact-follicle culture in studies to investigate steroidogenesis and protein synthesis in sheep follicles and oocytes.

There were those in the 1970s and 1980s who considered that culture of intact follicles might be the way forward in embryo production, but others pointed to such a system as being tedious and time-consuming and requiring skill; for such reasons, this route to large-scale embryo production was considered impracticable. With intact-follicle culture, for example, much longer culture periods are required and the progress of the oocyte cannot be followed stage by stage as it makes its way through to MII.

Modern-day interest in culturing oocytes in the intact follicle is still to be seen, particularly in horses, where primary oocytes are transferred into the antrum of the preovulatory follicle for *in vivo* maturation. In cattle, evidence has accumulated over the past decade to support the view that oocytes in preovulatory follicles have undergone 'prematuration' before the LH surge initiates final maturation; according to this view, the limited yield of embryos after conventional IVM may be attributed to the use of immature oocytes. The temporary hosting of primary bovine oocytes in a preovulatory follicle could provide the environment required for 'prematuration'. To that end, a study by Davies *et al.* (1997) sought to develop and validate a method of interfollicular transfer of oocytes using transvaginal ultrasonography; multiple oocytes were collected from donor heifers and transferred to the preovulatory follicle in inseminated recipient heifers. In a further paper by the group in Saskatchewan (Bergfelt *et al.*, 1998), it was reported that ovulation and formation of the corpus luteum occurred as normal and multiple embryos were produced (eight embryos from three heifers). Harkema *et al.* (2000) have also reported on limited attempts to develop a gamete recovery and follicular transfer (GRAFT) technique for use in cattle.

4.3.2. Simple and complex maturation media

Culture media employed in cattle IVM can be broadly divided into simple and complex. Simple media are usually bicarbonate-buffered systems containing basic physiological saline with the addition of pyruvate, lactate and glucose; the main differences between the various forms of simple media lie in differences in their ion concentration and in the levels of the energy sources. The media are usually supplemented with serum or albumin with trace amounts of antibiotics (penicillin, streptomycin, gentamycin). Complex media contain, in addition to the basic components of simple media, amino acids, vitamins, purines and other substances, mainly in the concentrations found in serum; fixed nitrogen is present as free amino acids.

Many experiments have been reported over the years that have been aimed at the systematic evaluation of the role of the different components of bovine IVM systems (serum from various sources or bovine serum albumin (BSA); follicular fluid (FF); gonadotrophins; steroids; growth factors and cytokines; antibiotic cover). It has also been essential to pay due regard to the management of an array of factors such as pH, osmolarity, temperature, gas phase, humidity and the timing of maturation. Progress over the years has been from complex media to chemically defined media in which there is a clearly

understood rationale for each ingredient employed.

The way in which the effectiveness of the maturation process is measured has also undergone changes over the years. A simple count of the number of blastocysts present at the end of a week is no longer acceptable. It is a question of how the blastocysts withstand freezing and thawing (or vitrification) and, even more importantly, how viable the blastocysts are after transfer and how normal and healthy the calves are when born.

Tissue culture medium 199

The complex culture medium TCM-199, buffered with bicarbonate or HEPES and supplemented with various sera and/or gonadotrophin and/or steroid (oestradiol-17β) hormones, has been the most widely used culture medium for the study of bovine oocyte maturation; it is also long established that the presence in the medium of follicular somatic cells can influence the subsequent competence of the oocyte. The fact that components such as serum may contain a whole array of amino acids, proteins, growth factors, hormones and other active substances has meant that it has been difficult, if not impossible, to know how far the response of the oocyte is due to the presence of unidentified contaminants in such supplements. Working in France, Lonergan *et al.* (1994d) demonstrated that TCM-199, a defined medium, was capable of supporting the maturation of bovine oocytes in the absence of serum; this enabled them to evaluate which serum components might be responsible for the improved developmental competence observed in a TCM-199 + serum medium.

4.3.3. Buffering systems, osmolarity and surface tension

The buffering system employed in maturation media will depend on whether the medium is exposed to air or to a CO_2 enriched atmosphere. The advantage of HEPES- or phosphate-buffered media for short-term work with oocytes and embryos is that they do not require a CO_2-controlled gas phase to maintain a relatively constant pH. In a study reported by Montagner *et al.* (2000), the use of HEPES in maturation and embryo development media was evaluated; the authors found that media with 25.0 mM HEPES were more efficient in minimizing the range of pH than those with 12.5 mM or without HEPES. A study reported by Edwards, L. *et al.* (1999) sought to determine whether pHi oscillated during maturation of the bovine oocyte; they found evidence that pHi correlates with meiotic progression and MPF activity in the maturing oocyte. On the question of osmolarity, the aim is to have the maturation medium isotonic with the natural tissue fluids (e.g. follicular fluid) that are in contact with the oocyte in the living animal; osmolarity is usually arranged to be between 270 and 285 mosmol, this being regarded as the optimum range.

In the USA, Agca *et al.* (2000) studied the effects of osmotic stress on GV- and MII-stage cattle COCs by exposing them to various anisotonic NaCl solutions for 10 min and then returning them to isotonic media (270 ± 5 mosmol/kg); they found oocytes at the GV stage to be significantly more sensitive to anisotonic stress than MII oocytes. Although MII oocytes were not affected, exposing GV oocytes to anisotonic conditions significantly increased polyspermic fertilization. Blastocyst formation rates of MII oocytes exposed to 75, 150, 600, 1200 or 2400 mosmol were similar (13–20%) but significantly lower than the rate for controls (29%).

The effect of surface tension on TCM-199 on bovine oocyte maturation and embryo development in culture was studied by Palasz *et al.* (1999) in Canada; they compared surface-tension measurements of commercial TCM-199 containing the surfactant Twin-80 and custom-prepared TCM-199 without the surfactant. From their results, they concluded that surface-active components in IVM media positively affect oocyte maturation.

4.3.4. Water-quality considerations

Water is a major component of the IVM medium and high-quality water is essential for its preparation. There are several basic groups of contaminants to be found in water; these include dissolved ionized and non-ionized solids and

gases, particulate matter, microbials and pyrogens. The usual recommendation is to prepare IVM media using reverse-osmosis, deionized and ultrafiltrated water. Rigorous quality-control testing is essential at regular intervals to ensure that water quality is within acceptable limits. In Japan, Nagano *et al.* (1999b) reported on the efficacy of a water-purification system with an ultraviolet lamp and ultrafilter (UV-UF) for the preparation of media for use with cattle embryos; they found that the rate of development of bovine zygotes to the blastocyst stage was significantly increased using medium prepared with UV-UF water. Results demonstrated that the UV-UF system was beneficial because it eliminated endotoxins from ultrapurified water.

4.3.5. Static and flux culture systems

In the IVM of sheep oocytes, Cambridge workers in the early 1980s employed a gentle agitation (flux) culture method; the flux method applied to the culture dish containing the oocytes was believed to prevent the attachment and subsequent differentiation of cumulus cells. The IVM method in question involved a 2 ml volume of medium containing 20 COCs and additional cumulus/granulosa cells; the gentle agitation was believed to maintain the correct state of somatic-cell differentiation during culture and to improve the quality of oocytes. Elsewhere, Galli and Lazzari (1996) were among those using a flux rather than a static culture system.

Although there are conditions in which the flux culture system may be appropriate, most reports in the literature now involve static culture systems. For the most part, such systems involve oocyte maturation in microdroplets (e.g. 10–20 COCs in 50–100 μl volumes). The system of microdroplets under oil has several advantages, such as minimizing the evaporation of water, protection from microbial contamination, attenuation of temperature and gas fluctuations and ease of examination during culture.

4.3.6. Effect of maturation time

During IVM, GVBD occurs from 3 h to more than 12 h of culture. Although some discrepancies exist in regard to the time of abstriction of the first polar body, it is usually recorded as occurring 18–24 h after the start of maturation. A study by Nakagawa *et al.* (1995) in Canada examined how the stage of nuclear maturation influenced subsequent embryo development after fertilization; there was a high percentage of pronuclear asynchrony among oocytes matured for > 28 h before fertilization. It was suggested that this might be an early cause of abnormal development that only becomes evident much later in embryonic development.

An experiment by Ocano Quero *et al.* (1999a) in Spain compared the effect of differing durations of incubation (24, 36 and 48 h) on the incidence of diploid bovine oocytes; the highest incidence of that defect (11.4%) was after 48 h and the lowest (2.6%) at 24 h. In Ireland, Enright *et al.* (2000a,b) investigated the effect of duration of IVM (16–32 h) on the developmental competence of the oocyte; for optimal quality, it was concluded that maturation should be carried out for 24 h. Such data were in agreement with earlier studies by Lu and associates in Ireland (see Table 4.2).

Table 4.2. Effect of maturation time on developmental competence of the bovine oocyte (from Ward *et al.*, 2002).

Duration of IVM (h)	No. of oocytes per treatment	Oocytes cleaved		Day 8 blastocyst yield	
		n	%	*n*	%
16	147	108	73.5^{b}	30	20.4^{b}
20	144	117	81.3^{ab}	38	26.4^{b}
24	140	120	85.7^{a}	55	39.3^{a}
28	136	114	83.8^{a}	36	26.5^{b}
32	145	89	61.4^{c}	36	24.8^{b}

Values in the same column with different superscripts (a, b, c) differ significantly ($P < 0.05$).

4.3.7. Antibiotic cover and oil overlay

An ideal antibiotic for oocyte culture should have broad-spectrum antibacterial activity as well as freedom from toxicity. The use of trace amounts of penicillin (50–100 iu per ml) and streptomycin (50–100 μg/ml) in flushing fluids and culture media has been routine practice in cattle embryo transfer (ET) for many years. In the IVM medium, antibiotics are usually included to provide cover against the growth and proliferation of microorganisms during the period of culture. The concentration of antibiotics is known to be non-toxic, but their inclusion in the medium must obviously be allied to rigorous standards of hygiene in the *in vitro* production (IVP) laboratory. In this context, the use of laminar-flow cabinets and the filtration and sterilization of all components of the culture medium are part of the essential precautions that must be taken in any cattle IVP laboratory.

A study in Japan reported by Uchinuno *et al.* (1996) found no bacteria in the antibiotic-containing media in which oocytes recovered from slaughterhouse ovaries were processed; in contrast, considerable numbers of bacteria were isolated from media without antibiotics. Bacteria isolated from fluid used in the transport of ovaries were mainly Gram-negative rods. The rates of cleavage and development to embryos were significantly higher in cultures with antibiotics than in those without antibiotics. In the Netherlands, Shirazi *et al.* (2000) examined the effect of penicillin–streptomycin on nuclear maturation of cattle oocytes. The presence of the 'pen–strep' affected the maturation rate; after 24 h of culture, the percentage of MII oocytes was significantly higher than in those cultured without antibiotic cover (86% vs. 81%).

The use of gentamycin sulphate (25–50 μg/ml) is an alternative to 'pen–strep' formulations; this antibiotic tolerates autoclaving and high temperatures and possesses biological and biochemical properties that make it particularly useful.

In some maturation systems where small droplets of medium are used, mineral or paraffin oil may be employed as a cover. It is known that, due to the high absorption capacity of such oils, concentrations of steroid hormones may be influenced by their presence. It is known that, during IVM of immature oocytes, both progesterone and oestradiol are secreted by bovine COCs (Armstrong *et al.*, 1996). In Japan, studies reported by Shimada *et al.* (2002) showed that the high concentrations of steroid hormones secreted by porcine COCs accelerated GVBD and activated p34 cdc2 kinase and MAPK in oocytes, in contrast to what occurred in oocytes cultured under mineral oil. The results suggested that mineral oil overlay may adversely affect the maturation of pig oocytes.

4.3.8. Temperature, gas phase and toxic factors

Temperature

Until well into the 1980s, research studies in cattle IVM were almost invariably carried out at a temperature of 37°C. Since the late 1980s, however, most laboratories carry out IVM of cattle oocytes at 38–39°C, as this is close to the known rectal temperature of the cow (38–39.3°C). Work in Ireland adopted the higher 39°C temperature as standard and produced evidence to support its use. There have been reports, however, showing that preovulatory follicles in cattle may be almost 2°C cooler than their adjacent stroma in the cow (Grondahl *et al.*, 1996; Hunter *et al.*, 1997); this has led to the suggestion that a lower temperature during IVM may be beneficial.

It is apparent that, in the cow, ovaries are always found to be cooler than the deep rectal temperature and mature follicles cooler than the adjacent stroma. The temperature gradient between rectal temperature, ovarian stroma and preovulatory follicles in the cow has been recorded as 38.7 ± 0.6, 37.6 ± 0.5 and 36.1 ± 0.6°C, respectively. Although a report by Greve *et al.* (1996b) suggested that the temperature decreases gradually from the small to the preovulatory follicles in cattle, the effect of this on follicular events is currently unknown. In Denmark, Shi *et al.* (1998) designed a trial in which they simulated the temperature changes that occur during bovine follicular development; such changes did not affect nuclear maturation (see Table 4.3).

Neither an increase in temperature from 37°C to 38.5°C at 10 or 18 h of IVM nor a decrease from 38.5°C to 37°C at 18 h of IVM had

Table 4.3. Effect of temperature gradients on bovine oocyte maturation (from Shi *et al.*, 1998).

Temperature (°C)	Oocytes	Cleaved (%)	Morulae (%)	BL (%)	Hatched BL (%)
37.0–37.0–37.0	401	280 (69.8)	125 (31.1)	93 (23.2)	54 (58.1)
37.0–37.0–38.5	443	321 (72.5)	139 (31.4)	109 (24.6)	60 (55.0)
37.0–38.5–38.5	392	291 (74.2)	130 (33.4)	99 (25.2)	51 (51.5)
38.5–37.0–37.0	365	289 (79.2)	159 (43.6)	100 (27.4)	61 (61.0)
38.5–38.5–37.0	417	318 (76.3)	135 (32.4)	82 (19.6)	49 (59.8)
38.5–38.5–38.5	401	298 (74.3)	128 (31.9)	88 (21.9)	51 (58.0)

Data based on six replicates. Chi-square test for independence: cleaved, $P = 0.0653$; morula, $P = 0.0019$; blastocyst, $P = 0.1568$; hatched blastocyst, $P = 0.7064$.
BL, blastocyst.

any significant effect on embryonic development. The studies in Denmark did suggest that a reduction in the IVM temperature during the second half of culture might be of marginal value; a decrease from 38.5°C to 37°C during the last 14 h of maturation improved the yield of embryos. The general conclusion from the Danish study was that temperature changes during IVM do not have dramatic effects on the developmental competence of the oocytes

Gas phase

A study reported by Pinyopummintr and Bavister (1994a) sought to determine the optimal gas phase for IVM of bovine oocytes; they concluded that low oxygen tension was detrimental and that the optimal atmospheric conditions were 5% CO_2 and 20% oxygen. In Arkansas, on the other hand, Miller and Rorie (2000) determined the effect of varying the oxygen atmosphere on IVM of cattle oocytes, maturing them for 24 h in a gas phase of 5% CO_2 and either 5, 10 or 20% oxygen; they found evidence, under their conditions, based on the development of blastocysts, that reducing the oxygen atmosphere to 5% during IVM could enhance oocyte developmental competence.

In Japan, Hashimoto *et al.* (2000b,c) found that low oxygen tension during IVM was beneficial for the subsequent development of cattle oocytes; they assessed the developmental competence of oocytes matured under 5% or 20% oxygen. The proportion of oocytes that matured to MII in their IVM medium (SOF with amino acids (SOFaa)) was found to increase with increasing glucose concentration (0–20 mM). The workers concluded that glucose can play an important role in supporting the completion of meiotic maturation under low oxygen tension and that this effect during IVM is beneficial in supporting the subsequent development of cattle oocytes.

Toxic factors – ammonium

The effects of high concentrations of ammonium during IVM and subsequent embryo development were the subject of a study reported by Hammon *et al.* (1999) in Utah. This was in the light of several reports that ammonium may be associated with negative effects on the development of the early bovine embryo when it is exposed to this agent during embryo culture. Although they used concentrations of ammonia varying from 175 μM to 1400 μM in the 24 h incubation period, they found no evidence of a significant adverse effect on oocytes. They concluded that bovine oocytes are capable of tolerating high concentrations of ammonia during maturation. Elsewhere, in Aberdeen, Sinclair *et al.* (2000a) showed that exposure to high concentrations of ammonium *in vivo* compromised the subsequent ability of oocytes to develop to the blastocyst stage *in vitro*; they noted that cattle oocytes recovered from medium-sized follicles were particularly sensitive to this effect. Further work in the same laboratory showed that prior exposure of bovine granulosa cells to ammonium reduced the capacity of such granulosa cells to support the subsequent maturation and development of sheep oocytes (Rooke *et al.*, 2002).

4.3.9. Bovine serum and bovine serum albumin

It has been common practice to include macromolecules from various sources in IVM

culture media; bovine serum or BSA has usually been used for that purpose. The maturation of the bovine oocyte is a complex phenomenon involving both the nucleus and the cytoplasm. Serum included in the medium has been shown by various groups to improve oocyte maturation and subsequent embryo development. Serum is known to contain a whole range of components, including hormones, growth factors, amino acids and binding proteins; it is also recognized as being a potential disease risk, under some conditions, when contaminated with viruses.

Bovine serum albumin (BSA)

BSA can be regarded as a variable component of a maturation medium, since it has been recognized for some time that the commercial preparation may be chemically impure. For that reason, it is unwise to regard a cattle oocyte maturation medium containing commercial BSA as being chemically defined. It had been recognized for some time that BSA was probably contaminated with some low-molecular-weight compound; attempts to isolate and identify this contaminant by Michael Kane in Galway eventually proved successful. Kane showed it to be citrate, which is known to enhance early embryonic development in rabbit embryos. As well as a possible growth-promoting effect, BSA may also have a role in chelating toxic metal ions or in promoting the uptake of ions such as Ca^{2+} or Fe^{2+} by the embryo in chelated form.

Sources of bovine serum

Bovine serum, in the form of fetal calf serum (FCS) or OCS has been used as the main protein source in cattle IVM studies for many years (see Fig. 4.7). Studies in Ireland in the late 1980s showed that OCS had a significant and marked effect, compared with FCS, on the subsequent developmental competence of oocytes; similar results were evident in the reports of laboratories elsewhere. There were some studies suggesting that pro-oestrous cow serum (collected on the day prior to oestrus) might be more effective in maturation media than OCS; analysis of the serum showed it to contain high levels of LH and prolactin. A useful addition to knowledge in the early 1990s was the finding that steer serum (SS) could be as effective as OCS; for large-scale embryo production, the practicalities of recovering and processing SS were much easier to organize than the collection of serum from cows in oestrus or at defined stages of their oestrous cycles.

Constituents of bovine serum

Many reports have shown that the effectiveness of bovine serum in cattle IVM may vary markedly from one batch to the next; this is probably a reflection of the differences in the composition of the batches used. Data on the amino acid content of serum is an example of this (see Table 4.4); similar variability may presumably occur with hormones, growth factors, cytokines,

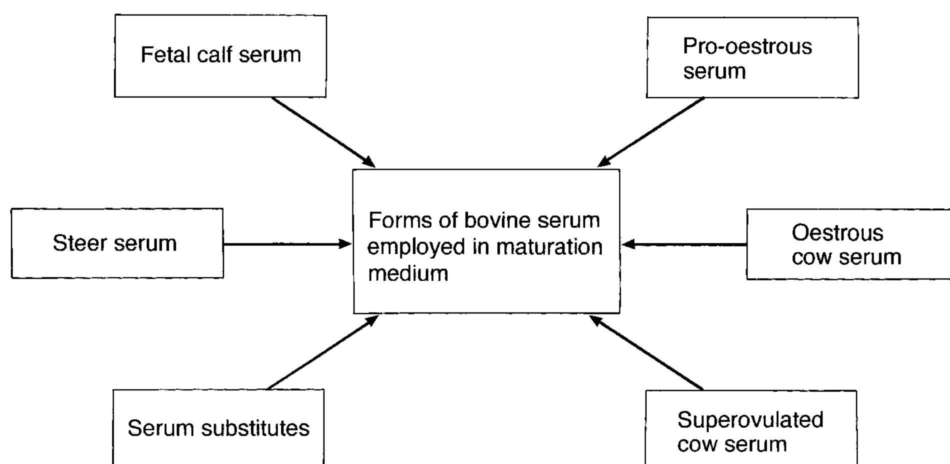

Fig. 4.7. Forms of bovine serum employed in maturation media.

Table 4.4. Amino acid concentrations in sera of clinically normal cows (from Fallon *et al.*, 1988).

Amino acid	Mean (μmol/l)	Range[a]	Coefficient of variation (%)	
			Survey	Method
Alanine	266	130–500	26.9	1.9
Arginine	246	80–620	36.1	19.6
Asparagine[b]	37	16–78	34.9	6.3
Citrulline	107	24–320	63.7	20.3
Glutamic acid[c]	524	369–722	17.5	4.1
Glycine	453	280–690	21.0	2.0
Histidine	70	20–160	55.7	40.0
Hydroxyproline	37	10–100	49.7	4.4
Isoleucine	116	60–200	26.4	3.5
Leucine	147	86–240	23.2	4.0
Lysine	133	70–230	28.4	7.3
Methionine	18	10–28	25.6	8.4
Ornithine	138	73–240	27.6	4.8
Phenylalanine	63	40–94	20.0	2.9
Proline	85	56–124	20.0	2.4
Serine	131	73–221	23.8	2.5
Threonine	78	32–170	31.7	1.3
Tryptophan	30	16–52	27.1	6.6
Tyrosine	95	43–190	32.3	7.5
Valine	257	140–440	25.9	1.6
Branched chain/aromatic amino acids	3.4	2.1–5.2	21.9	3.9

[a]Mean ± 2 SD (logarithmically transformed).
[b]Includes aspartic acid.
[c]Includes glutamine.
SD, standard deviation.

vitamins and the many other constituents of bovine serum.

In using OCS rather than other forms of serum, it was assumed that concentrations of hormones (steroids, gonadotrophins) differed from those in serum collected at other stages of the oestrous cycle or from other categories of cattle. Workers who have quantified the changes in peripheral plasma concentrations during the bovine oestrous cycle have recorded that oestradiol levels can increase threefold from a basal value of 5 pg ml to a peak value of 15 pg ml on the day of oestrus; the peak concentration of LH also occurs around the time when oestradiol is at its highest level. Such changes in hormone concentrations might go some way in explaining why OCS might genuinely differ in its effectiveness from other forms of sera.

Serum levels employed in IVM media

In many of the early Irish studies on IVM, OCS was included in the medium at a level of 20%; the usual range reported in the literature has been 10–20%.

Heat treatment of serum

At one time, in the late 1980s and early 1990s, the heat treatment of bovine serum was routinely practised before being used in IVM medium or in other media employed in cattle embryo culture. However, Bavister and associates in the early 1990s reported successfully using serum that had not been heat-inactivated; they drew attention to the lack of evidence in the literature for such a requirement (Pinyopummintr and Bavister, 1994b). Heat treatment usually involved exposing serum to a temperature of 56–60°C for 30 min; the treatment was believed to deactivate immunoglobulins and complements present in the serum. In human IVF, the treatment was believed to prevent the binding of spermatozoa and hence reduce the fertilization rate. There were those, however, who pointed out that, in the natural process of

conception in the human, the tubal environment, which both sperm and oocyte inhabit, is not devoid of immunoglobulins and complements; the same argument applies in cattle. With such thoughts in mind, Imoedemhe *et al.* (1994) carried out studies in which it was clearly shown that the absence of heat treatment did not adversely affect the outcome of human IVF. In fact, using medium supplemented with filter-sterilized, non-heat-treated serum resulted in a better fertilization rate than when the heat-treated preparation was employed. Moreover, the authors were unable to find any data in the medical literature to support the presumptions on which the heat treatment was based.

In cattle, Isachenko *et al.* (1994a) investigated the effect of the heat treatment on steroids and gonadotrophins in OCS; after heat treatment they found that the levels of FSH and LH fell to zero, whereas levels of oestradiol and progesterone showed no significant fall in concentration. Using the serum in IVM medium at the 20% level for 24 h, they found normal maturation rates (about 90%) with untreated serum and substantially lower levels with the serum subjected to deactivation.

4.3.10. Bovine follicular fluid

While there are researchers who see a completely defined maturation medium as the eventual route towards achieving high-quality cattle oocytes, others have explored the oocyte's natural environment (the preovulatory follicle) for clues by which they may formulate an appropriate medium (Ocano Quero *et al.*, 1997a,b,c). In Ireland, FF has been successfully employed on occasions as a source of fixed nitrogen at the 10–20% level; similar evidence was available from studies in France (Carolan *et al.*, 1996a), Japan (Kim *et al.*, 1996) and elsewhere. On some occasions, bovine follicular fluid (bFF) has been employed in IVP programmes for holding oocytes; to avoid possible disease risks, Pinto *et al.* (2002) used equine FF in this way without any evidence of detrimental effects on embryonic development. In their various stages of growth in the antral follicle, primary cattle oocytes are bathed in an extracellular fluid, which is the follicular fluid (FF). This fluid is a serum transudate modified by follicular metabolic activities, containing specific constituents such as steroids and glycoproteins synthesized by the cells of the follicle wall. During follicular growth, equilibrium is established between serum and follicular fluid. The metabolite concentrations in the two compartments are similar and are, in turn, similar to those in blood serum. FF contains several components of major physiological significance and buffers the internal environment of the follicle against the influence of external conditions; it maintains the oocyte in a state awaiting signals to resume meiosis and progress towards ovulation. Follicular development is assumed to be regulated by circulating pituitary gonadotrophins, the release of which is controlled by feedback mechanisms mediated by ovarian steroids and peptides. Within the preovulatory follicle, the final maturation of the oocyte occurs within an endocrine milieu characterized by a marked increase in the oestrogen-to-androgen ratio within the FF.

In France, Carolan *et al.* (1995a, 1996a) determined the effect of FF (10% level in the medium) taken from different categories of follicle on the outcome of IVM; there appeared to be no difference among fluids obtained from follicles of different sizes, but bFF from early atresic follicles appeared to be less effective. Other studies at Nouzilly in that year, this time with sheep oocytes, showed that oocyte quality was improved using FF from non-atresic follicles, but not by fluid from atresic follicles. The effectiveness of the IVM treatment was similar to that with 10% FCS. A study by Collins and Wright (1995) compared heat-treated and untreated bFF in an effort to optimize cattle IVM; their results indicated that factors found in bFF that improve the competence of the bovine oocyte may include heat-labile proteins inactivated by heat treatment or factors removed by filtration. Studies by Khatir *et al.* (1997a,b,c) sought to characterize calf FF and examine its ability to support cytoplasmic maturation of cow and calf oocytes. A report by Choi *et al.* (1998b) examined the developmental capacity of oocytes matured in two kinds of bFF (from small and large follicles); maturation rate in bFF was lower than in their control medium (TCM-199).

In Japan, Elmileik *et al.* (1999) examined the effect of adding bFF in varying concentrations (15–100%) from large, healthy follicles

(> 15 mm) to maturation medium from: (i) the start of culture (GV stage); (ii) at the completion of GVBD; or (iii) at the completion of the first meiotic division. Results indicated that bFF contains a substance or substances that apparently support cytoplasmic rather than nuclear maturation; the most favourable outcome (in terms of blastocyst yield) was with oocytes placed in 50% bFF after 18 h culture. Elsewhere in Japan, Ikeda *et al.* (1999b) sought to elucidate the beneficial effects of FF on IVM, using fractions of bFF separated by heparin-affinity chromatography; they found that the heparin-binding fraction of bFF (concentrations of 10–40%) was highly effective in enhancing the developmental competence of the cattle oocytes.

Inhibitory action of follicular fluid

Work in Ireland in the late 1960s by Sreenan used bFF as an embryo storage medium and in various studies on IVM; in IVM, FF in its undiluted form inhibited maturation and similar findings were reported elsewhere. In Colorado, Romero-Arredondo and Seidel (1994) found that bFF collected at 0 and 4 h after the LH surge appeared to possess a meiosis-inhibiting activity that is absent in fluid collected at 8 h or more after the LH surge; they also found that FF collected 8 or more hours after the LH surge is capable of overcoming the inhibitory effect of 0 h fluid. A paper by Dostal and Pavlok (1996) dealt with the isolation and characterization of maturation-inhibiting compounds in bFF. A paper by Kato and Seidel (1998) presented evidence that the composition of bFF changed between 8 and 20 h after the LH surge and that it contained a factor(s) by 20 h after LH that stimulated the resumption of meiosis. In Utah, Wang *et al.* (1999) investigated the effect of supplementing their maturation medium with 20% FF collected from various sizes of follicle (< 2 mm, 3–7 mm, 8–15 mm and > 15 mm); significantly fewer embryos developed in the medium supplemented with bFF from small follicles (< 2 mm and 3–8 mm) than with bFF from large follicles (> 15 mm). In the UK, Sfontouris and Campbell (2001) determined the effect of placing immature bovine oocytes into FF from follicles of differing oestrogenicity; they found that the culture of COCs in bFF from medium or highly oestrogenic large follicles had no effect on maturation, whereas fluid from less oestrogenic follicles had an adverse effect.

In Brazil, Goncalves *et al.* (2001) sought to determine the role of granulosa cells in explaining the inhibitory effect of FF on the maturation of cattle oocytes. They found evidence of an inverse relationship between follicle size and the ability of the fluid to inhibit nuclear maturation. Fluid from follicles smaller than 3 mm had the strongest inhibitory effect on the resumption of meiosis. FF from follicles of 3–8 mm was less potent but a significant percentage of oocytes failed to extrude the first polar body and less than 50% reached MII after 24 h culture; the fluid from follicles greater than 8 mm had no inhibitory action. The resumption of meiosis was inhibited in cattle oocytes cultured in bFF from small follicles containing mural granulosa cells but not cumulus cells. It was concluded that mural granulosa cells from small follicles are responsible for the factor(s) present in bFF that inhibit nuclear maturation.

Follicular fluid composition

FF has an important role in the nutritional and developmental support of the oocyte as well as serving as the vehicle in which the oocyte is transported to the oviduct at the time of ovulation. The fluid is known to contain many locally acting factors which regulate the development of the follicle as well as blood-borne factors that influence the actions of gonadotrophins. Studies by Peter *et al.* (1995) demonstrated that the glycoprotein vitronectin was present in bFF and that its concentration was influenced by the size of the follicle; the authors speculated that vitronectin, together with other related glycoproteins (fibronectin, laminin, etc.) may play a part in follicular growth, in the selection of the dominant follicle and in the ovulation process itself. Nitric oxide is an important intercellular messenger controlling many biological processes; it is known to be synthesized by enzymes found in granulosa cells. A study by Basini *et al.* (1998) in Italy demonstrated the presence of nitric oxide in bFF; they suggest that nitric oxide acts as a local modulator of granulosa-cell function. Other work by the Italian group indicated that intrafollicular factors modulate the effects of gonadotrophins at granulosa-cell level (Baratta *et al.*, 2000); they found evidence of

low-molecular-weight factors in bFF that reduced steroidogenesis in granulosa cells. Studies by Yoshimura *et al.* (1999) determined the levels of various hormones in bFF; they found that inhibin concentrations were higher in growing follicles than in atresic ones. In Utah, Hammon *et al.* (2000a,b) investigated the ammonia concentration in bFF at different follicle stages and how this affected the IVM of cattle oocytes; they found that ammonia concentrations decreased as follicle size increased but there was no evidence of an adverse effect on the subsequent developmental competence of oocytes.

Hyaluronic acid as a serum substitute

A general theme in many trials dealing with *in vitro* embryo production is the need to avoid using biological materials that pose any disease risk. To that end, a study reported by Marquant-Le Guienne *et al.* (1999) in France investigated the effect of HA as a substitute for serum during IVM on the production of blastocysts in different culture systems; the results obtained (see Table 4.5) indicated that similar embryo yields could be achieved as with their conventional IVM medium containing 10% of FCS.

Some workers have taken another route and used serum obtained from laboratory species rather than farm animals; a paper by Van Langendonkckt *et al.* (1997a) reported the use of media supplemented with rabbit-originated products. In Switzerland, Chanson *et al.* (2001) examined three protein supplements in their IVM medium (fetal bovine serum; BSA; synthetic serum substitute); they concluded that adequate protein supplementation was necessary and that the defined serum substitute could replace fetal bovine serum.

Hyaluronan in culturing oocytes in small groups

Under many cattle IVM culture systems, maturation is carried out with large groups of oocytes. There are occasions, however, when it is necessary to deal with single oocytes rather than groups. Although results vary according to methods used, single oocyte culture can sometimes give poor results. It is known that cattle COCs produce extracellular substances that promote maturation, among them the non-sulphated glycosaminoglycan hyaluronic acid (HA). In Sweden, de Vries *et al.* (2000) analysed the effect of exogenous HA in different group sizes (single to large numbers); COCs in small groups with HA reached metaphase II more often than controls (see Table 4.6).

Such results indicate that, when single or small COC groups are cultured, low concentrations of HA in the medium, caused either by low production or dilution, may be overcome by the addition of exogenous HA. In Canada, a study by Ali *et al.* (2002) sought to evaluate the effect of different concentrations of HA added to the maturation medium; they showed that the inclusion of high concentrations of HA (8–12 mg/ml) during IVM had a positive effect on blastocyst yield (35–37% vs. 29% in controls). They also showed that the addition of oestradiol to their synthetic oviductal fluid (SOF) medium in the

Table 4.5. Effect of hyaluronic acid in a serum-free maturation medium on blastocyst yield (from Marquant-Le Guienne *et al.*, 1999).

	Number of experiments	Bl and exp. Bl Day 7	Hatched Bl	
			Day 8	Day 9
Control	4	33.7% (183/542)	12% (53/441)	24.5% (108/441)
HA 0.25 mg/ml	3	26.7% (77/288)	8.3% (15/180)	22.2% (40/180)
HA 0.5 mg/ml	5	28.1% (136/484)	7.4% (28/377)	20.2% (76/377)
HA 1 mg/ml	4	23.5% (96/409)	10.2% (32/314)	20.7% (65/314)

Bl, blastocysts; exp. Bl, expanded Bl; HA, hyaluronic acid.

Table 4.6. Development of bovine oocytes in HA-supplemented medium (from De Vries *et al.*, 2000).

COC groups	Medium	No. COCs	MI	MII	Others
Single (1 per 10 μl)	Control	47	28	70	2
	CM	52	21	69	10
	HA	47	11	87	2
Small (5 per 20 μl)	Control	42	21	74	5
	CM	62	14	82	4
	HA	56	14	86	0
Large (15 per 50 μl)	Control	126	12	78	10
	CM	33	21	73	6
	HA	62	16	77	7

Development rates (MI or MII in per cent of *n*) of bovine oocytes after COC incubation in control (TCM-199), conditioned medium (50% CM) or HA (50 ng/ml).

presence of 8 mg HA resulted in a further significant increase in blastocyst yield (46%). The authors note that their results open the way to including HA in a completely defined maturation medium.

4.4. Somatic-cell Support

Cumulus cells are known to play a crucial role during oocyte maturation, in keeping the mammalian oocyte under arrest, in participating in the induction of meiotic resumption and in supporting cytoplasmic maturation (Tanghe *et al.*, 2002); it is also evident, at least in some species, that cumulus cells participate in the pick-up of the ovulated oocyte by the oviduct and create a microenvironment favourable to sperm capacitation and sperm penetration.

4.4.1. Cumulus–oocyte complex (COC)

It is evident from numerous reports in the literature that gonadotrophins, steroids and cellular factors all interact to provide essential support for the oocyte during maturation in the live cow; the preovulatory follicle can be viewed as a structure in which millions of somatic cells contribute to the welfare of one germ cell, the oocyte. Immature bovine oocytes are surrounded by several layers of tightly adherent cumulus cells, which provide nutritive factors and control the oocytes' nuclear and cytoplasmic maturation. There is a complex interplay between the oocyte and the cumulus cells during follicle growth and development, which is mediated by growth factors and hormones. The cumulus cells themselves communicate by way of an intricate network of transmembrane channels known as gap junctions, while the innermost layer of cells (corona radiata) extends cytoplasmic processes through the zona pellucida to form gap junctions with the oocyte (see Fig. 4.8). The importance of these processes and gap junctions is enhanced by the known impermeability of the oocyte membrane to various metabolites of low molecular weight; the entry of such molecules is believed to be exclusively via the corona cell processes and gap junctions.

Cumulus cells are linked to granulosa cells, which in turn are closely associated with the theca interna cells, even though physically they are separated by the basement membrane. It is believed that, despite this membrane, substances produced by thecal cells find their way into follicular cells and subsequently into the oocyte. There is certainly evidence that thecal cells can provide support for the oocyte during maturation, even though the means by which their secretory products reach the cell may not be well defined. It might also be mentioned that cumulus cells may have a role shortly after ovulation in ensuring the 'capture' of the ovulated bovine oocyte by the infundibulum of the oviduct.

4.4.2. Connexin 43 and oocyte meiotic maturation

In bovine follicles, cumulus cells play an important role in oocyte growth and differentiation by

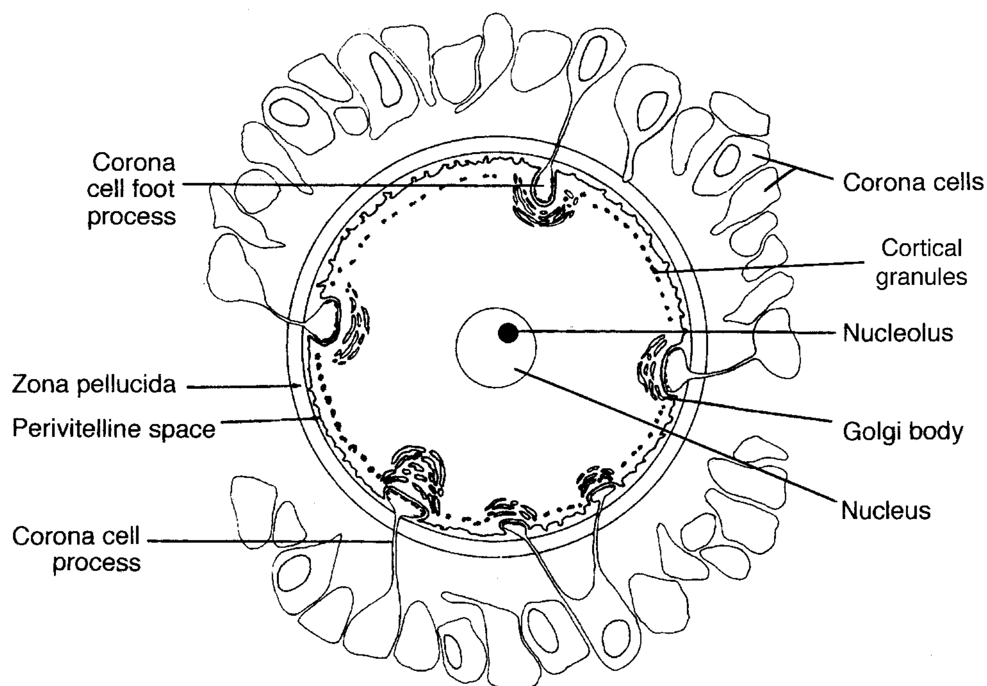

Fig. 4.8. Bovine oocyte and associated follicular cells.

providing the immature cell with nutrients and by controlling both nuclear and cytoplasmic maturation when the oocyte is selected for ovulation. It is known that there is a complex interplay between oocytes and cumulus cells during folliculogenesis, which is mediated by hormones and growth factors. The cumulus cells, which are linked between themselves and to the bovine oocyte by way of gap-junction channels, are believed to maintain meiosis-inhibiting levels of cyclic adenosine monophosphate (cAMP) in the oocyte by transferring cAMP to the oocyte, by regulating the production and hydrolysis of cAMP within the oocyte or perhaps by a combination of both mechanisms. A report by Gilchrist *et al.* (2001) in Australia reported that meiotic maturation and granulosa-cell cAMP levels are differentially regulated by type-specific phosphodiesterase inhibitors. It may be that the function of cumulus cells is more complex than the mere transfer of cAMP inhibition signals; they may also be transferring a positive signal that induces meiotic resumption, which can cancel the inhibitory signal by stimulating cumulus cells to produce a GVBD-inducing factor.

Workers in Switzerland, using gap-junction uncouplers, investigated the involvement of gap-junction channels between the cumulus cells in oocyte maturation in cultured cattle COCs (Vozzi *et al.*, 2000, 2001; Haefliger *et al.*, 2001); their data indicated that intercellular communication mediated by gap junctions between the cumulus cells are required for normal oocyte meiotic maturation and that connexin 43 (Cx43) plays an important role in this process. Gap junctions are composed of proteins belonging to the connexin (Cx) family and can mediate the exchange of ions and metabolites between neighbouring cells. It is believed that a gap-junctional network is essential for the induction of oocyte meiotic maturation. In knockout mice deficient in Cx37, it is found that they lack mature follicles, fail to ovulate and develop abnormal corpora lutea.

4.4.3. Additional cumulus/granulosa cells

The results of many studies show that the presence of cumulus cells during maturation is

essential for the acquisition of developmental competence by oocytes *in vitro* (Chian *et al.*, 1994; Yang *et al.*, 1994; Konishi *et al.*, 1995; Liu, Y. *et al.*, 1995; Kim *et al.*, 1997; Hill, J.R. *et al.*, 1998; Machatkova *et al.*, 2001). A study by Chian and Niwa (1994) sought to determine whether cumulus cells are necessary during the whole IVM period to achieve normal cytoplasmic maturation; they concluded that the presence of intact cumulus cells was necessary for at least 12 h of the 24 h culture period in TCM-199 supplemented with 10% FCS. The importance of maintaining an intact layer of corona cells was shown in studies in which oocytes were stripped of the cumulus-cell component of the COC. In Japan, Hashimoto *et al.* (1998b) cultured corona-enclosed oocytes and oocytes denuded of their somatic cells; addition of cumulus cells improved the development of corona-enclosed cells but had no effect on the denuded oocytes. A density of 1.6–3.2 million cumulus cells per ml gave the highest maturation rate and the highest blastocyst yield. It appeared that the developmental competence of oocytes surrounded by corona cells is supported in a cell density-dependent manner in the maturation medium; evidence supported the view that cumulus cells support oocyte development by secreting soluble factors that induce developmental competence or by removing an embryo development-suppressive component from the medium.

In terms of what additional follicular cells may add to the IVM medium, it should be noted that granulosa cells are able to produce oestradiol. In the USA, Roberts and Echternkamp (1994) studied the effect of cell location in the follicle on oestradiol production *in vitro*; they found that granulosa cells proximal to the basement membrane may be less differentiated with regard to oestradiol production than cells distal to the membrane. They also found that granulosa cells aspirated from small follicles appeared to continue to differentiate into oestradiol-producing cells during culture. In Brazil, Mingoti *et al.* (2002) investigated the ability of cattle COCs to produce steroids; considerable oestradiol accumulation was observed in the maturation medium during the 24 h period of oocyte incubation. The authors suggested that the addition of oestradiol to an IVM medium might be reviewed, in view of evidence showing cumulus cells secreting sufficient oestradiol to meet culture requirements.

4.4.4. Special needs of ovum pick-up (OPU) oocytes

It has been recognized for many years that the developmental capacity of sheep and cattle cultured *in vitro* is markedly influenced by the presence of both cumulus and parietal granulosa cells during IVM. However, the increasing use of the OPU technique in collecting oocytes for culture means that the opportunity to collect parietal granulosa cells is not available in the same way as in dealing with abattoir ovaries. In Italy, Galli and Lazzari (1995) demonstrated that parietal granulosa-cell supplementation during oocyte maturation improved blastocyst yield significantly, whereas cumulus cells did not provide the same input (Table 4.7). They concluded from their studies that, even though more time and effort may be required in organizing the collection of abattoir ovaries and recovering parietal granulosa cells, this was one way in which cattle embryo production could be optimized. Konishi *et al.* (1995, 1996a) in Japan also showed that co-culture with granulosa cells improved the ability of oocytes collected by OPU to develop to the blastocyst stage.

Table 4.7. Developmental capacity of cattle oocytes matured with and without granulosa-cell supplementation (from Galli and Lazzari, 1995).

	n oocytes	*n* cleaved (%)	*n* Grade 1	*n* Grade 2	Ratio G1 : G2	% G1 and G2 cleaved
IVM with GC	693	635 (91.6%[a])	104	59	1.76[a]	25.7%[a]
IVM without GC	615	572 (93.0%[a])	49	39	1.26[b]	15.4%[b]

Values within columns with different superscripts are different ($P < 0.05$).
GC, granulosa cells.

4.4.5. Use of non-follicular cells

Some studies have reported on the supplementation of the IVM medium with non-follicular cells. Methods of culturing cattle embryos from the zygote to the blastocyst stage have used oviductal cells, which are readiliy available from abattoir sources. Some have also used them in maturing oocytes. Durnford *et al.* (1994) in Canada used bovine oviductal epithelial cells (BOEC) in a comparison with granulosa cells but found no difference in outcome, although they noted that BOEC and granulosa cells behaved very differently in culture.

4.4.6. Action of theca cells

There are situations in which follicular cells promote the inhibition of meiotic resumption; this seems to apply to theca cells in cattle (see Fig. 4.9). Theca cells are known to synthesize various proteins that are different from those produced by granulosa cells (Richard and Sirard, 1995); the inhibition of meiotic resumption by theca cells might be associated with the synthesis of certain of such proteins. The action of theca cells is of interest to those who are attempting to provide a prematuration treatment for cattle oocytes (see Section 4.11).

4.5. Hormones and Growth Factors

It is well established that gonadotrophins play a major role in triggering the resumption of meiosis in the bovine oocyte and in the expansion of the cumulus oophorus. However, it is also well accepted that other hormonal factors may influence the meiotic maturation of the oocyte during the preovulatory period. The effects of hormonal supplementation during IVM of cattle oocytes have been investigated using serum-free and serum-containing media; the evidence obtained has not always been clear as to the need and the concentration of the hormones that should be added to the medium. A wide range of concentrations of FSH (0.05–20 μg/ml) and LH (0.5–100 μg/ml) have been employed in maturation studies. It has to be noted, however, that protein hormones are difficult to compare among studies because of the varying degrees of purity of many of the preparations. Studies in many laboratories have brought increasing evidence that growth factors play an important part in the maturation process.

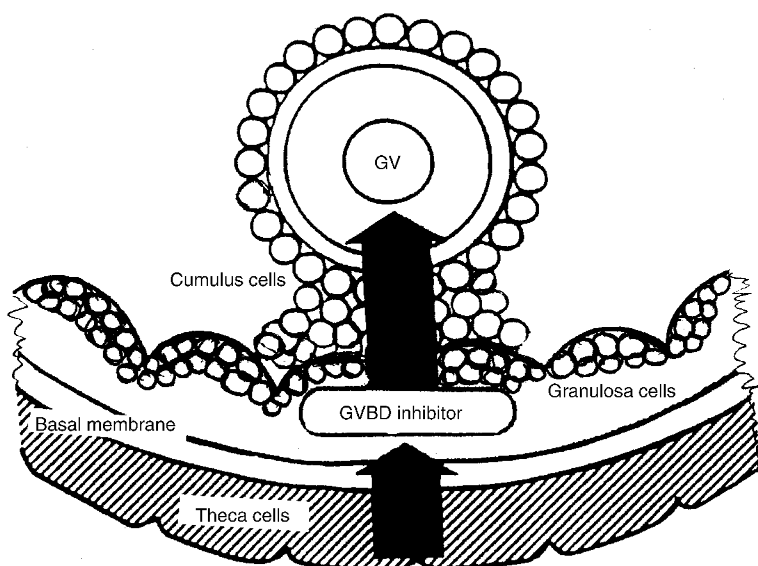

Fig. 4.9. Interaction between granulosa and theca cells in the inhibition of meiotic resumption.

The development of defined and semi-defined culture media now makes it possible to evaluate hormones and growth factors much more accurately. In Canada, for example, Ali and Sirard (2002a,b) matured bovine COCs in a defined maturation medium consisting of SOF. They found that the addition of LH (50, 500 or 5000 ng/ml) to the IVM medium did not increase the proportion of oocytes developing to the morula and blastocyst stages. The yield of morulae and blastocyts was significantly improved (30% vs. 18%) by the addition of epidermal growth factor (EGF) (30 ng/ml EGF); the addition of recombinant human FSH (r-hFSH) in the presence of oestradiol resulted in an even greater increase in the morulae/blastocyst yield (40% vs. 19%) in comparison with maturation medium alone. It was also apparent that the presence of BSA as the only protein supplement during IVM may be detrimental to oocyte maturation. Such work demonstrated the role of FSH, EGF and oestradiol in the kinetics of the nuclear and cytoplasmic maturation that are essential for the development of a competent bovine oocyte.

4.5.1. Hormones

It is well established that the key hormones in the regulation of ovarian function in the cow are FSH and LH; another pituitary hormone, prolactin, is also known to exert an effect on the ovary. Many of the reports in the literature on cattle IVM have dealt with the direct addition of gonadotrophins (FSH, LH) and steroids (oestradiol) to the maturation medium. In Ireland, Lu and associates found that oocyte maturation was possible in the absence of such additions; however, both the serum and the granulosa cells added to the maturation medium were sources of hormones. It is certain that OCS and SS contained gonadotrophins in some concentration; it is also known that granulosa cells are capable of steroidogenesis during culture, producing oestradiol.

Follicle-stimulating hormone and luteinizing hormone

Many reports have dealt with the important contribution of biological gonadotrophin preparations to cattle-oocyte maturation culture systems. Using recombinant gonadotrophins, Martins and Brackett (1998a,b) and Martins *et al.* (1998) demonstrated the effectiveness of both r-hFSH and recombinant human LH (r-hLH) to mature bovine oocytes, as evident in their subsequent embryonic development. In Leeds, Danfour *et al.* (1999) used preovulatory surge levels of LH (100 ng/ml) and FSH (50 ng/ml) in their IVM medium; they concluded that acceptable rates of maturation could be obtained from cattle oocytes under serum-free conditions in the presence of such surge levels. However, the effect of LH during IVM of cattle oocytes on their developmental competency to produce viable embryos has been controversial and there have been indications that it may inhibit embryonic development (Duszewska and Pienkowski, 1998). As noted earlier, in studies reported by Ali and Sirard (2002c), LH did not improve the developmental competence of oocytes. In Georgia, Martins and Brackett (1998) used recombinant LH and FSH, either alone or in combination, in their maturation medium; their data showed that cattle embryos can be produced *in vitro* using r-hFSH as the sole source of gonadotrophin in a completely chemically defined IVM culture system. In the live animal, the period when oocytes are exposed to a high LH concentration is limited to about 6 h. In Canada, Twagiramungu *et al.* (2000) examined the effect of adding LH to their IVM but found no evidence that a limited use of LH influenced subsequent embryo production.

In Italy, Modina *et al.* (2000) analysed the effect on oocyte developmental competence of r-hFSH and concluded that FSH is the only hormone supplement required to confer high competence. The same authors also found that a β-catenin mouse monoclonal antibody (Transduction Laboratories) can be used as an early marker of embryonic development. In Brazil, Alves *et al.* (2001) evaluated the use of differing doses of FSH-p in their maturation medium (1–40 μg/ml); they concluded that 20 and 40 μg/ml dose levels interfered with the nuclear maturation process. In Colorado, Choi *et al.* (2001b) sought to determine the optimal concentrations of relatively pure gonadotrophins when using a semi-defined medium for cattle IVM. They found that FSH and LH did not improve embryo development of bovine oocytes

in medium devoid of serum, indicating that growth factors such as EGF or growth hormone may be required. EGF and insulin-like growth factor I (IGF-I) potentially interact with gonadotrophins, steroids and other molecules to regulate oocyte follicular development in the live animal. Although the workers in Colorado did not find any positive effect of gonadotrophins on embryo development using their IVM system, there was a marked dose-dependent effect on cumulus cell expansion.

Prolactin

In contrast to what is known about the actions of FSH and LH, the role of prolactin in the mechanisms of meiosis and folliculogenesis regulation is not well understood; there is no general agreement as to how prolactin affects cattle oocyte maturation in the live animal. In Germany, Torner *et al.* (1996a,b) and Kuzmina *et al.* (1996, 1998) reported on the effect of bovine prolactin (bPRL) on cattle oocyte maturation; they provided evidence that bPRL both influenced the synchronous maturation of the cytoplasm and nucleus and led to an improved developmental competence. A further report on the influence of bPRL on the developmental competence of cattle oocytes came from Heleil *et al.* (1998) in Germany; under the influence of bPRL (50 ng/ml) during IVM, there was evidence that the sensitivity of COCs from follicles > 5 mm for prolactin was increased and this was followed by higher developmental competence after fertilization. A report by Kuzmina *et al.* (1999) examined the effect of prolactin on the dynamics of the Ca^{2+} content in cattle oocytes; they concluded that further studies were necessary to establish the complete chain of events binding the interaction of prolactin with its receptors in follicular somatic cells to Ca^{2+} intracellular stores in the oocytes. Studies conducted by Torner *et al.* (2000, 2001) in Rostock investigated nuclear and cytoplasmic maturation of cattle oocytes as influenced by origin (small or large follicles) and prolactin treatment; meoisis was prolonged by prolactin and there was a relationship between chromatin configuration and protein kinase activities.

There are reports in which substitutes for FSH and LH are employed. In Argentina, Alberio *et al.* (1998a) used highly purified human menopausal gonadotrophin (HMG) as a substitute for FSH successfully. Ocano Quero *et al.* (1999a,b) used pregnant mare serum gonadotrophin (PMSG) (4 iu/ml) and hCG (2 iu/ml) in their IVM medium; they presented evidence suggesting that such supplementation had a significant positive effect on maturation and fertilization rates.

Growth hormone (somatotrophin)

Growth hormone (recombinant bovine somatotrophin (r-BST)) has been employed in IVM medium in studies with cattle; there is evidence in several reports that the hormone accelerated the normal course of oocyte maturation (Izadyar *et al.*, 1996a,b); it has also been shown that growth hormone can enhance the yield of blastocysts after fertilization and embryo culture (Izadyar *et al.*, 1996c, 1997a,b,c). Papers by Izadyar *et al.* (1998a,b,c) showed that FSH and growth hormone act differently on nuclear maturation but that both enhance the developmental competence of IVM cattle oocytes. In Germany, Alberio *et al.* (1998b) found a tendency towards greater embryo development in oocytes treated with recombinant growth hormone and recombinant FSH during maturation. There is evidence in a report by Izadyar *et al.* (1999) that growth hormone can be synthesized by COCs during IVM; it was not clear whether the synthesis of the hormone was regulated by growth hormone-releasing hormone (GH-RH). It is believed that the stimulatory effect of growth hormone on bovine oocyte maturation and its beneficial effect on embryo development is not a direct effect but is mediated by IGF-I (Iga *et al.*, 1998a,b). In Germany, Kolle *et al.* (1998) reported results which suggested that growth hormone enhances the proliferation of cumulus cells during IVM. A later paper by Kolle *et al.* (1999) found evidence that growth hormone in combination with IGF-I significantly decreased apoptosis in cattle COCs in a maturation culture system (see Fig. 4.10).

Steroids

Various reports have dealt with the involvement of steroid hormones in the maturation of cattle oocytes. It is known that steroid hormones can be produced by the COCs during culture. A study

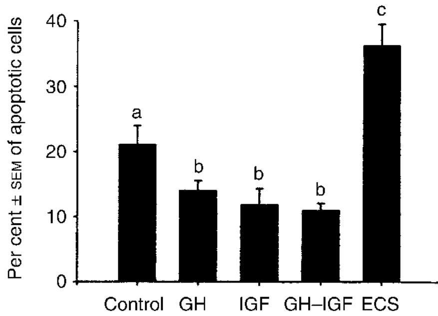

Fig. 4.10. Growth hormone and IGF-I effects on apoptosis in bovine cumulus–oocyte complexes during maturation. Percentage ± standard error of mean (SEM) of apoptotic cumulus cells of bovine cumulus–oocyte complexes after *in vitro* maturation (24 h) with different supplements. Means are expressed as percentages (number of apoptotic cumulus cells/total cell number). Means with different superscripts are significantly different (analysis of variance (ANOVA) followed by LSD post hoc test; $P < 0.05$) (from Kolle *et al.*, 1998).

by Ryan *et al.* (1999) in Scotland found that, in the presence of gonadotrophins, progesterone added to the IVM medium plays an important role in determining the developmental competence of bovine oocytes. A study by Silva *et al.* (1999) in Reading demonstrated a suppressive effect of the inhibin α-subunit on the developmental competence of IVM cattle oocytes. Further work in Reading by Silva and Knight (2000) examined the effects of androgens, progesterone and their antagonists on the developmental competence of cattle oocytes after IVM; they found evidence indicating that the negative effect of progesterone on blastocyst yield may be mediated by increased inhibin α-subunit expression by cumulus cells.

In the Netherlands, Beker *et al.* (2002) examined the effect of exposing bovine oocytes to oestradiol for varying intervals during the maturation process; the presence of the steroid during 8–22 h of culture (after GVBD) affected metaphase spindle formation and resulted in an increase in the percentage of oocytes showing nuclear aberrations. Such data agreed with other studies in their laboratory showing that the addition of oestradiol (1 μg/ml) to the TCM-199 maturation medium decreased the proportion of oocytes reaching MII after 22 h of culture. It may be mentioned that a paper by Kruip *et al.* (1988) some time ago presented evidence that oestradiol at certain dose levels could have an adverse effect on spindle formation and polar body extrusion.

Insulin and GH-RH

The influence of human and bovine insulin on IVM, IVF and cleavage rates of bovine oocytes was examined by Ocano Quero *et al.* (1998a) in Spain; there was a positive effect from the addition of the insulin.

A study by Beker *et al.* (2000) in the Netherlands investigated the effects of growth hormone-releasing hormone (GH-RH) on nuclear maturation, cortical granule distribution and cumulus expansion of the bovine oocyte; from their results, they concluded that GH-RH had no effect on nuclear maturation or cumulus expansion of bovine COCs but retarded cytoplasmic maturation, as reflected by delayed cortical granule migration. The authors suggest that the effects of GH-RH are exerted via a receptor localized on the plasma membrane of the oocyte. In Georgia, Freitas *et al.* (2002) evaluated the capacity of bovine oocytes to undergo IVM and subsequent embryonic development after replacing FSH (Folltropin) with GH-RH; although cumulus cell expansion was minimal with the releasing hormone, it was found that GH-RH and FSH gave comparable embryo production results.

4.5.2. Growth factors

The effects of supplementing TCM-199 with growth factors (EGF, transforming growth factors alpha and beta 1 (TGF-α and TGF-β_1), basic fibroblast growth factor (bFGH) and insulin), LH, FSH and FCS on cumulus expansion and fertilization of bovine oocytes after IVM were reported by Kobayashi *et al.* (1994) in Japan. EGF, TGF-α, LH and FSH increased cumulus expansion and fertilization rate; no significant effect was achieved using TGF-β_1 or bFGF. The Japanese workers reported no additive effect on cumulus expansion and oocyte fertilization when EGF was combined with LH or FSH; the addition of EGF or TGF-α to the maturation medium increased the yield of blastocysts. In France, data reported by Carolan *et al.*

(1995c) led them to conclude that IGF-II played a role in cattle oocyte maturation and that both IGF-II and insulin might be involved in embryonic development.

Epidermal growth factor (EGF)

It was shown by Lonergan *et al.* (1996) that, when the IVM medium was supplemented with EGF, there was a significant improvement in the maturation rate of cattle oocytes, an altered pattern of protein biosynthesis during maturation and improved subsequent embryo development; similar findings were evident in the data for oocytes recovered from young calves (Khatir *et al.*, 1996b). In Spain, Lorenzo *et al.* (1995) reported that EGF, with or without IGF-I, significantly stimulated cumulus expansion and meiotic maturation. A paper by Rieger *et al.* (1995) suggested that a combination of 50 ng/ml EGF and 100 ng/ml IGF-I was as effective in stimulating nuclear maturation and metabolic activity in cattle oocytes as FCS and gonadotrophin. In Italy, studies by Gandolfi *et al.* (1996a) indicated that EGF and IGF-I stimulated bovine oocyte metabolism during maturation and improved cleavage rate; evidence for a beneficial effect of EGF alone was provided by Im *et al.* (1994) in Korea and Kato and Seidel (1996) in the USA. A series of experiments in Japan reported by Anas and Terada (1999) examined the effect of EGF on nuclear and cytoplasmic maturation. They recorded evidence suggesting that EGF is capable of stimulating meiotic maturation in the bovine oocyte regardless of the presence or absence of cumulus cells and that PI3-kinase activity is essential for EGF-induced maturation. A report by Sakaguchi *et al.* (2000) showed that the progression of meiosis in bovine oocytes is accelerated by exposure to a combination of EGF and IGF-I in serum-free maturation medium without this improving their developmental competence; the acceleration effects were neutralized by the presence of FCS in the medium.

A report by Freitas *et al.* (2001) concluded that the addition of EGF to the maturation medium optimized embryo production, even in the presence of FSH and FCS. They also showed that EGF induces its positive maturation signal during the first 4 h of culture. In Canada, Goff *et al.* (2001) showed that the presence of EGF during maturation significantly enhanced cleavage rate and development to the blastocyst stage; their results indicated that EGF plays a physiological role in oocyte maturation and brings about marked changes in protein synthesis. Identification of the proteins induced by EGF could be important for improving our understanding of cattle oocyte maturation *in vitro*. Of interest to those dealing with cattle oocytes recovered by OPU, a study by Machatkova *et al.* (2001) has reported that the use of ECG may improve the oocyte's maturation and developmental potential in COCs with partially deficient cumulus cover.

IGF family

A review by Lackey *et al.* (2000) examined the IGF system as it relates to reproduction. IGF-I and II are ubiquitously expressed factors that regulate cell growth, differentiation and maintenance of differentiated cell function. IGF-binding proteins (IGFBP) are also ubiquitously expressed; these proteins are involved in transport and in increasing the half-life of IGF peptides. A report by Martins *et al.* (1997) investigated the apparent enhancement of oocyte quality after maturation in media supplemented with IGF-1 and FSH; their results confirmed a beneficial effect of IGF-I and FSH. There is evidence that a combination of EGF and IGF-I in a maturation medium accelerates the first polar-body extrusion of cattle oocytes; it is believed that this effect may be mediated by cumulus cells surrounding the oocytes. A report by Sakaguchi *et al.* (1999) indicated that the accelerating effect may be involved with increased H1-kinase and MAPK activities in the oocytes. Although studies reported by Quetglas *et al.* (2001) failed to record a beneficial effect of IGF-I in the IVM medium, there was a significant increase in cleavage rate when the growth factor was added to the *in vitro* culture (IVC) medium. In Georgia, Sirisathien and Brackett (2002) investigated the beneficial effects of IGF-I in their maturation medium on bovine embryo development and DNA fragmentation by TUNEL staining; IGF-I improved blastocyst development significantly.

The mechanism for the accelerating effects of EGF and IGF-1 on the meiotic cell cycle of cattle oocytes was investigated by Sakaguchi *et al.* (2002) in Japan; the growth factors significantly

increased the frequency of first polar-body extrusion in oocytes derived from small follicles (< 3 mm diameter) but did not increase the frequency of medium (4–6 mm) or large (7–10 mm) follicles. The workers suggested that this accelerating effect may be related to increased H1-kinase and MAPK activities during the early stages of maturation.

Midkine and other growth factors

The effect of midkine (MK), a heparin-binding growth/differentiation factor, is known to exist in bFF. In Japan, Ikeda *et al.* (1999a, 2000a,b) reported evidence that supplementation of the IVM medium with midkine enhanced the developmental competence of oocytes and that this effect might be mediated by granulosa cells. In Germany, Einspanier *et al.* (1999) examined the presence of vascular endothelial growth factor (VEGF) in the maturation medium; the authors concluded that VEGF may be an important factor necessary for oocyte maturation. Their findings were strengthened in a subsequent paper, which showed that the growth factor significantly improved cleavage rate and embryo development (Einspanier *et al.*, 2002).

The action of modulating growth factors bFGF and TGF-β_1 was examined by Bieser *et al.* (1998), who suggest that the action of such factors is essential for extracellular proteolysis during IVM of cattle COCs. In Brazil, Bortolotto *et al.* (2001) examined the effect of platelet-derived growth factor (PDGF), insulin and retinol and their interactions in IVM; insulin and PDGF accelerated nuclear maturation and this effect was enhanced by retinol.

Other farm animals

In other farm animals, reports have shown results similar to those found in cattle. In Canada, Ding and Foxcroft (1994) reported that EGF stimulated the resumption of porcine oocyte maturation and cumulus cell expansion *in vitro*; other work in that country indicated that EGF of follicular origin may have a physiological role in the regulation of follicular development and oocyte maturation in pigs. A paper by Coskun and Lin (1995) dealt with the mechanism of action of EGF in pig oocyte maturation. In India, working with buffalo oocytes, Chauhan *et al.* (1999c) found that EGF had a positive effect on cumulus expansion, nuclear maturation and postfertilization cleavage. In sheep, Choi *et al.* (2001) in the USA reported that EGF could be recommended as a supplement to maturation medium to enhance embryonic development *in vitro* in that species; blastocyst yield was significantly increased in comparison with controls (27% vs. 13%). Studies reported by Lorenzo *et al.* (2002) examined the effect of EGF and IGF-I on steroid secretions following IVM of equine oocytes; their results showed a positive influence of these growth factors. It was found that growth-factor supplementation of the IVM medium elevated steroid production by the cumulus cells; the findings provided further insight into growth factor-dependent steroidal effects on equine oocyte maturation.

4.5.3. Cytokines

A study reported by Spicer and Alpizar (1994) examined the effect of cytokines (tumour necrosis factor alpha; interleukin; interferon; bovine trophoblast protein 1) on FSH-induced oestrogen production by granulosa cells from small and large cattle ovarian follicles; their results showed that less differentiated granulosa cells (small follicles) are more responsive to cytokines than highly differentiated cells (large follicles). A paper by Margawati *et al.* (1997) dealt with the effect of leukaemia inhibitory factor (LIF) during maturation and culture on blastocyst cell number, inner cell mass and trophectoderm.

4.5.4. Oocyte-derived growth factors

It is well established that growth factors synthesized by ovarian somatic cells directly affect oocyte growth and function, but much less information is available on how oocyte-secreted factors play a reciprocal role in modulating somatic cell functions in the cow. Eppig and colleagues have shown that in some species, such as mice, the oocyte can promote the organization of the follicle, the proliferation of granulosa cells and the differentiation and function of cumulus cells (see Fig. 4.11).

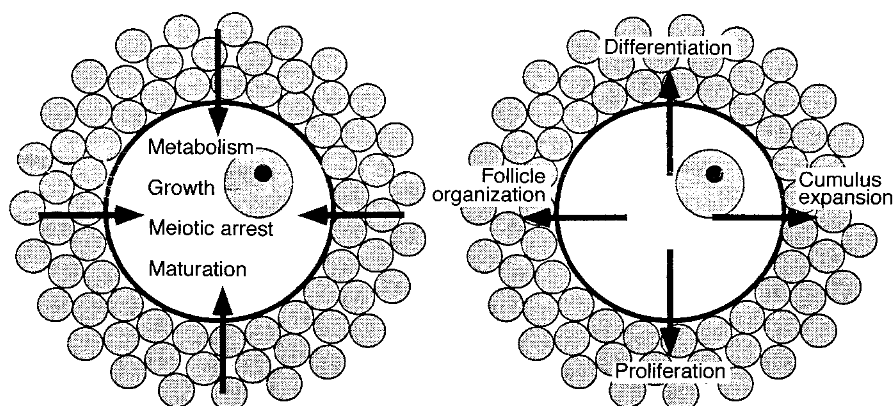

Fig. 4.11. Oocyte–cumulus interactions in the mouse (from Eppig *et al.*, 1993).

A paper by Dong *et al.* (1996) reported that a growth factor, growth differentiation factor 9 (GDF-9), plays an important role in folliculogenesis. The evidence implicated GDF-9 in sustaining the growth and differentiation status after the primary one-layer follicle stage in the mouse. In cattle, Ralph *et al.* (1995a) found, in contrast to the story in mice, that bovine oocytes do not secrete a factor responsible for cumulus cell expansion; the cattle oocyte did, however, secrete a factor that induced cumulus cell expansion in mice. In Australia, Li, R. *et al.* (2000) have reported that oocyte-secreted factor(s) determine functional differences between mural granulosa cells and cumulus cells; their results provided evidence that mural granulosa cells and cumulus cells have distinctive phenotypes and that the oocyte is responsible for some of the characteristic features of cumulus cells. It is clear that bovine oocytes secrete factors that simultaneously promote growth and attenuate steroidogenesis in follicular somatic cells. There is evidence suggesting that the pig oocyte secretes a factor or factors that affect granulosa cell steroid synthesis and granulosa cell number (Brankin *et al.*, 1999).

4.6. Oocytes Cultured Singly or in Groups

Although the laboratory production of cattle embryos from slaughterhouse ovaries is a well-established technique, on most occasions the oocytes are treated in groups rather than individually. With the increasing use of OPU from live donor cattle, however, there has been the need to look more closely at dealing with individual oocytes (O'Doherty *et al.*, 1997; Nolan *et al.*, 1999). In commercial practice, where limited numbers of oocytes may be coming from genetically valuable animals by OPU, there is an obvious requirement to have IVC systems capable of supporting the single oocyte.

4.6.1. Single-oocyte culture systems

In France, Carolan *et al.* (1996b,c) at Nouzilly and Peynot *et al.* (1996) at Jouy-en-Josas reported that under certain culture conditions a large proportion of bovine oocytes can develop to the blastocyst stage when matured, fertilized and cultured individually; as well as being useful to commercial operators, the authors suggest that such systems may permit studies that will expand knowledge of events in late folliculogenesis. In the same French studies, it was found that serum (FCS) had a deleterious effect on the maturation of individual oocytes but that the addition of EGF helped to compensate for this effect; it was suggested that poor embryo development previously reported after individual oocyte maturation may have been the result of the adverse effects of serum. In New Zealand, Hagemann *et al.* (1998b) reported on a single-oocyte culture system that resulted in developmental rates comparable to those achieved with group culture. A novel technique, known as the 'well of the well' (WOW) system, was developed

by Vajta *et al.* (2000a,b) and gave embryo yields similar to those from group culture, although oocytes were only processed singly for their maturation. In Ireland, Ward *et al.* (2000) found that single culture of the oocyte during fertilization or culture had an adverse effect on blastocyst yield; when they cultured oocytes in groups of 5, 10 or 25, there was no difference in blastocyst yield among groups but there was a significant decrease in those cultured singly.

A study by Mizushima and Fukui (2001) in Japan used a serum- and protein-free medium supplemented with polyvinyl alcohol (PVA) in the culture of individual oocytes. In the control medium containing FCS, their single-culture system gave similar results to their group-culture system, in terms of maturation and fertilization, although the blastocyst yield tended to be lower (single 16.5% vs. group 24.5%). In their chemically defined medium, supplemented with PVA, the addition of beta-mercaptoethanol (β-ME) or both hypotaurine and β-ME, resulted in single- and group-culture systems giving similar maturation, fertilization and cleavage rates, although blastocyst yield was still low in the defined medium. The Japanese workers concluded that β-ME added to a chemically defined medium increased the intracellular glutathione level of oocytes cultured singly and improved the maturation rate.

4.7. Single-culture Medium Systems

There are many questions relating to the optimization of embryo production systems. It may be a question of labour or of materials employed in the media. As more is learnt of the limits within which it is possible to operate in cattle embryo production, then more thought can be devoted to possible ways of simplifying the production process. The preparation of media is an exacting and time-consuming process, which often calls for weekly or biweekly attention to avoid the risk of degradation of certain components. With a single medium system, the media for maturation, fertilization and embryo culture can all be made up from the same basic stock solutions, thereby saving time.

4.7.1. Synthetic oviductal fluid (SOF) formulations

In conventional cattle embryo production *in vitro*, oocytes and embryos may be exposed to as many as four different media during their progress through the production system. Typically, different media are used for each phase of embryo production (maturation medium, fertilization medium and embryo culture medium), although there have been those who believe that the potential exists to develop an entire culture protocol from oocyte maturation through to blastocyst development in a single medium. This would avoid the possible stress effects arising from the embryo being transferred between different culture environments, which may vary in their pH and osmolarity and in the levels of ions, energy substrates and other factors. As noted in an earlier context, the standard medium for IVM is TCM-199, either with or without serum supplementation. In France, Lonergan *et al.* (1994d) compared SOF with TCM-199 for the maturation of oocytes but found it to be less effective. At a later date, in Ontario, however, Watson *et al.* (2000) reported studies in which oocyte maturation with amino acid-supplemented synthetic oviduct fluid (cSOFMaa) resulted in blastocysts of a quality comparable to those matured in TCM-199 plus newborn calf serum; their results led them to conclude that this formulation of the SOF medium could be an effective base medium for cattle oocyte maturation. SOF was a medium originally based on the biochemical analysis of the ewe's oviductal fluid; over the years, SOF was changed by researchers by the addition of amino acids as well as other modifications, such as the addition of citrate, the removal of glucose and the addition of ethylenediamine tetra-acetic acid (EDTA).

In Indiana, using several modifications of SOF, Gandhi and Krisher (1999) and Gandhi *et al.* (2000) were able to demonstrate that SOF could be used successfully to mature and culture cattle oocytes and embryos, resulting in development comparable to that in a standard multiple-media system (see Table 4.8). However, for the practical implications of such work to be evaluated, it is necessary to have a great deal of information about the ability of IVP embryos to give rise to normal pregnancy rates and normal young.

Table 4.8. SOF formulation employed as a single culture system for bovine embryo production (from Gandhi *et al.*, 2000).

Reagent (mmol l^{-1})	SOFM (maturation)	SOFF (fertilization)	SOFS (sperm wash)	SOFCI (culture 1)	SOFC2 (culture 2)
Sodium pyruvate	0.33	0.33	1.0	0.33	0.33
Glutamine	1.0	–	–	1.0	1.0
Glucose	1.5	–	–	1.5	3.0
Sodium lactate	–	–	18.3	–	–
HEPES	–	–	12.5	–	–
Bovine serum albumin	–	6 mg/ml fatty acid free	3 mg/ml fraction V	8 mg/ml crystallized	8 mg/ml crystallized
EDTA	–	–	–	0.1	–
Taurine	–	–	–	0.1	–
MEM. NEAA	1x	2x	–	1x	1x
MEM. EAA	1x	–	–	1x	1x
MEM. vitamins	–	–	–	–	1x

MEM, Eagle's minimum essential medium; NEAA, non-essential amino acids; EAA, essential amino acids. Supplements added to base SOF medium for specific developmental stages.

4.8. Chemically Defined Culture Systems

The evaluation of culture media for IVM of cattle oocytes has steadily progressed over the past decade towards more defined conditions. It is now commonplace to use defined or semi-defined media in evaluating the effects of protein supplementation of the medium on maturation and subsequent embryonic development (Aurich and Hahn, 1994a; Tornesi *et al.*, 1995; Wright and Collins, 1995).

4.8.1. Using synthetic oviductal fluid (SOF)

A report by Calder *et al.* (1999) indicated that serum supplementation of SOF medium was not required; amino acid supplementation of SOF and culture under a 5% CO_2 atmosphere during IVM significantly enhanced blastocyst yield. A study by Ali and Sirard (2002a,b) evaluated the effect of FCS, BSA, purified BSA, essential fatty acid-free BSA and chicken-egg albumin as protein supplement during IVM in SOF. They also evaluated the replacement of protein with macromolecular-like polyvinyl pyrrolidone (PVP) or PVA. They found that SOF alone could support maturation and that embryos could be produced after oocytes were cultured in medium containing PVP; the authors were able to show that the effect of FSH on the maturation process was dependent on substrates present in the medium.

4.8.2. TCM-199

A study by Lonergan *et al.* (1994c,d) showed that TCM-199 could be regarded as a completely defined IVM medium capable of supporting the maturation of cattle oocytes in the absence of serum; they considered this to be an important step forward in the development of culture systems useful for studying the specific metabolic requirements of maturing bovine oocytes. Rose-Hellekant *et al.* (1998) modified a basic culture medium (TCM-199) to produce six media containing PVA, gonadotrophins, EGF and oestradiol as well as various energy substrates and amino acids. The authors drew several conclusions from their results: (i) the type of energy substrate or nutrient supplied during IVM affected the subsequent developmental competence of the oocytes; (ii) oocyte maturation in simple medium containing glucose with lactate or 11 amino acids or glutamine or lactate and glutamine can support development as readily as TCM-199; (iii) media supporting at least moderate cumulus-cell expansion during oocyte maturation also supported subsequent blastocyst development. A study by Palma *et al.* (1999) evaluated the effect of a defined maturation medium on subsequent embryo production;

TCM-199 was used in IVM, with or without OCS supplementation. The authors concluded from their data that maturation without protein did not adversely affect embryo yield. As noted earlier, in France, Marquant-Le Guienne *et al.* (1999) studied the effect of replacing serum with HA in their IVM medium; blastocyst yields and hatching rates with HA were comparable to those found with serum.

4.9. Oxidative Stress in Oocyte Maturation

Reactive oxygen species (ROS) production is a normal process of cell metabolism. *In vitro* environments usually increase cell production of ROS, which are often implicated as the main cause of cell damage. There is ample evidence that the ROS in oocyte-maturation and embryo-development culture affect the IVP of cattle embryos (Cetica *et al.*, 1998; Geshi *et al.*, 2000). Different metabolic paths, which are mediated by enzymes such as superoxide dismutase (SOD) and glutathione (GSH), control ROS cellular levels. GSH is a non-protein sulphydryl compound in cattle cells; it serves as a reservoir for cysteine. It is believed that the presence of glutamine (Gln) favours the utilization of cell metabolic paths that prevent or reduce the oxidative damage and influence subsequent embryo development.

4.9.1. Role of glutathione (GSH)

It is known that GSH synthesis during IVM of cattle oocytes plays an important part in subsequent fertilization and embryo development. It is also recognized that cysteamine in the IVM medium stimulates GSH synthesis and improves embryo development. Results reported by Iudica *et al.* (1999) in Argentina suggested that the addition of GSH (150 μg/ml) or Gln (150 μg/ml) to their maturation medium improved embryo development. In the same year, Furnus and De Matos (1999) in the same country presented evidence suggesting that in cattle oocytes the rate-limiting factor for GSH synthesis is cysteine; it should be noted that the constitutive amino acids involved in GSH synthesis are glycine, glutamate and cysteine (see Fig. 4.12).

Papers by De Matos and Furnus (2000) and De Matos *et al.* (1995, 1997, 2000a,b,c, 2002) served to emphasize the importance of inducing GSH synthesis during IVM; they concluded that the supplementation of bovine IVM medium with thiol compounds (β-ME, cysteine, cystine) to stimulate the synthesis of GSH is a useful method of increasing the efficiency of embryo production. The authors concluded that the high

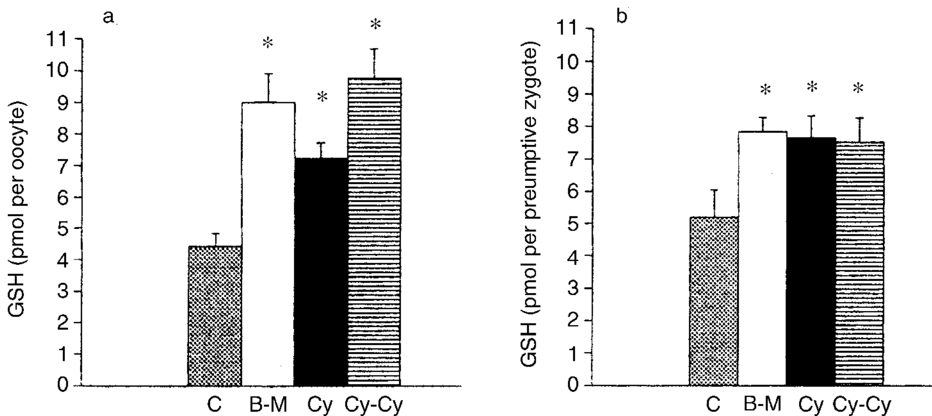

Fig. 4.12. Role of glutathione (GSH) in the *in vitro* maturation medium. (A) Intracellular glutathione levels after IVM of bovine oocytes, and (B) after IVF (presumptive zygotes), matured in IVM medium alone (control) (C) or supplemented with 100 μm β-mercaptoethanol (B-M), 0.6 mM cysteine (Cy) or 0.6 mM cystine (Cy-Cy). Bars with an asterisk (*) are significantly different from control ($P < 0.05$; mean ± SE). SE, standard error. (From De Matos and Furnus, 2000.)

intracellular GSH levels after the induction of GSH synthesis during IVM by thiol compounds remain during IVF and are still present at the start of embryo culture. In Japan, Hashimoto *et al.* (2000b) investigated the effect of varying levels of glucose in their maturation medium (SOF supplemented with 20 amino acids); they also examined intracellular ROS and the GSH content of oocytes; they found that excessive glucose (20.0 mM) in the medium impaired the development of oocytes to the blastocyst stage, possibly due to the increase in ROS and the decrease in the intracellular GSH content of the oocytes.

In Canada, Ali *et al.* (2001) examined the effect of cysteine, catalase and SOD on IVM and IVF of cattle oocytes. Significant improvements in the developmental competence of the oocytes were achieved when cysteine was added to the maturation medium but not when SOD or catalase was added. It was suggested that cysteine supplementation permeates the COCs during IVM, whereas antioxidants effective outside the cells (catalase) had no effect. A review by Nagai (2001) deals with evidence indicating that GSH is a crucial intra-oocyte factor for events after maturation in cattle.

Despite reports of positive effects, the role of ROS in oocytes in IVM culture systems has been controversial; in most cells, enzymatic antioxidant systems can attenuate the effect of oxidative stress by scavenging ROS. In Argentina, Cetica *et al.* (2001) found that antioxidant enzyme activity diminished in cumulus cells and increased in oocytes as a result of maturation; they concluded that oocytes might be capable of controlling the increase in ROS because of the action of their own enzymatic antioxidant system.

In other farm species, it is also now recognized that a major factor affecting *in vitro* oocyte and embryo welfare is increased oxidative stress. A study by Gasparrini *et al.* (2000) on the effect of cysteamine during IVM demonstrated that the addition of a thiol compound (such as cysteamine) to the medium improved embryo production efficiency. In pigs, it has been shown that the GSH content of matured pig oocytes is correlated with subsequent fertilization and developmental progress. In work reported by Jeong and Yang (2001) in the USA, the presence of cysteine in the maturation medium significantly improved blastocyst development. A study by Brad *et al.* (2002) showed that oocytes matured *in vitro* had lower levels of GSH compared with *in vivo*-matured pig oocytes; the authors suggested that there is a need for an improved IVM medium for pigs.

4.10. Two-step Culture Systems

The stepwise acquisition of developmental competence by the bovine oocyte during its progress through folliculogenesis is now well established. Until the recent decade, it was not fully appreciated that this acquisition included particularly important events that occurred in the days and hours immediately prior to the preovulatory LH surge.

4.10.1. Background information

In the cow, the ability of the oocyte to develop into a viable embryo is acquired gradually during its progressive differentiation throughout folliculogenesis, a process that culminates in a state of developmental competence, which some term 'oocyte capacitation', a state finally attained in the preovulatory follicle of the cow in the hours immediately preceding ovulation (see Fig. 4.13). In recent years, two-step culture protocols for oocyte maturation have appeared on the scene. Such protocols may have value in increasing the meiotic competence of cattle oocytes isolated from small antral follicles; they may also have relevance in attempts to bring about 'capacitation' of the oocyte in the way that apparently occurs *in vivo* in the final hours before ovulation of the preovulatory follicles. Although a culture period of 24 h is sufficient for the completion of nuclear maturation of bovine oocytes, cytoplasmic maturation may not occur in this time period in a way optimal for subsequent embryo development (see Dieleman *et al.*, 2002).

It is clear, after more than a decade of research that many cattle oocytes fail to become fully competent after conventional IVM, despite the use of a wide range of culture techniques. There appears to be a missing link in the chain of events that occurs during cytoplasmic

maturation when oocytes are matured *in vitro* rather than in the live cow. It is evident that the bovine oocyte undergoes various changes during the final days and hours before ovulation that are not adequately simulated in the 24 h maturation treatments currently employed. The need is for attention to be focused on follicular signalling and mechanisms involved in the acquisition of full developmental competence (see Fig. 4.14). For such reasons, there may be a case for the artificial reversible arrest of nuclear maturation, providing the oocyte with sufficient time and opportunity for certain crucial changes. To that end, culture systems using follicular components or specific cell cycle inhibitors are currently being used by researchers in attempts to improve oocyte quality.

4.10.2. Maintenance of meiotic arrest

Although the oocytes of certain mammalian species, such as the mouse, can undergo GVBD in the absence of protein synthesis, the oocytes of other species, including the cow, require active protein synthesis for meiosis to be resumed (Tatemoto and Horiuchi, 1995). It is

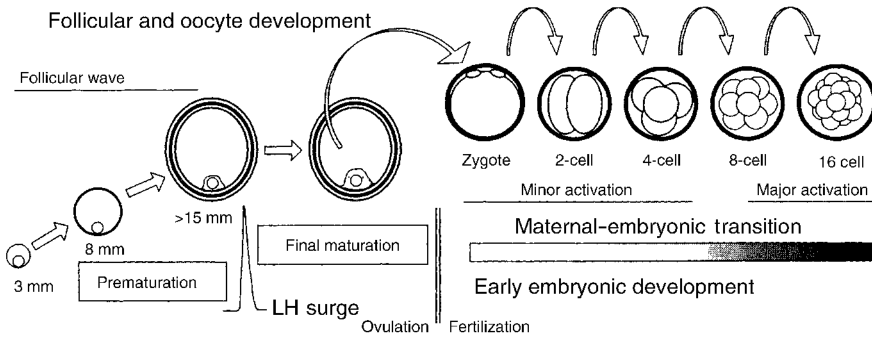

Fig. 4.13. Prematuration essential for developmental competence (based on Dieleman *et al.*, 2002).

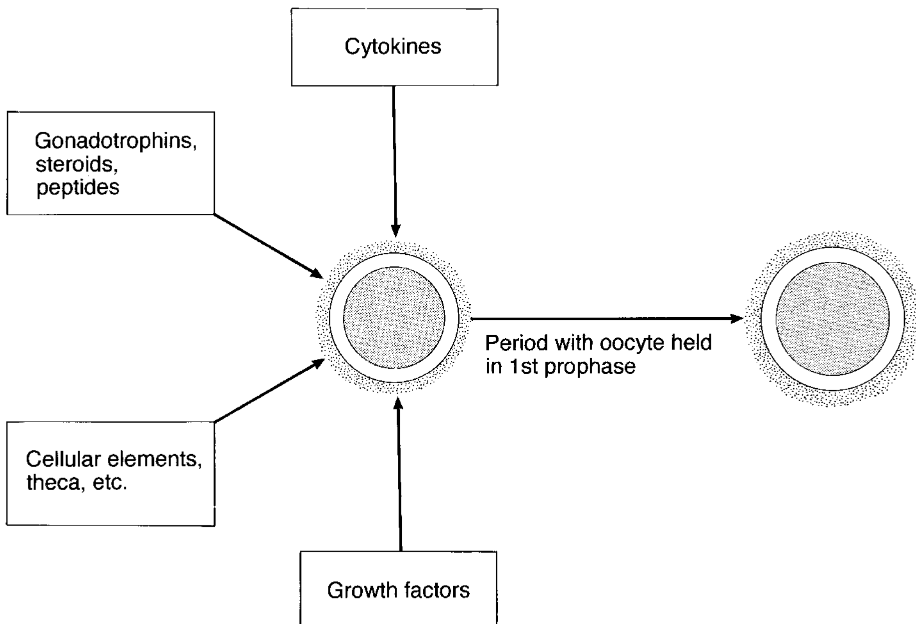

Fig. 4.14. A pre-IVM treatment for improving bovine oocyte quality.

well established that follicle-enclosed oocytes do not resume meiosis, either *in vivo* or *in vitro*, in the absence of appropriate signals. The implication is that there is an inhibitory agent(s) within the antral follicle responsible for maintaining the bovine oocyte in the GV stage; a range of substances, including nucleotides, gonadotrophins, steroids, peptides, amino acids and growth factors have been implicated.

Cattle oocytes

It is known that bovine oocytes are arrested at the G2/M border of the first meiotic division (GV stage) and that the arrest at this stage is determined by the status of the MPF, which is composed of two subunits (p34cdc2 kinase and cyclin B). Studies by Levesque and Sirard (1996) sought to identify the protein initiating meiosis; they found that the limiting factor to the appearance of MPF was the accumulation of cyclins. Wu, Bin *et al.* (1996a,b, 1997) examined the dynamics of MPF activity and concluded that *de novo* synthesis of p34cdc2 kinase and cyclin B takes place during maturation.

Several other groups working with cattle oocytes have reported investigations dealing with factors regulating meiotic resumption (Carbonneau and Sirard, 1994; Chian and Sirard, 1996; Rose-Hellekant and Bavister, 1996; Tatemoto and Terada, 1996a; Evans *et al.*, 1997; Sirard and Coenen, 1997; Sirard *et al.*, 1998) and MII arrest (Dominko and First, 1996). Reviews by Dekel (1996) and Whitaker (1996) dealt with various biochemical events that are known to participate in the control of meiosis in mammalian oocytes.

4.10.3. Biological inhibitors

Almost a half-century ago, Chang suggested that FF may contain an oocyte maturation inhibitor. In more recent times there has been evidence of a heat-stable polypeptide in FF that maintains meiotic arrest in cattle oocytes; this factor is apparently derived from the somatic cells of the follicle and released into the FF. It is believed that the structural integrity of the bovine COC and the mural granulosa-cell layer is an important factor in the maintenance of this arrest.

Influence of granulosa–theca cells

In some mammalian species, granulosa cells are known to exhibit meiosis-arresting activity and under certain IVC conditions are capable of maintaining bovine oocytes in the GV stage for up to 24 h. The same granulosa cells are also known to be essential for the normal maturation of the bovine oocyte. Such apparently contradictory observations are explicable on the basis of the concentration of granulosa cells employed *in vitro*. Inhibition does not appear until cattle oocytes are co-cultured with high concentrations of cells; lower cell concentrations appear to enhance the quality of the oocyte during the maturation process.

Cell-to-cell contact between the COC and the granulosa cells is known to be important in enhancing the inhibition of meiosis in a dose-dependent manner and it is also apparent that the culture of granulosa cells together with theca cells serves to augment the meiosis-arresting effect (Kotsuji *et al.*, 1994). In the Netherlands, De Loos *et al.* (1994) demonstrated that contact with the follicular wall was capable of maintaining meiotic arrest in bovine oocytes and similar evidence came from Canadian studies in which oocytes were co-cultured with follicular hemisections (Sirard and Coenen, 1995; Richard and Sirard, 1996a). There is also evidence from Canadian work that theca cells can maintain the inhibition of meiosis in cattle oocytes without direct contact with the COC (Richard and Sirard, 1996b); under similar conditions, granulosa cells proved incapable of such an effect. Other work has evaluated the effects of cAMP modulators on the ability of the theca cells to maintain meiotic arrest in bovine oocyes (Richard *et al.*, 1996); results suggested that cAMP isomers could not reverse the effect of theca cells.

A study reported by Yada *et al.* (1999) examined the role of theca- and granulosa-cell interaction during the development of the cow's ovarian follicle; it was concluded that theca cells secrete factors that inhibit the differentiation of immature while promoting that of matured granulosa cells, and that granulosa cells secrete factors that promote both the differentiation and growth of theca cells during follicle development. In Utrecht, a study by van Tol and Bevers (2002) attempted to characterize one of the factors secreted by theca cells; they concluded that the

factor that inhibits FSH-induced resumption of meiosis in COCs attached to membrana granulosa cells is a small, stable, polar molecule that is not a peptide.

It is known that all components of the renin–angiotensin system are present in the reproductive system and that angiotensin II (Ang-II) is present in the mammalian ovary and has been correlated with oocyte maturation, ovulation and follicular atresia. The way in which Ang-II can mediate the meiosis-inhibiting effect of theca cells was demonstrated in Brazil, where Giometti *et al*. (2002) investigated the role of Ang-II in bovine oocyte nuclear maturation. Cattle COCs were cultured with or without theca cells in the presence or absence of Ang-II; they showed that Ang-II blocked the inhibitory effect of the theca cells.

4.10.4. Biochemical inhibitors

As well as cyclic nucleotides, there are several other chemically defined factors that are known to delay or prevent the resumption of meiosis in bovine oocytes cultured *in vitro*. These inhibitors include hypoxanthine, steroid hormones, such as oestradiol, androstenedione and progesterone, inhibitors of phosphodiesterase, such as 3-isobutyl-1-methylxanthina (IBMX) and theophylline and inhibitors of protein synthesis, such as cycloheximide and puromycin.

Role of cyclic 3, 5′-adenosine-monophosphate (cAMP)

Spontaneous resumption of meiosis *in vitro* can be reversibly inhibited if bovine oocytes, on release from their follicles, are cultured in a medium containing membrane-permeable analogues of cAMP or agents that serve to increase intracellular cAMP concentrations. There is the view that cumulus cells hold the oocyte in the GV stage by transferring inhibitory molecules to it, such as cAMP, via the gap junctions linking the two cell types. In the preovulatory follicle, in response to LH, cAMP levels are elevated. Such increased concentrations of the nucleotide affect the cumulus cells and interrupt the communications within the COC; under such conditions, it is believed that the flow of cAMP to the oocyte declines, inhibition is decreased and meiosis resumes. As noted by Richard *et al*. (1997), the fact that the intracellular level of cAMP may be implicated in the process of meiotic resumption in cattle oocytes has been recognized for some time. In contrast to rodents, high levels of cAMP exert only a transient inhibition of GVBD in cattle oocytes; for that reason, compounds that influence cAMP levels in oocytes, such as hypoxanthine, are not suitable.

Studies reported from Canada by Mayes (2000) produced evidence suggesting that cattle oocytes have an active type 3 phosphodiesterase (PDE) enzyme, which, when inhibited, prevents meiotic resumption through increased levels of cAMP; they also showed that the inhibitory effect of theca cell monolayers on meiotic resumption is enhanced by type 3 PDE inhibitors (cilostamide, milrinone, rolipram).

Experiments reported by Guixue *et al*. (2001) in Italy aimed to use cAMP to induce differentiation in bovine oocytes and their cumulus cells without inducing arrested meiosis; results suggested that the intracellular cAMP concentration during the interval between oocyte isolation from the follicle and the start of IVM may be critical in ensuring optimal developmental competence.

Manganese

Work by Bilodeau-Goeseels (2001) in Canada examined the effects of manganese concentration on the nuclear maturation of bovine COCs and denuded oocytes; it was concluded that manganese chloride inhibited GVBD but only the effect of the lowest concentration tested (50 μM) was reversible.

4.10.5. Pretreatment of donor cattle

Providing the bovine oocyte with a longer period for its development to greater competence is now known to be possible in dealing with the donor animal prior to its slaughter; this may yield many useful clues about the factors that need attention in the prematuration treatment of oocytes collected from abattoir ovaries. A review by Sirard (2001) makes it clear that to obtain competent oocytes it is necessary to stimulate

the ovaries with exogenous hormones before recovering the oocytes; he notes that, when the mechanisms by which the follicle induces oocyte competence are understood, the appropriate conditions may be simulated *in vitro*. This will require arrested-meiosis culture conditions for the oocytes and the appropriate signals to re-create the microenvironment of the dominant/ preovulatory follicle.

Clear improvements in oocyte developmental competence have been achieved by appropriate pretreatment of donor cattle, either by ovarian stimulation with FSH followed by slaughter of the animal and the incubation of ovaries before oocyte aspiration or by the transvaginal collection of oocytes from FSH-stimulated cattle followed by a 'coasting period' of 48 h.

4.10.6. Two-step treatment in the laboratory

Although the culture period of 24 h has been regarded as sufficient for the completion of nuclear maturation of cattle oocytes in conventional IVM systems, it is now clear that cytoplasmic maturation may not have reached the stage optimal for full developmental competence. This has led researchers to suggest that the artificial reversible arrest of nuclear maturation may provide the bovine oocyte with sufficient time for the generation of vital factors necessary for further development. Although the molecular events responsible for oocyte developmental competence are not known, it seems possible that transcripts and proteins are synthesized and stored in a stable form during this time period (see Fig. 4.13). The required reversible arrest may be achieved by culture systems using either follicular components or specific cell cycle inhibitors. In the first step of the culture system, the GV-arrested oocytes would remain in the transcriptionally active state and synthesize dictyate-stage-specific proteins; in the second step, meiotic maturation would resume and continue to metaphase II.

Although studies with rodent oocytes have shown that GVBD can be reversibly blocked by several biochemical inhibitors, mainly agents that increased or sustained high intracellular levels of cAMP, such inhibitors were not suitable for cattle. Although papers by Farin and Yang (1994) and Martus and Farin (1994) showed that 5, 6-dichloro-1-β-D-ribosfuranosylbenzimidazole (DRB), a reversible inhibitor of RNA polymerase II, was effective in preventing GVBD in cattle oocytes, the agent had to be replaced at frequent intervals, which made its use rather impracticable. It has also been shown that the addition of cycloheximide or 6-dimethylaminopurine (6-DMAP) to the culture medium may inhibit GVBD effectively, but that this had an adverse effect on the developmental potential of arrested oocytes after fertilization (Lonergan *et al.*, 1997, 1998). In Denmark, Avery *et al.* (1998b) studied the developmental competence of cattle oocytes arrested with 6-DMAP for 24 h; exposure to the agent before IVM induced asynchronous cytoplasmic maturation, which adversely affected the fertilization process. In Brazil, working with zebu oocytes, Dode and Adona (2001) found that 6-DMAP had a reversible effect on the maintenance of meiotic arrest but reduced embryo yield markedly and significantly.

Roscovitine

Much more promising candidates for use in two-step culture systems have been the new generation of specific cell cycle inhibitors. In France, Mermillod *et al.* (2000a,b) tested the ability of roscovitine, a potent inhibitor of M-phase-promoting factor (MPF)-kinase activity to maintain cattle oocytes at the GV stage for a 24 h culture period; they showed for the first time the feasibility of culturing oocytes under meiotic inhibition without decreasing their subsequent developmental potential (see Table 4.9). The French workers found a high reversibility after culture for 24 h, using a concentration of 25 μM; at the end of this period, most oocytes remained arrested. After washing and culture for a further 24 h in a permissive medium (TCM-199 supplemented with EGF), almost all the oocytes completed meiosis to MII; cleavage rates, blastocyst yields and cell numbers were similar in treated and control groups. In the USA, McCann *et al.* (2001) reported that the culture of cattle oocytes with roscovitine for 48 h after recovery from follicles did not compromise the subsequent development of embryos after maturation and fertilization.

In Canada, Calder *et al.* (2001a,b) found that roscovitine-induced inhibition of cattle oocyte maturation was enhanced in BSA-supplemented media and that progression to MII was accelerated after the oocytes were released from the inhibition; the authors noted that it may require an earlier fertilization time. As observed by Avery *et al.* (2002), prior to the *in vivo* maturation of the bovine oocyte in the cow's preovulatory follicle, there is probably a period of 4–5 days of 'capacitation' during which there is synthesis and modification of a large number of different molecules, such as RNA and proteins. The workers attempted to evaluate the distribution of mitochondria after using roscovitine to arrest meiotic resumption for periods of 24–48 h; they found that the ooplasm of inhibited oocytes collected from all follicle sizes (2 to > 6 mm diameter) mimicked the ooplasm appearance of OPU oocytes from larger follicles.

One report that has shown evidence of an enhancement of developmental competence of cattle oocytes in a two-step culture system is that of Gomez *et al.* (2002) in Spain (see Table 4.10). They employed a treatment in which oocytes were pretreated with retinoic acid while they were arrested with roscovitine. Retinoic acid was used on the basis of evidence showing that blastocyst yield in superovulated cattle and sheep was improved by retinol treatment of donor animals. The Spanish study was able to demonstrate that the presence of retinoic acid during a 24 h pretreatment period improved embryonic development, cryopreservation tolerance and embryo quality (as assessed by cell number).

Butyrolactone I

In Ireland, Lonergan *et al.* (2000b) showed that butyrolactone I (BL-I), a potent inhibitor of cyclin-dependent kinases, inhibited meiotic resumption in cattle oocytes by blocking GVBD in a dose-dependent manner; a concentration of 100 μM arrested > 60% of oocytes, while 150 μM inhibited almost all oocytes compared to the control in which > 80% resumed meiosis. After the second 24 h culture period under conditions permissive to normal maturation,

Table 4.9. Culturing cattle oocytes under roscovitine meiotic inhibition (from Mermillod *et al.*, 2000).

			Blastocysts			
Treatment	*n*	% Cleaved	% Day 7	% Day 8	% Day 9	Cell number
Control	284	82 ± 8	23 ± 6	34 ± 2	41 ± 4	130 ± 41
Roscovitine	293	90 ± 7	18 ± 5	31 ± 8	36 ± 7	122 ± 61

Cleavage, development and cell numbers in blastocysts (means ± SD) from oocytes matured either directly (control) after collection or after 24 h culture in the presence of roscovitine at 25 μM for inhibition of meiosis. No significant difference between treatments.
SD, standard deviation.

Table 4.10. Effect of retinoic acid on meiotically inhibited bovine oocytes (from Gomez *et al.*, 2002).

(RA) PM	(RA) M	*n*	Morulae	Blastocysts day 6	Blastocysts day 8	Blastocysts expanded	Re-expansion 4 h	Post-warming 72 h
(–)	(+)	215	31.1 ± 5.1^{c}	4.9 ± 1.3^{c}	19.3 ± 3.1^{b}	11.5 ± 3.7^{b}	77.5 ± 13^{a}	12.5 ± 12
(+)	(+)	211	28.5 ± 3.6^{c}	6.9 ± 1.2^{bc}	20.3 ± 2.9^{b}	11.8 ± 1.7^{b}	50.0 ± 22	0.0^{b}
(+)	(–)	225	40.5 ± 3.9^{b}	11.8 ± 3.3^{ab}	28.2 ± 3.3^{a}	21.3 ± 2.3^{a}	75.0 ± 7^{a}	21.9 ± 7^{a}
(–)	(–)	199	29.3 ± 4.4^{c}	5.7 ± 2.3^{c}	18.0 ± 2.5^{b}	13.5 ± 3.3^{b}	44.4 ± 16	5.5 ± 5
No	(–)	205	54.9 ± 3.7^{a}	15.2 ± 1.3^{a}	32.7 ± 3.2^{a}	25.0 ± 1.8^{a}	39.0 ± 13^{b}	11.5 ± 6

Development data are percentages from matured oocytes ± SEM (six replicates) and from vitrified/warmed embryos. ANOVA and Duncan's test. Different superscripts show significant differences: ^{a,b,c}($P < 0.05$).
SEM, standard error of the mean; ANOVA, analysis of variance; RA, retinoic acid; PM, prematuration medium; M, maturation medium.

almost all (95%) of arrested oocytes resumed meiosis and progressed to MII. In terms of developmental competence, oocytes maintained in meiotic arrest for 24 h with 100 μM exhibited a similar capacity to develop to the blastocyst stage as non-blocked control oocytes following maturation, fertilization and culture *in vitro*. It was concluded that it is feasible for cattle oocytes to be maintained in artificial meiotic arrest without compromising their subsequent developmental competence; this may represent a tool for improving the development of less competent oocytes. Elsewhere, similar results showing full reversibility associated with normal fertilization rates and a low incidence of polyspermy after using BL-I were reported (Imai *et al*., 1999; Kubelka *et al*., 2000a,b; Motlik *et al*., 2000). In pigs, studies reported by Wu *et al*. (2002) examined the effects of BL-I on porcine oocytes in a serum-free maturation system; they found the agent to be a potent inhibitor and the inhibition to be fully reversible.

In Italy, Ponderato *et al*. (2001) used a combination of roscovitine (12.5 μM) and BL-I (6.25 μM) to block GVBD in cattle oocytes; they recorded 93% of oocytes at the GV stage during a 24 h culture period and 92% resuming meiosis and reaching MII in the second 24 h period. In agreement with data reported by Calder *et al*. (2001a), who used roscovitine only, the authors of the Italian study found that MII kinetics was different for inhibited and control oocytes; 50% of treated oocytes reached MII at 13–14 h in contrast to 18 h in controls. This led them to fertilize inhibited oocytes at 16 h, which resulted in a significantly better outcome than fertilizing at 22 h. Using cryopreservation as a test of viability, they noted that the outcome was similar for both groups. A study by Faerge *et al*. (2001) used transmission electron microscopy to assess the nuclear ultrastructure of cattle COCs after 8 h of meiotic inhibition with roscovitine, butyrolactone and other inhibitors. In Japan, Hashimoto *et al*. (2002) investigated the effects of BL-I on the developmental competence of bovine oocytes following their release from meiotic arrest; they showed that blastocyst yield was significantly higher in oocytes arrested in fetal blood serum (FBS)-supplemented medium under 5% oxygen (37%) than in oocytes arrested in other ways (5–24%) or matured directly after aspiration (23%). They also showed that BL-I-treated oocytes started GVBD and reached the MII stage about 6 h earlier than non-arrested oocytes; it was concluded that the oocytes had acquired higher developmental competence during the period of their meiotic arrest.

In Belgium, Genicot *et al*. (2002) found that mitochondrial activity in cumulus cells was reduced when prematuration of bovine COCs was attempted with BL-I; they suggest that this could impair cytoplasmic maturation and subsequent embryonic development after fertilization. Other work in the same laboratory showed that prematuration of calf oocytes with meiotic inhibitors (BL-I and roscovitine) resulted in a lower developmental potential subsequently, something that was not found in oocytes from adult animals (Donnay and Verhaeghe, 2002).

In Japan, Anas *et al*. (2001) examined the effect of different doses of mono-(2-ethylhexyl)-phthalate (MEHP) in their TCM-199 maturation medium; the percentage of oocytes reaching MII was significantly reduced. In testing the reversibility of the inhibitory effect of MEHP, oocytes were cultured for 24 h before being transferred to the IVM medium and cultured for a further 24 h; the authors concluded that MEHP can reversibly arrest bovine oocyte maturation *in vitro*.

Other farm animals

In other farm animals, there have been attempts to develop two-step maturation culture systems. A study in France by Marchal *et al*. (2001) examined the effectiveness of roscovitine in preventing meiotic resumption in porcine oocytes and tested their subsequent fertilization and developmental competence; they found that a concentration of 25 μM was sufficient to prevent meiosis in 93% of oocytes after 22 h in the presence of EGF and FSH. The authors concluded that roscovitine treatment could be useful in studying the final stages of maturation in pig oocytes. A report by Pimentel *et al*. (2002) dealt with a study aimed at verifying whether a delay of meiotic resumption by treatment with roscovitine would increase the developmental competence of equine oocytes; horse COCs were maintained in IVM medium supplemented with 100 μM roscovitine for 24 h, followed by an additional 24 h in maturation medium alone. The treatment was apparently effective in

blocking meiosis without compromising the subsequent development of the oocytes.

Elsewhere, results reported by Franz *et al.* (2002a,b) in Colorado also showed that roscovitine could be used to maintain equine oocytes in the GV stage for up to 24 h without decreasing their developmental potential; in this study, where oocytes were classified by cumulus morphology, roscovitine suppressed meiosis in oocytes with compact but not with expanded cumulus-cell morphology. In Missouri, Wu *et al.* (2002) demonstrated that pig oocytes could be held at the GV stage with BL-I and still retain high developmental competence; the authors concluded that BL-I treatment may prove useful in producing high-quality oocytes for embryo production, cloning and transgenic research.

4.10.7. Enhancing the quality of oocytes from small follicles

It is possible that agents such as BL-I may have a useful role to play in improving the meiotic and developmental competence of those bovine oocytes that are isolated from small follicles. A study reported by Pavlok *et al.* (2000) has shown that cattle oocytes isolated from 1–2 mm diameter follicles, which rarely mature to MII in the conventional IVM culture systems, became more competent when incubated with BL-I for 48 h. During their subsequent culture for 24 h under conditions permissive for normal maturation, more than 80% of the oocytes matured to MII, in contrast to 30% in the control group; changes typical of oocytes coming from larger follicle classes could be observed in the BL-I-treated oocytes. It seems possible that prolonging the period of RNA and protein synthesis in oocytes collected from small antral follicles may contribute to the acquisition of greater developmental competence. This type of two-step culture treatment may have useful practical implications in utilizing a greater number of antral follicles recovered from abattoir ovaries.

4.10.8. Synchronizing germinal vesicle development

A paper by Park, K.W. *et al.* (1999) in Japan reported that bovine oocytes recovered from larger-diameter follicles were at a more advanced stage of GV development than those from smaller follicles. They devised a classification scheme for the nuclear morphology of bovine oocytes at the GV stage, identifying five stages based on the chromatin and nucleolus stages (see Table 4.11). They showed that GV morphology differed among immature oocytes, with those from larger follicles being at relatively later stages; only 6% of oocytes from small follicles (1.0–1.9 mm diameter) were at the GV-V stage compared with 46% from follicles 4–6 mm diameter. It is possible that immature cattle oocytes that have advanced to the GV-V stage have a higher developmental competence when they are matured and fertilized. It was also shown that co-culture of oocytes with theca cells was a good method of promoting their progress to GV-V. The authors suggest that a two-step culture system designed first to promote progress to the GV-V stage and then to promote meiotic maturation may be a method for improving the developmental competence of oocytes.

Table 4.11. Stages of germinal vesicle (GV) development based on changes in the nuclear morphology of bovine oocytes (based on stages shown by Park, K.W. *et al.*, 1999).

Stage	Nuclear morphology
A	GV-I, nuclear membrane and nucleolus are distinctly visible; condensed filamentous chromatin clumps visible around the nucleolus and a few are situated near the nuclear membrane
B	GV-II, filamentous chromatin clumps are localized mainly around the nucleolus
C	GV-III, filamentous chromatin clumps are evenly distributed around the nucleolus and the nucleolus has disappeared completely
D	GV-IV, chromatin is condensed into thick clumps
E	GV-V, chromatin is condensed into a single clump; the nuclear membrane is still visible

4.11. Other Factors Influencing Oocyte Maturation

There are numerous factors, hormonal and otherwise, that have been used in maturation media and reported on by researchers. It should be appreciated that the importance of such factors can only be evaluated in the context of the culture conditions employed.

4.11.1. Energy sources and second messengers

Glucose

A study reported by Chung *et al.* (2002) in Canada sought to verify the effect of glucose on oocyte maturation and subsequent fertilization and embryonic development, using a bovine model; they showed that oocyte maturation rates and blastocyst yields were significantly higher in the presence of glucose, using a chemically defined maturation medium. Such results confirmed the importance of glucose, which is a constituent of regularly used maturation media, such as TCM-199.

cAMP and analogues

The second messenger, cAMP, is a differentiating factor found in follicular cells and oocytes. In pigs, where there may be a large variation in the meiotic progression of oocytes cultured *in vitro*, Funahashi *et al.* (1997) found that the exposure of COCs to dibutyryl cAMP for the first 20 h of culture increased the homogeneity of nuclear maturation and improved the efficiency of embryo production. In China, Shi and Lu (1996) showed that the addition of dibutyryl cAMP (100 μM) to the maturation medium increased the proportion of oocytes developing to blastocysts in comparison with the control medium (46.5 vs. 38.5%). In a study reported by Luciano *et al.* (1999), the intracellular concentration of cAMP in cattle COCs was manipulated by the addition to the collection and maturation medium of invasive adenylate cyclase (iAC). They found that iAC has a biphasic effect on oocyte maturation. High concentrations in the IVM medium inhibited the resumption of meiosis, whereas low concentrations (0.1 or 0.01 μg/ml) resulted in high rates of maturation to metaphase II. The same low concentrations of iAC resulted in either similar or significantly higher blastocyst yields. It was concluded that maintenance of an optimal intracellular concentration of cAMP before and during IVM could ensure a high level of developmental competence of cattle oocytes matured in a medium without serum and hormones. Further studies reported by Guixue *et al.* (2001) attempted to induce differentiation of the early maturing oocyte in conditions of meiotic arrest or during normal maturation. Results supported the view that intracellular cAMP concentration during the interval between oocyte isolation from the follicle and the start of IVM is critical for achieving optimal developmental competence; the study appeared to confirm the importance of the priming period with iAC and held out the possibility of replacing FSH–LH and oestradiol by cAMP only.

4.11.2. Hormones and vitamins

Prostaglandins and steroids

The effect of prostaglandins on the maturation of cattle oocytes was the subject of a study in Portugal reported by Marques *et al.* (1997); the reported evidence suggested that prostaglandin E_2 (PGE_2) may stimulate the resumption of meiosis by antagonizing other arachidonate metabolites or by directly enhancing the meiotic competence of treated oocytes.

Although it is well established that cattle oocytes require a specific steroid environment to achieve full maturation and developmental competence in the live animal, limited information is available on the effects of steroids on *in vitro* oocyte maturation. In Reading, Silva and Knight (2000) examined the effects of androgens, progesterone and their antagonists on cattle oocytes; they also measured concentrations of total α-inhibin, inhibin A, activin A and follistatin in a COC-conditioned medium to assess any possible influence of progesterone and androgens on the secretion of inhibin-related peptides by cumulus cells. They found that the addition of progesterone or the anti-progestin mifepristone to COCs did not affect cleavage rate but that progesterone had a marked and significant adverse effect on blastocyst yield (40%

reduction), which was only partially reversed by mifepristone. It appeared that the negative effect of progesterone on blastocyst yield may be mediated by increased inhibin α-subunit expression by cumulus cells.

Retinoic acid

It is known that retinol and its metabolites, especially retinoic acid, play an important part in controlling events during the cell cycle. There have also been reports of retinol increasing blastocyst yield in superovulated cattle and sheep. In Spain, Duque *et al.* (2002) examined the effects of retinoic acid and vitamin A on maturation; they found evidence that retinoic acid improved the development and quality of blastocysts. In the USA, Livingston *et al.* (2002) reported studies that demonstrated that supplementation of 5 μM retinol in the IVM medium (modified synthetic oviductal fluid (mSOF)) improved the developmental competence of bovine oocytes; they also noted that similar supplementation of the embryo culture medium may result in higher-quality blastocysts.

4.11.3. Opioid antagonists and chemical agents

Endogenous opioid peptides

It has been shown that endogenous opioid peptides regulate follicle development in pigs and are involved in meiotic resumption in rodent oocytes. In Italy, Albrizio *et al.* (2000) demonstrated dose-dependent effects of naloxone, an opioid antagonist, on the maturation rate of cattle oocytes *in vitro* and the presence of the μ-opioid receptor transcript in the COC. A further study by Dell'Aquila *et al.* (2000) examined the effects of β-endorphin added to the maturation medium; from their results, they concluded that endogenous opioid peptides play a role in cattle oocyte maturation and that their effects may be mediated by the presence of gonadotrophins (FSH/LH) and oestradiol.

Dimethylsulphoxide and ethanol

A report by Tsuzuki *et al.* (1998b) in Japan dealt with the effects of dimethylsulphoxide (DMSO) on the maturation and fertilization of cattle oocytes matured in TCM-199. Using a dose of 50 μM led to a significant increase in the maturation rate (85% vs. 67%) and the same dose levels significantly increased blastocyst yield when added to both maturation and culture media. In Denmark, on the other hand, where Avery and Greve (2000) examined the effect of small doses of DMSO and ethanol on IVM, either after spontaneous resumption of meoisis or where it was inhibited with 6-DMAP or IBMX; they concluded that even small doses of DMSO or ethanol caused profound negative effects on oocyte maturation and subsequent embryo development.

Selenium

A paper by Elhassan and Wright (1995) investigated whether selenium and vitamin E have a beneficial antioxidant or toxic effect on the fertilization and early development of artificially matured oocytes; they concluded that the addition of these antioxidants to the maturation medium was not necessary. A study reported by Bowles and Lishman (1998) involved the culture of cattle COCs in media containing insulin (10 μg/ml), selenium (10 ng/ml) and/or transferrin (10 μg/ml). The addition of selenium to the IVM medium significantly improved maturation rate, fertilization rate and the yield of blastocysts. The authors concluded that embryo production could be improved by adding selenium to the maturation medium and transferrin to the culture medium.

4.11.4. Simplifying maturation culture systems

Many factors are relevant to the effectiveness and cost of the oocyte maturation systems currently used. The incubation system, which usually requires an expensive piece of equipment, is one area that has received attention, with some efforts being directed towards reducing the expense associated with that part of the embryo production process. In work reported by Palma *et al.* (1998a,b), IVM was performed in a bag system, in which four-well Nunc dishes were placed in sterile polyethylene bags, which

were then filled with a mixture of 5% CO_2 plus 95% synthetic air; the authors concluded that maturation and other stages (IVF and IVC) could be successfully carried out in the bag system. An alternative method that was claimed to be successful involved IVM in 10 ml polystyrol tubes placed in a water-bath (Olivier *et al.*, 1998a,b). In Japan, Suzuki *et al.* (1997, 1999) reported on the development of a simple, portable CO_2 incubator for embryo production; COCs were matured (22 h), fertilized (5 h) and cultured (7 days) using effervescent granules in amounts suitable for generating a CO_2 atmosphere comparable to that in their standard CO_2 incubator. A negative air pressure resulted in a significantly higher blastocyst yield than a positive pressure.

In a study reported by Avery *et al.* (2000a), they describe the ability of a standard CO_2 Heraeus incubator and the Oxoid CO_2 Gen atmosphere-generating system to support IVM, IVF and embryo culture. The Oxoid CO_2 Gen gas-generating system, originally designed for the growth of bacteria, is based on the chemical reaction of ascorbic acid and air; when a sachet with ascorbic acid is placed in the confined volume of the AnaeroJar, an atmosphere of 6% CO_2 in 15% oxygen is created, which is comparable to the 5% CO_2 and 20% oxygen used for conventional IVM of cattle oocytes. The authors concluded that the Oxoid system was a cheap, convenient and stable alternative to expensive CO_2 incubators for the production of cattle embryos.

4.12. Evaluating the Maturation Process

Various studies have dealt with ways of assessing the developmental competence of cattle COCs after they have undergone IVM; the emphasis is on trying to make available objective tests that can identify factors in the oocyte that are crucial for its continued progress through fertilization and early embryonic development.

4.12.1. Stages in nuclear maturation

During maturation of the bovine oocyte, chromatin structures within the immature cell proceed through a series of morphological arrangements, starting at prophase of the first meiotic division and continuing to metaphase of the second division.The various features associated with the mature cattle oocyte after the surrounding cumulus cells are removed are illustrated in Fig. 4.15. Early work in Ireland showed that 80–90% of oocytes reached maturity by 24 h after initiating maturation culture; the nuclear membrane disappeared after 5–6 h (GVBD) and MI was reached after about 12 h. Reports in the literature from other laboratories showed a range of times for the occurrence of the peak period for MII, varying from about 20 h to 30 h; such differences were presumably the result of variations in the

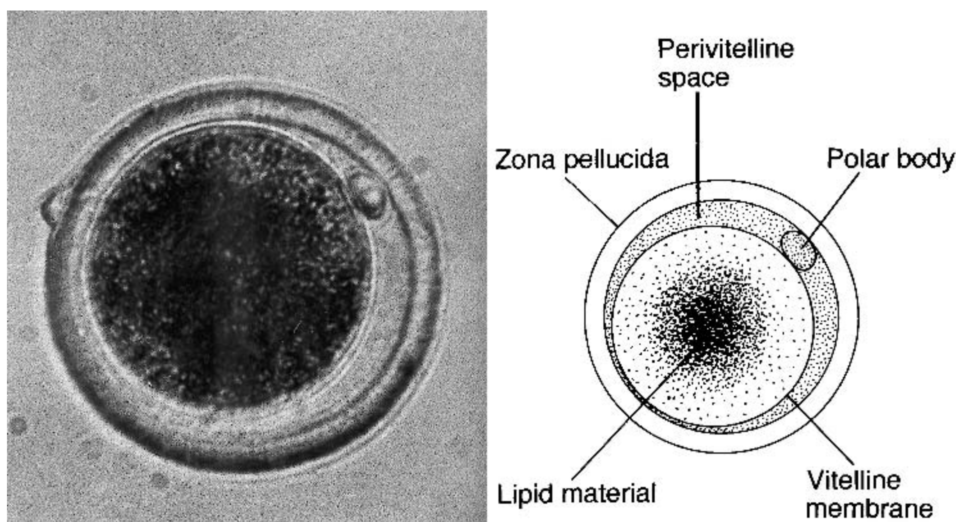

Fig. 4.15. The mature bovine oocyte.

culture systems employed. In Ireland, an effect was evident due to the presence or absence of a hormone supplement in the IVM medium; a delay in the resumption of meiosis was recorded when oocytes were cultured with gonadotrophins, possibly the result of an increase in the intracellular cAMP level.

4.12.2. Cumulus-cell expansion

Maturation of the bovine oocyte *in vitro* involves well-defined changes in the cumulus cells that surround it; the cumulus cells expand to form a spherical mass in three dimensions and the COC appears to float in the culture dish. In the live animal, such changes are part of the process whereby the COC separates itself from the follicle wall just prior to ovulation; the stickiness may be important in ensuring the pick-up of the COC by the ciliated epithelium of the fimbriated infundibulum of the oviduct. An article by Greve and Callesen (2001) contains the observation that the mucification of *in vivo*-produced cattle COCs is much more pronounced than their IVP counterparts.

A typical classification scheme employed in assessing the degree of cumulus cell expansion and mucification is given in Table 4.12. It is believed that cumulus cells are stimulated by gonadotrophin (FSH) and growth factor (EGF) to produce and secrete HA, which results in the expansion process; cells of the membrana granulosa within the ruptured follicle do not undergo expansion but become luteal cells in the subsequent formation of the corpus luteum.

In assessing oocyte maturation, due regard is paid to the degree of cumulus-cell expansion in the belief that this is a reflection of physiological normality. Certain of the constituents of the maturation medium may influence cumulus cell expansion, glutamine, which is a constituent of TCM-199, being an example. Studies by Furnus *et al.* (1997, 1998b) in Argentina indicated that the optimal concentration of glutamine in TCM-199 for cumulus cell expansion should be 2 mM rather than the 1 mM concentration normally present in this medium; the authors suggest that the higher concentration of glutamine may enhance the fertilizability and developmental competence of cattle oocytes. In Yorkshire, Danfour *et al.* (2002) found cumulus cell mass and expansion to be good predictors of blastocyst potential after IVF in an embryo production system in which single oocytes were cultured in a serum-free maturation medium. In France, Nuttinck *et al.* (2002) used an immunodetection procedure to demonstrate that cyclooxygenase-2 (cox-2) expression in cumulus cells may be involved in the differentiation of cattle COCs that occurs during oocyte maturation. In Brazil, Cordeiro *et al.* (2002) evaluated protein synthesis by cumulus cells during IVM; their results indicated a change in the pattern of protein synthesis in the cells during maturation and that some proteins were stage-specific.

Mitochondrial distribution

The pattern of mitochondrial distribution may be a useful indicator of developmental competence in cattle oocytes. The mitochondria in GV-stage cattle oocytes are generally arranged in a cortical distribution, but relocate during IVM. According to Bavister (2000), two distinct patterns of mitochondria distribution may be observed in mature oocytes, depending on the IVM culture conditions. After maturation of oocytes in a medium with glucose and amino acids, which supports a high proportion of

Table 4.12. Assessing bovine oocytes after maturation: classification scheme for assessment of bovine oocytes after maturation, based on the degree of cumulus-cell expansion (from Hunter and Moor, 1987).

Grade 1: full cumulus cell expansion	This is characterized by the extremely adhesive nature of the cumulus cells and the enlargement of the cumulus cell mass to at least $\times$ 3 diameters (> 300 μm) away from the zona pellucida
Grade 2: moderate cumulus cell expansion	This is where the observed expansion of the cumulus cells would be of the order of $\times$ 2 diameters (> 200 μm) away from the zona pellucida
Grade 3: slight expansion of cumulus cells	This category would be characterized by cumulus cells being tightly adherent to the zona pellucida

blastocyst development after IVF, mitochondria became located mainly in the centre of the oocyte; in GV oocytes matured with glucose and lactate, on the other hand, which have poor embryo development after IVF, mitochondria are usually located in the cortical region. It would appear that the distribution pattern and location of mitochondria are correlated with the oocyte's developmental competence. In Denmark, Avery *et al.* (2002) used confocal microscopy to evaluate the normality of the distribution of mitochondria in roscovitine-inhibited oocytes.

4.12.3. Morphological assessment and staining methods

Many morphological and ultrastructural features have been described by workers that may prove useful in assessing the normality or otherwise of the maturation process in cattle. After IVM of the bovine oocyte, the cell is surrounded by an expanded cumulus, there are changes in the dimensions of the perivitelline space, expulsion of the first polar body into that space and the establishment of the second metaphase spindle tangential to the surface of the vitelline membrane. It has been observed that the first polar body may not always be in evidence in oocytes that have a chromosomal configuration of MII; this does not necessarily indicate an abnormality. A cytogenetic study of the artificially matured bovine oocyte was reported by Ectors *et al.* (1995).

Various workers have described changes occurring in the oocyte of the live cow in organelle structure and location in relation to the occurrence of the preovulatory LH surge. One of the first effects visible is the formation of the perivitelline space, with the simultaneous loss of intercellular contact between the cytoplasmic processes of the corona radiata cells and the ooplasm. In the course of maturation, there is a migration of vesicles, mitochondria and lipid droplets to the centre of the oocyte, resulting in an almost organelle-free cortical region characterized by the presence of nests of smooth endoplasmic reticulum (SER) and cortical granules beneath the vitelline membrane. Nucleolar changes at the ultrastructural level have been described for cattle oocytes matured both *in vivo* and *in vitro*.

In any discussion of oocyte evaluation, it must be emphasized that normality cannot be assured by any morphological examination. As enunciated by Cambridge workers in the 1970s, the only valid guide to complete oocyte maturation was the capacity of the oocyte to develop after fertilization to at least the blastocyst stage of development. The same workers stressed the importance of not using earlier cleavage stages as a measure of normal maturation, noting that many abnormalities induced during culture are not detectable until later in embryonic development. It is also now clear that evaluating the normality of both oocyte and embryo calls for a much more sophisticated approach than mere morphological examination; emphasis now is upon detecting the expression of genes that indicate their normality or otherwise.

4.12.4. Gene expression and oocyte competence

Studies reported by Robert (1999) in Canada have demonstrated the presence of BCL-X and Bax mRNA in bovine COCs; the author notes that the level of pro-apoptosis mRNA versus the anti-apoptosis mRNA may be important for the survival of the embryo subsequently. It is known that, in cattle, the maternal RNA stock is the only source of RNA to be translated during the initial stages of embryo development; the RNA stock in the COC is what may decide the fate of the embryo after fertilization. A further report by Robert *et al.* (2000) dealt with the isolation of specific messengers from granulosa cells in the COCs of competent oocytes, using cDNA library subtraction and differential screening; markers of developmental competence may be useful in improving the selection of cattle oocytes for use in embryo production.

5

Capacitating Bovine Sperm

5.1. Introduction

The production of sperm by the bull (spermatogenesis) is a lengthy process, occurring in the testes, whereby stem cell spermatogonia at the base of the seminiferous tubules divide by mitosis to maintain their own numbers and to cyclically produce primary spermatocytes, which undergo meiosis to produce haploid spermatids, which differentiate into spermatozoa released into the tubular lumen. The three major divisions of spermatogenesis, which are characterized by the development of spermatogonia (spermatocytogenesis), spermatocytes (meiosis) and spermatids (spermiogenesis), each occupy about one-third of the total duration of sperm production. The bull's sperm is a small, highly condensed cell, which is only about one-twenty-thousandth the volume of the bovine oocyte.

When sperm are produced in the bull's testes, they are immobile; it is only after they reach their storage site, the epididymis, that they acquire the ability to move forwards (progressive motility), an ability that is crucial for successful fertilization. The spermatozoa do not actually move in the epididymis; they become motile only after ejaculation or when diluted in a suitable medium. It is possible that, within the epididymis, sperm signalling mechanisms are primed, but the sperm remain inactive during storage, ready for activation when appropriate (see reviews by Mortimer, 1997; Fazeli *et al.*, 2000). After ejaculation and as they enter the isthmus of the cow's reproductive tract, bull sperm slow down and resume their migration only when ovulation occurs; eventually they reach the ampullary region of the oviduct, where fertilization takes place. As well as acquiring the ability to move forward, sperm must undergo a further important maturation process, termed capacitation.

5.1.1. Historical

The phenomenon of capacitation was first recognized and described independently by Austin in Australia and Chang in the USA in 1951; they demonstrated that rabbit sperm finally become competent to penetrate the mature oocyte only after having spent some time in the female tract. Of historical interest in cattle *in vitro* fertilization (IVF) are the reports of Iritani and associates in Japan in the late 1970s; they examined the possibility of capacitating bull sperm in the isolated bovine oviduct and uterus and in the reproductive tract of the live rabbit.

Much is now known about the cellular mechanisms involved in the control of bovine sperm function. Bull spermatozoa are highly specialized cells that are incapable of fertilizing the cow's oocyte until they have undergone a series of biochemical changes resulting in the state of 'capacitation'. Capacitation is the term used to cover various events, which include the expression of a hyperactivated pattern of motility and the acquisition of a capacity to respond to unique signals emanating from the oocyte. It is

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believed that a glycoprotein constituent of the zona pellucida (ZP3) acts both as a recognition site for bull sperm and as a physiological trigger, stimulating the sperm to undergo an exocytotic event known as the 'acrosome reaction'. The second messengers generated as a result of sperm–ZP3 interaction include an influx of extracellular calcium and cytoplasmic alkalinization. The components of the sperm plasma membrane responsible for recognizing ZP3 and transducing the recognition signal remain unclear at this time.

It is clear from the information emerging in recent years that the cow's reproductive tract plays a far more complex role in controlling sperm function, particularly in modulating motility and fertilization, than has hitherto been appreciated; a greater understanding of the oviduct's role is likely to help in improving cattle embryo production systems.

5.1.2. The capacitation process

Capacitation is now clearly recognized as a process involving the spermatozoon in a complex series of biochemical and physiological reactions (Breitbart *et al.*, 1995). It is also now known that the capacitation process can be reversed by re-exposure of the capacitated sperm to seminal plasma. The vesicular glands of the bull are known to secrete a variety of proteins that bind to the surface of sperm at the time of ejaculation. There is evidence that capacitation is involved in the release of bull sperm from the oviductal epithelium, thereby enabling sperm to leave the sperm reservoir (at the uterotubal junction) and progress towards the site of fertilization in the ampullary section of the oviduct (Lefebvre and Suarez, 1996). It is believed that the lipid changes that occur in the plasma membrane of the spermatozoon during capacitation are related to the intrinsic membrane properties, such as permeability, adhesiveness and fusibility. In cattle, as in other mammals, the role of calcium ions is essential for the reaction to occur (Mahmoud and Parrish, 1996). A paper by Dragileva *et al.* (1999) in Israel suggests that cytosolic Ca^{2+} is actively transported into the acrosome by an ATP-dependent Ca^{2+} pump and that the accumulated Ca^{2+} is released from the acrosome via an inositol-1, 4, 5-triphosphate (IP_3)-gated calcium channel. It is believed that the intracellular Ca pump is active during capacitation and that this pump may have a role in regulating intracellular calcium ions during capacitation and the acrosome reaction.

It is known that large numbers of leucocytes colonize the uterus and that these cells can produce considerable quantities of hydrogen peroxide and other reactive oxygen species (ROS) that are toxic to sperm; in the oviduct, however, it has been shown that oviductal fluid contains a catalase activity that prevents decreased sperm motility (Lapointe *et al.*, 1998). A study by O'Flaherty *et al.* (1999) examined the influence of ROS on capacitation and the acrosome reaction in frozen–thawed bull sperm; they concluded that ROS is required in the capacitation process and that hydrogen peroxide may participate as an inductor of the acrosome reaction. A review by Medeiros *et al.* (2002a) notes that the ROS-generating mechanism of sperm has not been characterized; the effects of ROS are prevented or diminished by the detoxifying enzymes superoxide dismutase (SOD), catalase, peroxidases and reducing agents, such as glutathione, ascorbic acid, taurine and hypotaurine, present in sperm cells and in their microenvironment. A recent commentary on semen quality by Aitken and Marshall Graves (2002) notes that an important mechanism by which DNA damage is induced in the male gamete is oxidative stress; spermatozoa are particularly vulnerable to this because they generate ROS and are rich in targets for oxidative attack. The authors also draw attention to the fact that, because spermatozoa are transcriptionally inactive and have little cytoplasm, they are deficient in both antioxidants and DNA-repair systems. Oxidative stress may be a cause of male infertility and contribute to DNA fragmentation in sperm.

Based on their studies with epididymal sperm and bovine seminal plasma (BSP), Therien *et al.* (2001) have proposed the sequence of events that is set out in Fig. 5.1. According to this, at ejaculation sperm are exposed to seminal-fluid (BSP) proteins, contributed by the seminal vesicles. During this brief exposure, BSP proteins remove a significant amount of cholesterol accompanied by the release of some phospholipids. This lipid efflux may slightly destabilize the sperm membrane (priming). At the same time,

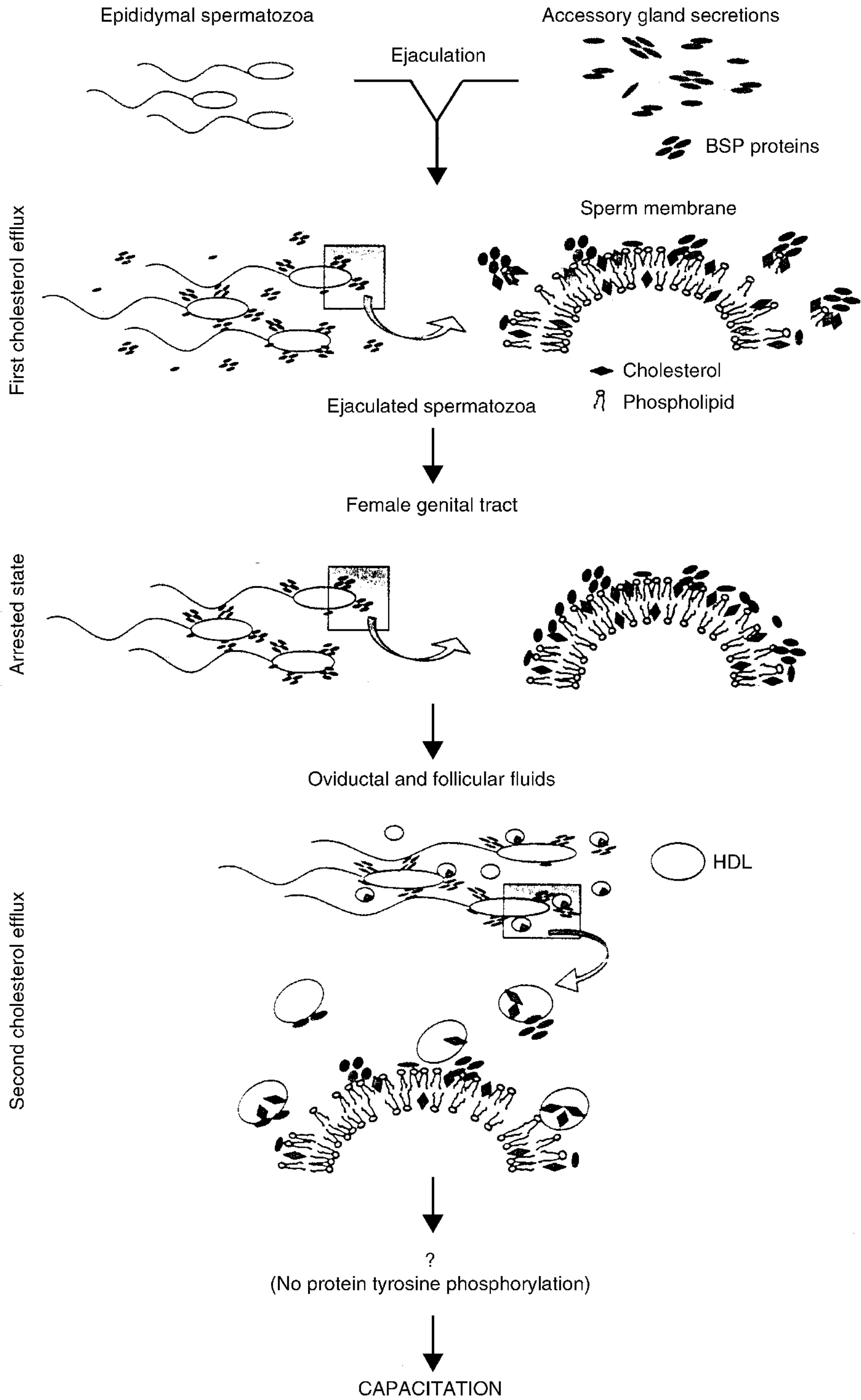

Fig. 5.1. Mechanism of sperm capacitation by bovine seminal plasma proteins and high-density lipoprotein. HDL, high-density lipoprotein. (From Therien *et al.*, 2001.)

BSP proteins coat the sperm surface via their interaction with choline phospholipids; this coating of BSP proteins prevents free movement of phospholipids and thereby stabilizes the sperm membrane (arrested state).

In the second step, the oviduct/follicular fluid high-density lipoprotein removes BSP proteins from the sperm membrane. As a result, sperm membrane lipids are free to move; in addition, high-density lipoprotein may induce a second efflux of cholesterol. Since cholesterol is known to have an important stabilizing effect on membranes, its efflux would probably provoke further destabilization of the membrane and trigger certain unknown signal-transduction pathways. Such events could regulate the surface expression of sperm zona pellucida (ZP) receptors; the adhesion to the ZP would then trigger the acrosome reaction. It may also be noted that factors in seminal plasma influence the motility of bull sperm, as well as taking part in the capacitation process. Studies reported by Henricks *et al.* (1998) in South Carolina were able to identify insulin-like growth factor I (IGF-I) in BSP and its receptor in the acrosomal region of the spermatozoon. They also showed that treatment of bull sperm with IGF-I or IGF-II increased motility and straight-line velocity; the conclusion was that the IGF system may be involved in fertilization in the cow.

5.1.3. Hyperactivation

Hyperactivated movement was first reported by Rudi Yanagimachi in hamster sperm more than 30 years ago; he suggested that the vigorous beating of flagella played a vital part in the penetration of the ZP, a view subsequently confirmed by workers elsewhere (Stauss *et al.*, 1995). It is also possible that hyperactivated sperm may be more effective at penetrating oviductal mucus and the cumulus matrix in the live animal.

The changing motility patterns shown by bovine sperm during capacitation have been described by various workers; accelerated progressive sperm movement was detected after incubation of bovine sperm with ampullary oviductal fluid (Grippo *et al.*, 1995). It is also known that ram sperm exhibit hyperactivated motility under capacitating conditions; in Australia, Mortimer *et al.* (1999) determined the characteristics of flagellar beat pattern in sperm of that species. Hyperactivated motility of the bovine spermatozoon is characterized by high-amplitude, asymmetrical flagellar movements. It has been shown that Ca^{2+} is essential for the initiation and maintenance of sperm hyperactivated motility; data reported by Ho and Suarez (2001a) in Cornell suggested that there is an IP_3 receptor (IP_3R)-gated Ca^{2+} store in the neck region of the bull spermatozoon that plays a role in regulating hyperactivated motility. It is also believed that the vigorous movement of sperm requires ATP and cyclic adenosine monophosphate (cAMP) (see review by Ho and Suarez, 2001b).

Evidence supports the view that Ca^{2+} is the most important factor regulating hyperactivation and that cAMP is required but is not sufficient for expression of hyperactivation; a hypothetical model proposed by Ho and Suarez (2001b) for the regulation of hyperactivation is shown in Fig. 5.2. In the USA, Ren *et al.* (2001) discovered a channel that allows calcium ions to enter the sperm cell.

5.1.4. The acrosome reaction

The acrosome reaction involves multiple fusions of the outer acrosomal membrane with the overlying plasma membrane, a reaction that allows the dispersal of acrosomal contents, which is essential for penetration of the ZP (see Fig. 5.3); the acrosomal enzymes enable the spermatozoon to cut a narrow cleft in the zona matrix, sufficient for the gamete to gain access to the oocyte plasma membrane (vitelline membrane; oolemma), to which it fuses, triggering the events that constitute fertilization. A study by Dalvit *et al.* (1995) indicated that the presence of oxidative substrates is required to produce acrosome reactions; the acrosome reaction is believed to occur in response to certain stimuli. According to Tesarik (1996), two physiological acrosome-reaction inducers are known; progesterone and ZP3; despite their molecular differences, both inducers can apparently activate the same signal-transduction pathways.

Successful induction of the acrosome reaction can be assessed either by oocyte penetration *in vitro* or by way of differential staining

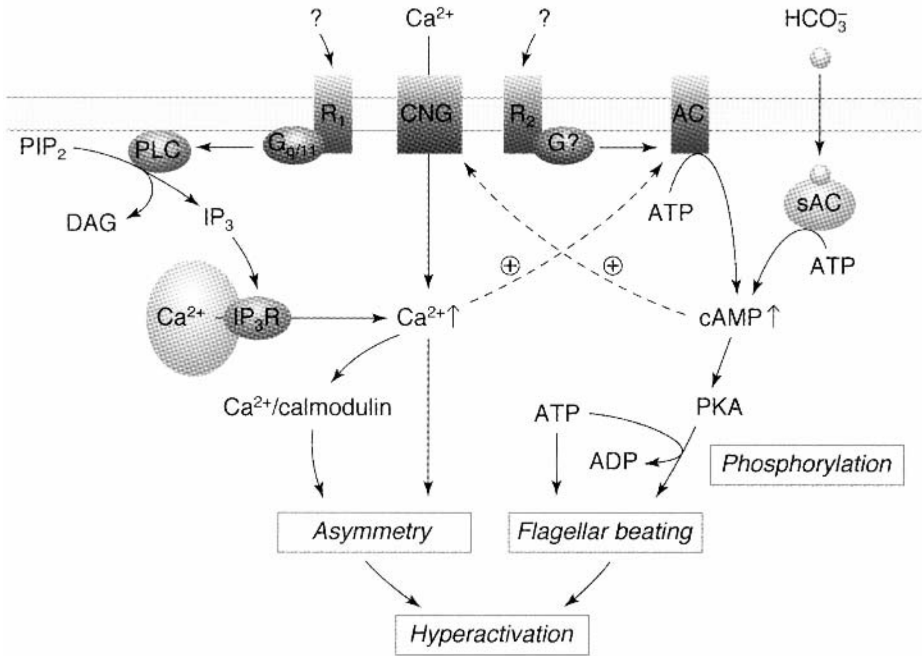

Fig. 5.2. Regulation of hyperactivation in bovine sperm (from Ho and Suarez, 2001b).

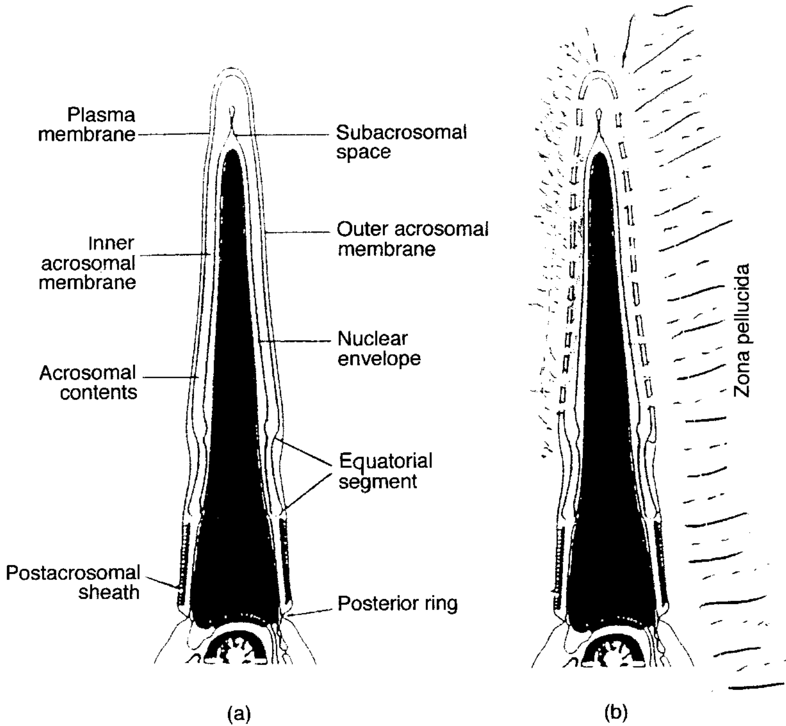

Fig. 5.3. Schematic drawing of the bovine acrosome reaction: (a) intact sperm; (b) sperm during fusion of the plasma and outer acrosomal membranes.

techniques. The acrosomal status of bull sperm was evaluated by Nishikimi *et al.* (1997) using a lectin (concanavalin). A study by Khorasani *et al.* (1999) sought to determine the effects of exogenous progesterone and cholesterol on the zona-binding ability of bovine sperm and the outcome of IVF; it is known that the acrosome reaction can be induced by progesterone and that cholesterol can inhibit this effect. The authors concluded that progesterone treatment of bull sperm prior to fertilization may improve the outcome of IVF. Protein kinase C (PKC) has been implicated in the acrosome reaction. In Israel, Garbi *et al.* (2000) demonstrated the induction of the acrosome reaction and activation of sperm PKC-α by lysophosphatidic acid, which is known to induce signal transduction cascades in many cell types by binding to specific cell-surface receptors.

In Germany, Pereira *et al.* (2000) studied the effect of heparin, caffeine and calcium-ionophore A23187 on the induction of the acrosome reaction in frozen–thawed bull sperm; a 30 min incubation of sperm with calcium ionophore resulted in a 20% acrosome reaction. In Georgia, Dinkins and Brackett (2000) demonstrated that previously cryopreserved bull sperm could be capacitated in chemically defined conditions devoid of heparin or other biological components. In Madison, Parrish and Susko-Parrish (2001) have shown that calcium increases in the anterior bovine sperm head during capacitation; their results suggested that calcium is accumulated in the acrosome during capacitation and the more calcium accumulated, the faster the acrosome reaction in response to the ZP.

5.1.5. Artificial induction of capacitation

Many reports appeared in the 1980s dealing with efforts to achieve capacitation of bovine sperm, using either chemical agents or biological fluids; some of these are detailed in Table 5.1

Table 5.1. Approaches to the artificial capacitation of bovine sperm *in vitro*.

Year	Method	Researcher(s)
1982	High ionic-strength (HIS) medium	Brackett *et al*
1983	Bovine follicular fluid	Fukui *et al.*
1984	Standard ionic-strength medium	Iritani *et al.*
1984	Heparin	Parrish *et al.*
1985	Elevated pH	Cheng
1985	Ionophore A23187	Hanada
1986	Liposomes	Graham *et al.*
1988	Percoll gradient/ hypotaurine	Utsumi *et al.*
1988	Caffeine	Niwa *et al.*
1989	TEST yolk	Ijaz and Hunter
1989	Oviductal-cell monolayer	Guyader *et al.*

5.2. Capacitation in the Cow

In the cow, the time required for capacitation of ejaculated sperm *in vivo* has been estimated as about 6 h, based on the first penetration of oocytes after artificial insemination (AI); many factors in the bovine reproductive tract that are involved in sperm transport and capacitation remain to be identified and defined.

5.2.1. Sperm transport

The movement of bull sperm through the oviduct is known to be carefully regulated, so that relatively few sperm reach the site of fertilization (ampulla) at any one time; this may be regarded as a safeguard against the possibility of polyspermic fertilization. There has been a suggestion that sperm transport may differ between the two oviducts of the cow according to the site of ovulation; apparently the oviduct ipsilateral to the cow's ovulating ovary may exhibit greater muscular and secretory activity than the contralateral tube. A knowledge of the mechanisms involved in sperm migration through the cow's reproductive tract is necessary in understanding how the capacitation process may affect such mechanisms.

Of the countless millions of sperm released into the cow's reproductive tract at mating, only thousands pass through the uterotubal junction into the oviductal isthmus, where they form a reservoir (Fig. 5.4). Oviductal sperm reservoirs have been found in cattle and other mammalian species; in the cow, the reservoir is evidently formed when bull spermatozoa bind to ligands on the surface of oviductal epithelium. In pigs,

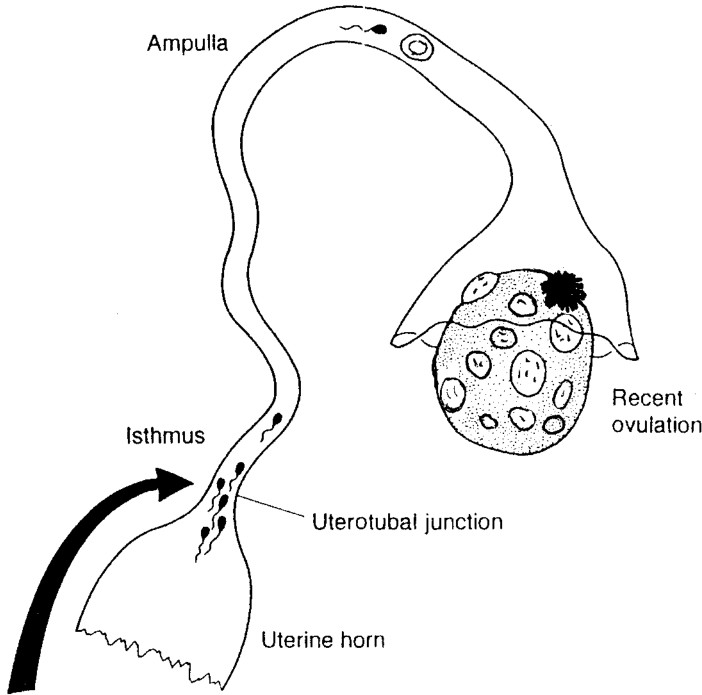

Fig. 5.4. Sperm reservoir in the isthmus and site of fertilization in the cow.

Petrunkina *et al.* (2001a,b) suggested that the attachment of boar sperm to oviductal epithelial cells might represent a mechanism for selecting functionally competent sperm and prolonging their lifespan by delaying capacitation in the oviductal reservoir. Similarly, adhesion to oviductal cells is believed to select high-quality sperm and to be responsible for extending the fertile life of bovine sperm. Sperm binding to oviductal epithelium is believed to be an important mechanism regulating sperm reservoir formation in the isthmus; various studies suggest that capacitation is involved in the loss of sperm capacity to bind to the epithelial cells and could be responsible for sperm release from the reservoir (Lefebvre and Suarez, 1995; De Pauw *et al.*, 2000; Gualtieri and Talevi, 2000a,b, 2001; Boilard *et al.*, 2001; Ignotz *et al.*, 2001a,b). Such studies have demonstrated: (i) only acrosome-intact sperm bind to specific oviductal epithelial cells; (ii) acrosomes of bound sperm are preserved intact over time; and (iii) release of unreacted sperm is likely to be due to changes of the sperm surface, probably triggered by capacitation.

There is evidence that the binding of bull sperm to oviductal epithelium is mediated by a seminal-plasma protein that has been identified as PDC-109 (Gwathmey *et al.*, 2001; Ignotz *et al.*, 2001a,b; Suarez and Ignotz, 2001); these workers have examined the role of PDC-109 and suggest this protein plays an important role in creating the sperm reservoir in cattle.

5.2.2. Oviductal secretory cells

Fertilization occurs in the oviduct of the cow's reproductive tract; the oviduct and the fluid it contains provide the microenvironment appropriate for sperm motility, capacitation, final oocyte maturation, fertilization and early embryo development (Lauer *et al.*, 1995; Buhi, 1996, 1998; Boatman, 1997; Lefebvre *et al.*, 1997; Boilard, 1999). A paper by Anderson and Killian (1994) concluded that isthmic secretions may play a major role during *in vivo* sperm capacitation, given that bull spermatozoa may reside in the oviduct isthmus for up to 18 h

before fertilization. It is known that oviductal fluid is a complex mixture of various constituents, some derived from blood plasma, together with certain specific proteins that are synthesized by the oviductal epithelium (King and Killian, 1994; Lapointe *et al*., 1994; Abe *et al*., 1995a,b; Lapointe and Sirard, 1995, 1996; Remy *et al*., 1995; Rodrigues and Killian, 1995; Staros and Killian, 1995, 1997, 1998; Way and Killian, 1995; Hill *et al*., 1997; Medeiros and Parrish, 1997; Suriyasathaporn *et al*., 1997; Van Langendonckt *et al*., 1997b; Vansteenbrugge *et al*., 1997; Walter, 1997; Way *et al*., 1997; Martus *et al*., 1998a,b; Rodrigues and Killian, 1998; Roh *et al*., 1998, 1999a; Boquest and Summers, 1999; Boquest *et al*., 1999; Wijayagunawardane *et al*., 1999a,b; Reischl *et al*., 2000; Boilard *et al*., 2002).

It is clear, from many reports, that the secretions of oviductal cells may be influenced by the stage of the oestrous cycle and by the particular region of the oviduct under examination. A report by Gabler *et al*. (1999), for example, demonstrated that an acidic glycoprotein, osteopontin, which functions in cell adhesion and other activities, is synthesized by the oviduct and that the relative amounts of different forms of osteopontin in oviductal fluid vary with oviduct region and stage of cycle. In Italy, Talevi and Gualtieri (2000) reported preliminary data indicating that cleavage rates achieved after IVF in co-culture with bovine ampullary monolayers were significantly higher than those achieved in co-culture with isthmic monolayers; it appeared that the fertilization rate was enhanced by the ampullary monolayer. The same authors noted that the bovine model might be useful in studying the role of the oviduct in human sperm selection and fertilization. In the Netherlands, Sostaric *et al*. (2002) presented evidence suggesting that endocrine changes around the time of ovulation induced a decrease in sperm binding; the same workers noted that oviduct explants were superior to cell cultures in studying the sperm-binding process.

There is research interest in the role of carbohydrates in the fertilization process. Carbohydrates attached to proteins on the surface of many types of cells act as important recognition markers; it is believed that a specific carbohydrate recognition system is in place on the isthmic oviductal epithelium. This system, which is thought to include the monosaccharide fucose, is recognized by a lectin carried by the bull spermatozoon. Evidence reported by Lefebvre *et al*. (1997) indicated that the attachment of bull sperm to the oviductal mucosa may be mediated by such a system. A study by Grippo *et al*. (2000) sought to test the hypothesis that free monosaccharides are available in oviductal fluid and that their concentrations and profiles change with the stage of cycle; their data, however, led them to conclude that bovine fluid does not contain fucose or similar monosaccharides at any time of the cycle.

Among growth factors known to be present in oviductal fluid are fibroblast growth factor 1 and 2 (FGF-1 and FGF-2), IGF-I and vascular endothelial growth factor (VEGF). A report by Viuff *et al*. (1994) supported the view that platelet-derived growth factor (PDGF-B) and basic fibroblast growth factor (bFGF) are synthesized and released into the lumen of the cow’s oviduct where they exert regulatory functions on early embryonic development. As shown in Fig. 5.5, members of the IGF family, including the ligands IGF-I and II, the receptor type I and binding proteins, are known to be expressed in the bovine oviductal epithelium and in the early-cleavage embryo (Winger *et al*., 1997a; Watson *et al*., 1999b); VEGF enhances the *in vitro* maturation of oocytes and accelerates the development of early embryos (Einspanier *et al*., 1999). A study by Gabler *et al*. (2001) found evidence suggesting that, especially around the time of ovulation, there is a well-balanced protease and inhibitor system within the bovine oviduct, which protects the gametes and the developing embryo from proteolytic degradation in the oviductal lumen.

In the Czech Republic, Slavik and Fulka (1999) provided evidence suggesting that the properties of the ZPs of ovulated and *in vitro*-matured cattle oocytes are not identical and may be modified by contact with oviductal fluid from oestrous animals; they showed that most (70%) *in vitro*-matured cattle oocytes were penetrated by ram sperm, in contrast to a small proportion (38%) when oocytes were matured in the presence of bovine oviductal fluid. It appeared that oviduct cell secretions contributed to the establishment of a species-specific barrier preventing penetration of cattle oocytes by ram sperm.

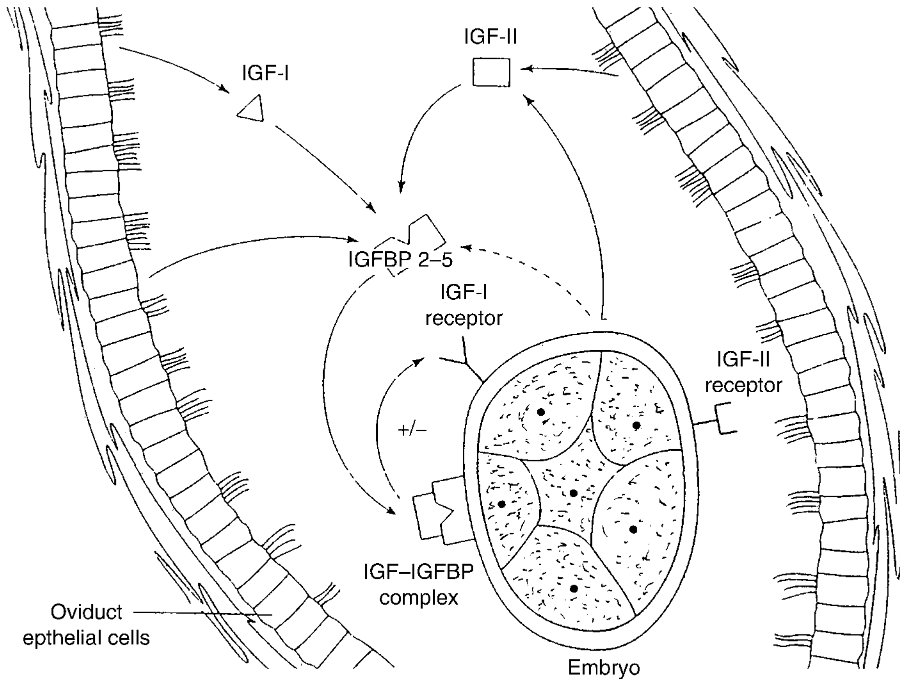

Fig. 5.5. Maternal and embryonic IGF circuits. Detectable amounts of immunoreactive IGF-I and IGF-II are released from primary bovine oviduct cell cultures, and detectable amounts of IGF-II are released from bovine blastocysts. Bovine oviduct primary cultures express transcripts encoding IGFBPs 2–5 and release IGFBPs 2–5 into conditioned media. Bovine zygotes express mRNAs encoding IGFBPs 2–4 through to the blastocyst stage. mRNAs encoding IGFBP-5 have been detected in bovine blastocysts. The detection of IGF-I, IGF-II and IGFPBs 2–5 in the culture environment indicates that IGF paracrine and autocrine regulatory circuits are present and may contribute to the events that regulate bovine early development. (From Watson *et al.*, 1999b.)

5.2.3. Glycosaminoglycans

Glycosaminoglycans (GAGs) are reputed to be among the most intricate structures in living organisms; they include heparin, heparan sulphate, chondroitin sulphate, dermatan sulphate, hyaluronic acid and keratan sulphate. The most familiar member of the GAGs is heparin, discovered as far back as 1916, whose action in the prevention of blood clotting has long been widely recognized. Techniques currently available permit the physiochemical and biological properties of GAGs to be examined in detail.

GAGs that have been identified as effective inductors of capacitation *in vitro* are known to be abundant in the follicles of the cow and to be released by the ovulating follicle into the ampullary region of the oviduct at the time of ovulation. Dealing with molecules involved in sperm adhesion and release from bovine epithelial cells, Gualtieri and Talevi (2000a,b) note that heparin-like GAGs are present in the female reproductive tract and their concentration varies, reaching a peak during the periovulatory phase, which may well enable them to act simultaneously as inducers of sperm release from the oviduct and enhancers of the fertilization process. In the USA, Bosch *et al.* (2001) found evidence that heparin was able to promote sperm displacement from oviductal epithelial-cell attachment; they suggested that such sperm were likely to be the most competent ones.

5.2.4. Simulating oviductal events *in vitro*

There have been many studies *in vitro* showing the effect of oviductal cells on various aspects of sperm function. A study by Ijaz *et al.* (1994) of

in vitro-cultured oviductal cells showed that they secreted factors that supported and maintained sperm viability and motility up to 30 h. The fertilizing ability of bull sperm co-cultured with oviductal epithelial cells was studied by Chian *et al.* (1995a,b); they showed that such cells secrete a capacitating factor into the medium. Studies reported by Chian and Sirard (1995a,b) showed that sperm capacitation was induced by attachment to bovine oviductal epithelial monolayers and that sperm attachment to the ampullary epithelium improved capacitation. They also suggested that isthmus epithelial cells are important in maintaining sperm motility and that attachment of sperm in the isthmus may reduce the number of sperm reaching the ampullary region in the live animal. It appears that oviductal cells can induce capacitation of bull sperm under various hormonal conditions; isthmus oviductal cells secrete sperm-motility-maintaining factors under the influence of oestrogen and ampullary cells do so under the influence of progesterone (Lapointe *et al.*, 1995). In Australia, a study by Boquest *et al.* (1999) examined the effect of bovine oviductal proteins on bull sperm; they suggested that proteins secreted by the oviduct promote sperm viability, delay the acrosome reaction and suppress sperm motion. Other work reported from Queensland that year showed that pretreatment of oviductal cells with oestradiol-17β prolonged the motility of bull sperm (Boquest and Summers, 1999).

Results from *in vitro* studies with oviductal cells reported by Wijayagunawardane *et al.* (1999a,b) suggested that the preovulatory LH surge, together with locally recirculated high levels of oestradiol from the mature follicle and progesterone from the regressing corpus luteum induced a stimulatory effect on oviductal prostaglandins (PGE_2 and $PGF_{2\alpha}$) and endothelin 1 (ET-1) during the periovulatory period. It is suggested that ET-1 may act as a local amplifier for oviductal prostaglandin production, stimulated by luteinizing hormone (LH) and ovarian steroids.

Although osteopontin, BSA and lipocalin-type prostaglandin-D synthase (L-PGDS) have been identified in cow oviductal fluid, their role in tubal events has been unclear. Now, studies by Way *et al.* (2002), who treated bovine oocytes with antibodies to these compounds, have demonstrated that such antibody treatment inhibited sperm–oocyte binding and fertilization. Other studies by the same group have also shown that the same antibody treatments inhibited embryonic development *in vitro*, suggesting a role for these same three proteins in early bovine embryo development (Killian and Goncalves, 2002).

5.3. Capacitation Procedures

Artificial capacitation procedures are aimed at simulating the sequence of events that normally occurs in the cow's reproductive tract. Experience has shown that it is more difficult to capacitate sperm artificially in some species than in others; methods used in dealing with the spermatozoa of farm mammals have usually been based on modifications of techniques used in laboratory species, which have been studied more intensively.

5.3.1. Historical

Since the initial discovery of the need for sperm capacitation if fertilization is to succeed, considerable research activity has been devoted to the development of methods to artificially induce capacitation and the subsequent acrosome reaction *in vitro*. The first authentic demonstration of mammalian fertilization *in vitro* was that recorded by Chang in the late 1950s in rabbits, using sperm recovered from mated does; it was later found that epididymal sperm could be prepared to fertilize (capacitated) without having to undergo preparation in the female tract.

In Ireland, attempts were made in the late 1960s by Sreenan to fertilize *in vitro*-matured cattle oocytes, using bull sperm exposed to various procedures. In the USA, Brackett and colleagues in the early 1970s used immunological methods to show that the capacitation process involved removal or alteration of seminal plasma components that normally coat the surface of the rabbit spermatozoon. Brackett's work with mice and rabbits went on to show that capacitation could be achieved in media of high ionic strength (Brackett–Oliphant (BO) medium).

5.3.2. Modifying osmolarity and pH

In the early 1980s, IVF using artificially capacitated bull sperm was demonstrated and verified by the birth of calves (Brackett *et al.*, 1982); the technique employed to capacitate sperm involved the use of a hypertonic medium (380 nosmol), as previously used with success in capacitating rabbit sperm. It later became evident from studies in the USA and Japan that the treatment of ejaculated bull sperm with a chemically defined medium of normal osmolarity (290 mosmol) could be effective. However, whether using high ionic strength (HIS) or normal medium, the fertilization rates achieved with ejaculated bovine sperm were usually low, with considerable bull-to-bull variation in evidence. The HIS capacitation treatment that resulted in the birth of the first IVF calf showed 26–36% penetration of cattle oocytes. Some workers saw the use of the HIS medium as being suitable for certain bulls (those that responded well) but not as a method suitable for general application.

pH values

In cattle, the studies of Lu in Ireland in the late 1980s recorded the optimal pH value for the modified Tyrode/albumin/sodium lactate/sodium pyruvate (TALP) medium used for capacitation to be 7.4 and for fertilization 7.8 (Table 5.2).

5.3.3. Evaluating sperm-capacitation systems

Several research groups have devoted time and effort to examining various capacitation systems for use with bull sperm and testing their efficiency in various ways. In Germany, Markle-Rutz (1994) investigated the motility of bull sperm using different capacitation procedures (Brackett and Oliphant medium; modified Tyrode's solution; modified Krebs–Ringer solution; modified tissue-culture medium 199 (TCM-199)); all the treatments increased the percentage of motile sperm. In Minnesota, Iqbal and Hunter (1995a) compared calcium-free modified Tyrode's solution with bovine follicular fluid or heparin and TEST-yolk buffer at two incubation temperatures (37 and 39°C). Other work in the same laboratory compared various capacitation systems for their ability to alter the net negative surface charge of bovine sperm; they found that capacitation reduced the net negative surface charge (Iqbal and Hunter, 1995b). In a third report, they showed that none of their capacitation systems induced sperm motility changes that were related to capacitation or the efficiency of capacitation (Iqbal and Hunter, 1995c). Other studies in

Table 5.2. Effect of pH on capacitation and fertilization in cattle (from Lu and Gordon, 1987).

pH of capacitation medium		pH of fertilization medium			Total
		7.0	7.4	7.8	
7.0	Oocytes examined	44	35	40	119
	% fertilized[a]	43.2[b]	57.1[bc]	72.5[c]	57.1
	% cleaved	4.5[A]	37.1[B]	35.0[B]	24.4
7.4	Oocytes examined	40	32	34	106
	% fertilized[a]	42.5[d]	68.8[e]	91.2[f]	66.0
	% cleaved	22.5[c]	9.4[c]	70.6[D]	34.0
7.8	Oocytes examined	44	48	48	140
	% fertilized[a]	54.4[g]	70.8[gh]	79.2[h]	68.6
	% cleaved	22.7	35.4	29.2	29.3
Total	Oocytes examined	128	115	122	365
	% fertilized[a]	46.9[E]	66.1[FG]	80.3[G]	64.1
	% cleaved	16.4[H]	28.7[IJ]	42.6[J]	29.0

[a]No. of fertilized oocytes including no. of cleaved oocytes.
The percentage of fertilization or cleavage of treatment groups with different superscripts within column is statistically different: $P < 0.05$ for b–h and $P < 0.01$ for A–J.

Minnesota by Alfonso and Hunter (1995) led them to conclude that a TALP swim-up procedure was superior to the Brackett–Oliphant method for capacitating frozen–thawed bull sperm.

It is known that BSP proteins potentiate bovine sperm capacitation induced by heparin and high-density lipoprotein (HDL). In Canada, a study by Lane *et al.* (1999a) sought to clarify the mechanisms involved; they concluded that heparin and HDL mediate capacitation by different mechanisms. The ROS requirements for bovine sperm capacitation and the acrosome reaction were the subject of a study by O'Flaherty *et al.* (1999). In Brazil, using thawed semen from 20 different bulls, Assumpcao *et al.* (2000) tested *in vitro*-capacitation protocols, using heparin (100 μg/ml) for 6 h and calcium ionophore (5 μM) for a 1 min incubation period; heparin treatment resulted in greater sperm capacitation rates than the calcium ionophore treatment.

5.4. Heparin and Heparin-like Glycosaminoglycans

Of the various methods that have been examined and evaluated in capacitating bull spermatozoa for IVF, that involving the use of the GAG heparin has received the greatest research attention (Parrish *et al.*, 1994; Miller and Ax, 1995; Ijaz *et al.*, 1996). Capacitation of bull sperm by heparin first requires that the molecule binds to sperm. Although heparin can bind specifically to both epididymal and ejaculated sperm, it also binds to seminal plasma proteins; it is not clear whether the GAG's ability to capacitate ejaculated sperm is by binding to proteins already present on epididymal sperm or to seminal plasma proteins absorbed on to the surface of sperm at ejaculation (Braundmeier and Miller, 2001). It is possible that heparin-binding proteins are among the accessory gland proteins that may influence fertility in cattle (see Table 5.3).

5.4.1. Actions and interactions of heparin

In the *in vitro* production (IVP) of cattle embryos, the primary limiting factors to success are the number and quality of oocytes collected and the variable fertilizing ability of bovine sperm. In a study reported by Markkula *et al.* (1999a) in Finland, they first optimized IVF with the semen of five bulls, using oocytes recovered from abattoir ovaries; optimization of the heparin concentration (2.5–20 μg/ml) in the fertilization medium did not remove the differences between bulls but did improve the effectiveness of the embryo production process. The *in vitro* fertility of the five bulls, measured in terms of embryo production, could not be predicted on the basis of non-return rates (NRR) or on embryo production after conventional superovulation treatment. As observed by Parrish *et al.* (1999), the discovery of heparin as a capacitating agent made it possible to make major advances in bovine gamete biotechnology. Although alternative methods exist, heparin-induced

Table 5.3. Summary of molecules proposed as markers of sperm fertility. The authors stress that most of the listed functions of molecules are speculative; some may be indicators of general defects in spermatogenesis or in the function of the male reproductive tract. (From Braundmeier and Miller, 2001.)

Molecule	Location	Proposed function
Fertility-associated proteins (Osteopontin and prostaglandin-D synthase)	Seminal plasma	Unknown
Heparin-binding proteins	Accessory-gland fluid and spermatozoa	Increases affinity for heparin and enhances capacitation
Fertility-associated antigen (FAA)	Accessory glands and spermatozoa	A heparin-binding protein that increases affinity for heparin and enhances capacitation
Heat-shock protein (Hsp2A)	Spermatozoal tail membrane	Measures spermatozoal maturity
Acrosin	Within acrosome	Measure of intact acrosomes

capacitation is believed to be superior in being able to capacitate sperm *in vitro* from many different bulls. The authors noted that this was reflected in the 350 and more citations to the original work of Parrish and Susko-Parrish in the mid-1980s.

The fact remains, however, that, despite the knowledge gained over the 16 years, the mechanisms involved in heparin-induced capacitation are still not fully understood (see McCauley *et al.*, 1996). Although it is believed that regulation of Ca^{2+} during capacitation is complex and may involve the loading of an intracellular Ca^{2+} store, how this loading relates to events surrounding the acrosome reaction is unclear. In a report by Parrish *et al.* (1999), it was found that the influx of calcium ions into bull sperm during the first 2 h of incubation was critical to heparin-induced capacitation; the initial influx into the sperm is believed to fill an intracellular Ca^{2+} store in the acrosome.

Understanding the regulation of calcium ions may have relevance to the cryopreservation of sperm. It is known that, after thawing, cryopreserved sperm take up Ca^{2+} faster than fresh sperm and initiate the sequence of capacitation that may result in their premature death before reaching the oocyte. It is also known that three acidic seminal-plasma proteins represent the major proteins in the plasma; upon ejaculation, these proteins bind to the sperm membrane and potentiate heparin-induced capacitation. A loss in sperm-bound seminal-plasma proteins was observed by Nauc *et al.* (1999) after cryopreservation; such post-thaw loss in sperm-bound proteins may trigger destabilization of sperm membranes and premature sperm capacitation. In sheep, a species in which the use of frozen–thawed semen has been notoriously difficult, Maxwell *et al.* (1999) found that treatment of frozen–thawed ram semen with seminal plasma markedly improved fertility after AI and surmised that this may have been due in part to its decapacitation effect. In a review of the current status of sperm cryopreservation, Medeiros *et al.* (2002a,b) note that the identification of possible seminal-fluid components which, added to semen diluents, would decrease cryopreservation-induced capacitation is an area worthy of examination.

In the USA, Bosch *et al.* (2000) demonstrated the ability of heparin to induce sperm release from their attachment to bovine oviductal epithelial cells. The effect of heparin on motility parameters and protein phosphorylation during capacitation was dealt with in a report by Chamberland *et al.* (2001) in Quebec; on the basis of their findings, they suggested that capacitation of bovine bull sperm and capacitation-associated motility changes may be regulated by a mechanism that includes protein phosphorylation and that some unknown protein kinase is involved.

5.4.2. Practical application of heparin treatment

There are those who suggest that heparin treatment is the most effective method for the *in vitro* capacitation of bull sperm; the agent is believed to be in the oviducts of the cow at the time of fertilization (Mahmoud and Parrish, 1996). The *in vitro* capacitation of bull spermatozoa by heparin is known to be associated with an increase in intracellular pH (Vredenburgh-Wilberg and Parrish, 1996), calcium and cAMP (Medeiros and Parrish, 1996). It is believed that any level of fertilization can be achieved in the cow by adjusting either sperm concentration or heparin dose in the fertilization medium.

The standard IVF procedure, as initially employed in Ireland by Lu and associates, involved a preincubation period of 15 min, during which sperm, in a Ca^{2+} modified TALP medium, were exposed to heparin. Spermatozoa were then added in a small volume of capacitation medium (5 μl) to droplets of fertilization medium (45 μl). An alternative strategy was to add heparin directly to the fertilization medium without any preincubation treatment.

In Germany, Streicher (1998) dealt with experiments that determined the fertilizing ability of different bulls after heparin treatment. The author recorded that the addition of heparin to the fertilization medium significantly increased cleavage rate (72.3% for 1 μg/ml heparin vs. 34.3% for no heparin); blastocyst yield was also significantly increased by heparin. It was also found that incubation with heparin for 15 min gave better results than incubation for 2 min; longer incubation with heparin decreased the

cleavage rate but tended to increase the blastocyst yield.

In the USA, McHugh and Rutledge (1998) inseminated matured cattle oocytes with heparin treated sperm from various members of the genus *Bos* (*Bos bison*, *Bos grunniens*, *Bos javanicus* and *Bos gaurus*); they were able to show that the sperm of all four species fertilized the cattle oocytes and produced hybrid embryos. Each of the non-domestic *Bos* species did, however, have a different heparin and sperm concentration requirement for successful IVF.

5.5. Use of Fresh or Frozen Semen

The storage of bull spermatozoa was revolutionized a half-century ago with the discovery by Chris Polge and associates that glycerol could act as a very effective cryoprotectant; the discovery opened the way to bull sperm being frozen and stored for prolonged periods before being thawed out and used successfully in AI. Although a vast literature on bull-sperm cryopreservation has built up over the years since the first calf was born after the insemination of frozen–thawed semen, there are still many important questions requiring research, particularly in terms of the factors responsible for differences among bulls in the ability of their spermatozoa to survive the freeze–thaw process. A review by Holt (2000) has drawn attention to studies in mice that reveal genetically based trends in the success of sperm cryopreservation; it may be that an examination of genetic differences between bulls could offer a similar genomically based approach in cattle.

5.5.1. Fresh semen

Various workers have employed both fresh and frozen bull semen in their cattle IVF studies. In some reports, it was apparent that fresh semen required a longer capacitation period than frozen; there was also evidence that ejaculated bull spermatozoa required a longer heparin treatment period than frozen. One problem in using fresh bull sperm in IVF is likely to be the unpredictable quality of semen; when using frozen–thawed bull semen, there is at least the knowledge that the semen had passed through an initial screening before freezing. On the debit side, there is the possibility that the quality of frozen–thawed semen is likely to deteriorate more rapidly than that of fresh semen. In Cornell, Goldman *et al.* (1998) examined the reaction of fresh and frozen bull sperm incubated with bovine oviductal epithelial cells; at examination after 5–24 h of incubation, the percentage of motile fresh sperm exceeded that of frozen sperm and more fresh sperm attached to the epithelial-cell monolayer. In New Zealand, Krzyzosiak *et al.* (2001) attempted to use in IVF sperm that had been stored for up to 11 days at ambient temperature in a citrate-based commercial diluent; not unexpectedly, the fertility of the sperm declined with storage time.

In Belgium, De Pauw *et al.* (2002a) investigated the *in vitro* fertilizing ability of freshly diluted bull semen after 4 days of storage at room temperature and examined whether limiting the contact of sperm with seminal plasma, by collecting semen immediately into a Tris–egg-yolk diluent, could improve IVF. It is known that prolonged storage of bovine spermatozoa at ambient temperature can lead to a deterioration in the integrity of the cell membrane, in motility and in fertilizing ability. The Belgian workers found that fresh sperm coated immediately after ejaculation for 5 min with the Tris–egg-yolk diluent yielded significantly higher fertilization rates than uncoated sperm after 4 days of storage (see Table 5.4).

5.5.2. Frozen semen

The majority of dairy cattle in many countries around the world are currently bred by AI, usually with semen frozen in plastic straws of 0.25–0.5 ml capacity. It is likely to be much more feasible for an IVF laboratory to obtain supplies of frozen semen rather than keeping a bull for the provision of fresh semen ejaculates. Despite the 50 years since the first calf by frozen semen was born in Cambridge, today no more than about 50% of sperm survive current cryopreservation techniques (Medeiros *et al.*, 2002a,b); that is a measure of the need for a fuller understanding of the factors influencing cell preservation by low-temperature storage.

Table 5.4. Effect of sperm collection method on IVF in cattle (from De Pauw *et al.*, 2002a).

Spermatozoa	Storage	No. of oocytes examined	No. (%) of fertilized oocytes: No. (%) of normal fertilization	No. (%) of polyspermy	Total no. (%) of penetrated oocytes
Fresh uncoated	4 days in HEPES-TALP	163	32 (20)[a]	0 (0)[a]	32 (20)[a]
Fresh coated	4 days in HEPES-TALP	146	98 (67)[b]	15 (10)[b]	113 (77)[b]
Frozen–thawed control	Not stored	121	70 (58)[c]	0 (0)[c]	70 (58)[c]

[a-c]Significantly different values in the same column ($P < 0.05$).

In Poland, Katska *et al.* (1994) sought to determine whether removal of seminal plasma from bull ejaculates immediately after collection and before semen freezing could influence the outcome of *in vitro* embryo production; they found that this procedure did increase the yield of blastocysts. Such results would support reports in several species, including cattle, that seminal plasma contains factors that may adversely affect male fertility; these factors are believed to inhibit sperm capacitation, the acrosome reaction and the action of acrosomal enzymes.

5.5.3. Semen diluents

The diluents and cryoprotectants employed in the freezing of bull semen may be factors influencing the viability and capacitation of bull sperm; for those involved in IVF and embryo production, there is need to keep such factors in mind. It is well established that with current diluents and cryopreservation techniques, some 50% of sperm are likely to die or become immotile. The components of egg yolk and milk and the mechanisms by which they affect and protect the membranes of the bovine spermatozoon are not well understood. The protective mechanism of egg yolk apparently relies on the phospholipids and low-density lipoprotein components; the protection afforded by skim milk apparently lies with the protein fraction. In terms of the general vitality of frozen–thawed bull sperm, in comparison with those freshly collected, it appears that semen dilution and freeze–thaw procedures do not substantially impair their energy metabolism, which makes such sperm acceptable for IVF purposes. Various studies have shown the beneficial effects of adding antioxidants to cryopreserved bull sperm. In one such study in Canada, Bilodeau *et al.* (2001) demonstrated that thiols prevented hydrogen peroxide-mediated loss of sperm motility in frozen–thawed bull semen. A further study reported by Bilodeau *et al.* (2002) indicated that the addition of low amounts of catalase and millimolar concentrations of pyruvate greatly improved the antioxidant properties of a commonly used diluent (egg-yolk Tris glycerol) and was beneficial to the motility of frozen–thawed semen.

As to the thawing of semen, appropriate guidelines about temperature control should be carefully observed; sperm quality after thawing shouild be assessed on the basis of the percentage of progressively motile sperm, the rate of progression and the percentage of sperm showing normal morphology. Before IVF and to minimize the risk of semen contamination, straws should be carefully wiped with 70% ethanol prior to cutting and releasing their contents.

5.6. Bulls as a Source of Variability

Almost all authors reviewing cattle embryo production remark on the variability in outcome due to the male factor. There are numerous studies indicating that the selection of bulls producing sperm with a high fertilizing capacity is an important factor in achieving successful and consistent IVP results. Available evidence suggests the existence of a bull effect on embryonic development that may not be related to sperm penetration rate or the apparent normality of fertilization. In Ireland, several reports showed that, although differences in fertilization rates were non-significant among bulls after

IVF, the blastocyst yield could differ markedly and significantly (see Gordon, 1994); results reported elsewhere have generally been in agreement (Avery and Quetglas, 1996; Brackett and Keskintepe, 1996; Kurtu *et al.*, 1996; Larocca *et al.*, 1996; Palma *et al.*, 1996; Schneider *et al.*, 1996).

5.6.1. High- and low-fertility bulls

Evidence of bull fertility differences has been available in reports from AI stations for many years. Workers in the 1950s reported fertilization rates of 100% for bulls of high fertility and 70% or so for those of low fertility. Studies in California, reported many years ago, showed that conception rates varied from 34 to 70% in a large dairy herd according to the bull used to breed the cows; the financial cost to dairy farmers of low-fertility bulls can be substantial. Among those working in embryo transfer, it is well known that the fertilization rate and the yield of embryos after insemination of the superovulated donor with frozen–thawed semen may be markedly influenced by the choice of bull. It is believed that the differences seen between bulls in normal AI may be accentuated in the breeding of superovulated animals; this led to the suggestion that potential AI bulls with above-average fertility might be selected on the basis of fertilization rates achieved with superovulated cattle.

A report by Palma and Brem (1994) presented data on the suitability of frozen–thawed semen from 40 bulls for *in vitro* embryo production. The high variation in embryo production rate due to the various bulls, which ranged from 10 to 51%, is clearly evident. In Germany, Graule *et al.* (1995) used frozen–thawed semen from 14 Simmental bulls with widely varying NRR (43–75%) to show that bulls with low NRR rates had low sperm penetration rates *in vitro* when used in embryo production systems. Whether semen quality varies with different batches from a particular bull has been investigated by several groups. Zhang *et al.* (1997) found evidence of great variability among batches; this was in contrast to the findings of Astiz Blanco *et al.* (1999). In Italy, Vincenti *et al.* (2001) analysed data from a commercial laboratory producing *in vitro* cattle embryos and found that batch did not affect embryo yield.

5.6.2. Bull variability

The marked variability that occurs among bulls in their suitability for *in vitro* embryo production may be due either to factors affecting the efficiency of sperm penetration or to the part played by the spermatozoon once it gains entrance to the ooplasm.

In terms of the first possibility, there are those who have found seminal-plasma proteins to be a source of variation. It is now well established that seminal-plasma proteins become associated with the sperm membrane at the time of ejaculation; as noted above, an important part of bovine sperm capacitation involves the removal or modification of such proteins. A study by Henault and Killian (1995) found evidence that seminal plasma is important in determining the fertility of ejaculated spermatozoa and that these seminal proteins differ between bulls. Results of studies by Katska *et al.* (1994) in Poland showed that the absence of seminal plasma during capacitation improved the *in vitro* fertilizing ability of spermatozoa in bulls that produced gametes with low fertilizing ability.

The second possibility is highlighted in evidence showing that more rapidly cleaving bovine embryos give rise to higher blastocyst yields than later-cleaving embryos (Van Soom, 1996; Dinnyes *et al.*, 1999; Lonergan *et al.*, 1999a,b,c; Ward *et al.*, 2001a, 2002; Rizos *et al.*, 2002a,b). Studies in the USA have shown that embryos sired by bulls of high fertility enter the zygotic S phase earlier, cleave to the two-cell stage faster and develop more rapidly to the blastocyst stage than embryos sired by low-fertility bulls (Eid *et al.*, 1994); it is believed that an increase in the duration of the G2 phase may be due to increased sperm DNA damage in low-fertility bulls or to a higher proportion of zygotes sired by such bulls failing to complete DNA replication during the S phase (Eid and Parrish, 1995). In France, Comizzoli *et al.* (2000b) fertilized *in vitro*-matured cattle oocytes with bull spermatozoa that resulted in either a high or a low blastocyst yield; they showed that the onset of the S phase and the first cleavage

occurred earlier in the high-yield group than in the low-yield group. The data demonstrated that the precocity of the onset of the first S phase stemmed from a paternal control exerted during a transient period of the G1 phase.

One of the consequences of poor sperm quality may lie in the inability of the oocyte to recognize damaged chromatin. Under such conditions, meiosis may well continue, leading to a situation where normal and damaged metaphase plates are present in the same cell. As observed by Fulka *et al.* (1998), the inability of the oocyte to recognize damaged chromatin in all instances may have important implications for fertilization and early development. It could lead to an abnormal sperm nucleus being present in an oocyte containing a normal female pronucleus. In the embryo, however, each daughter cell in every cell division should acquire the correct chromosome complement. Such events are under the control of the cell cycle checkpoints, which ensure that such events as DNA replication and chromosome segregation are completed with high fidelity.

Quite apart from the bull's role in efficient embryo production systems, there is interest in using *in vitro* tests to predict the *in vivo* fertility of bulls. In the Netherlands, Lansbergen *et al.* (1997) analysed the predictive values of cleavage and embryo production rates in an ovum pick-up (OPU)/IVP system for *in vivo* characteristics; they concluded that the percentage of transferable embryos produced could have a predictive value. In Ireland, Ward *et al.* (2001a) have demonstrated that the kinetics of early embryonic development, as measured by the timing of the first cleavage division, varied among different bulls and that such differences could be used to discriminate between bulls of high and low field fertility.

5.6.3. Methods of assessing bull fertility

In animal agriculture, despite a vast literature on semen analyses for the various farm species, no single useful test has apparently emerged to meet the needs of those involved in AI or fertility testing. Over a period of many years, bull semen quality has been based on a subjective evaluation of parameters such as wave motion and progressive sperm motility and an objective evaluation based on semen concentration and morphological abnormalities. At this point in time, there is a considerable array of tests available, which range from simple visual assessments to detailed tests that assess molecules involved in the spermatozoon–oocyte interaction. Various new tests became available based on the use of sperm in the IVP of cattle embryos; such tests have been shown to be significantly related to the field fertility of the assayed semen, especially when combined with other features, such as sperm-motility patterns. A review by Braundmeier and Miller (2001) divided semen traits into those that are compensatable and those that are not. Spermatozoa with defective compensatable traits (motility and morphology) may have difficulty reaching the site of fertilization but this problem may be overcome by using high numbers of sperm. Uncompensatable traits are those where the defects affect the function of the sperm during the later stages of fertilization and in early embryonic development. Uncompensatable traits include nuclear vacuoles, morphological deficiences that do not inhibit movement and defective chromatin structure. The authors note that there are now promising new molecular tests that may be added to the standard tests employed in semen evaluation.

A review by Verstegen *et al.* (2002) in Belgium has dealt with computer-assisted semen analysers (CASA) in research; although cost is a major factor limiting the use of such equipment in cattle, the use of analysers has increased considerably over the past 15 years, particularly in human fertility clinics. It is clear that such analysers can provide an efficient, precise and reliable tool for evaluating fertility objectively, improving assisted-reproduction technologies and developing physiological or toxicological studies.

5.6.4. Enhancing semen quality

The low penetrability of sperm from individual bulls during *in vitro* embryo production is a factor that must be considered in any commercial

application of IVP technology. With that in mind, Kreysing *et al.* (1995) sought to examine the effects of casein phosphopeptides (CPPs) (a mixture of α and β-caseins) on bull sperm to test whether blastocyst yield might be improved. Although the mechanism whereby CPPs achieve their effect is unclear, their incorporation into the IVP system accelerated and enhanced penetration of oocytes. As noted in an earlier context, the early removal of seminal plasma from ejaculated semen may be an approach worthy of consideration.

5.7. Efficiency of Capacitation Procedures

5.7.1. Staining methods

Various staining techniques have been developed to assess the occurrence of the acrosome reaction in the bovine spermatozoon. In the UK, Christensen *et al.* (1994) assessed the suitability of bright-field microscopy and two stains (naphthol yellow S + erythrosin B; naphthol yellow S + aniline blue); they found naphthol yellow S plus aniline blue a suitable stain, easy to undertake and to evaluate. A study reported by Cross and Watson (1994) assessed the acrosomal status of bovine sperm using fluoresceinated lectins; after screening 12 lectins for their ability to label the acrosomal region of ethanol-fixed bovine sperm, they concluded that labelling with *Pisum sativum* agglutinin (PSA) can serve as a rapid assay for acrosomal status.

5.7.2. Oocyte penetration tests

The acrosome reaction of the bovine spermatozoon is the ultimate proof of capacitation and is usually the first step towards penetrating the oocyte. In the cow, the evidence suggests that the acrosome reaction only occurs at the surface of the ZP. The penetration of the ZP is therefore used in some tests as the end-point of capacitation. In Hiroshima, Tatemoto *et al.* (1994a) sought to determine whether the ZPs of dead follicular oocytes recovered from cattle ovaries and stored in liquid nitrogen for varying periods (1 day to 3 months) permitted sperm penetration; they were able to demonstrate that the ZPs of dead oocytes only permitted penetration by capacitated and/or acrosome-reacted sperm.

5.7.3. Sperm–zona binding

A sperm–oocyte binding assay was used by Topper *et al.* (1999) to determine whether the ability of bull sperm to bind to the ZP was altered during *in vitro* capacitation by heparin or oviductal fluid or by treatment of sperm from the cauda epididymidis with accessory-sex-gland fluid; the evidence suggested that increased zona binding ability is an essential part of the process of capacitation. The authors found that incubation with heparin or oviductal fluid increased the ability of sperm to bind to the ZP; exposure of epididymal sperm to accessory-sex-gland fluid resulted in a direct increase in zona binding ability, followed by a further increase during capacitation *in vitro*.

6

In Vitro Fertilization

6.1. Introduction

Fertilization is a complex process resulting in the union of the spermatozoon and the oocyte; it signals the start of the transition from oocyte to embryo. Fertilization is also a dual process, during which the oocyte both is activated and has the hereditary material from the sire introduced into it; activation can be regarded as an obligatory event for the start of the oocyte's developmental programme after fertilization.

Successful cattle *in vitro* fertilization (IVF) requires appropriate preparation of both gametes, as well as favourable culture conditions. Many of the basic events of mammalian fertilization have been clarified and the molecular mechanisms underlying these events identified (Wassarman, 1994). In addition, many regulatory molecules, cytokines, growth factors, enzymes and inhibitors that influence cell growth and differentiation and other responses have been identified in the oviduct (see review by Buhi, 1996); such agents may act, in either an autocrine or a paracrine way, to regulate events in the oviduct, including the progress of early cleavage-stage embryonic development.

6.1.1. *In vitro* maturation and fertilization: early reports

Less than 20 years ago, no more than a handful of IVF calves had been reported in the literature, most of the births occurring in North America; until 1986, the calf was the result of the fertilization *in vitro* of an ovulated or *in vivo*-matured oocyte. The first report of successful *in vitro* maturation (IVM) and IVF of cattle oocytes leading to the birth of calves was that of Hanada and associates in Japan in 1986, using calcium ionophore to capacitate bull sperm; the three calves born represented 21% of the embryos transferred to recipient animals. The type of IVF system employed by Iritani and associates in Japan at that time is illustrated schematically in Fig. 6.1.

One group of calves born in the late 1980s was the result of work by Lu and associates in Ireland; in this, 18 calves were born alive and healthy to 13 recipient cattle after non-surgical transfer of embryos (Fig. 6.2); this represented an embryo survival rate (59%) no different from that normally achieved with embryos produced by conventional superovulation technology. The work of Lu over the next few years was to firmly establish the effectiveness of his *in vitro* embryo production and to encourage others to follow this route to the large-scale production of low-cost cattle embryos. The main factors in Lu's successful technique were: (i) the use of follicle dissection and rupture for the collection of high-quality cumulus–oocyte complexes (COCs); (ii) the provision of additional granulosa cells (3–5 million per ml) to support oocyte maturation; (iii) the use of oestrous cow serum as a protein source and to supply physiological levels of gonadatrophins and oestrogen; and (iv) the use of a flux culture system to prevent unwelcome differentiation of follicular cells. There were also

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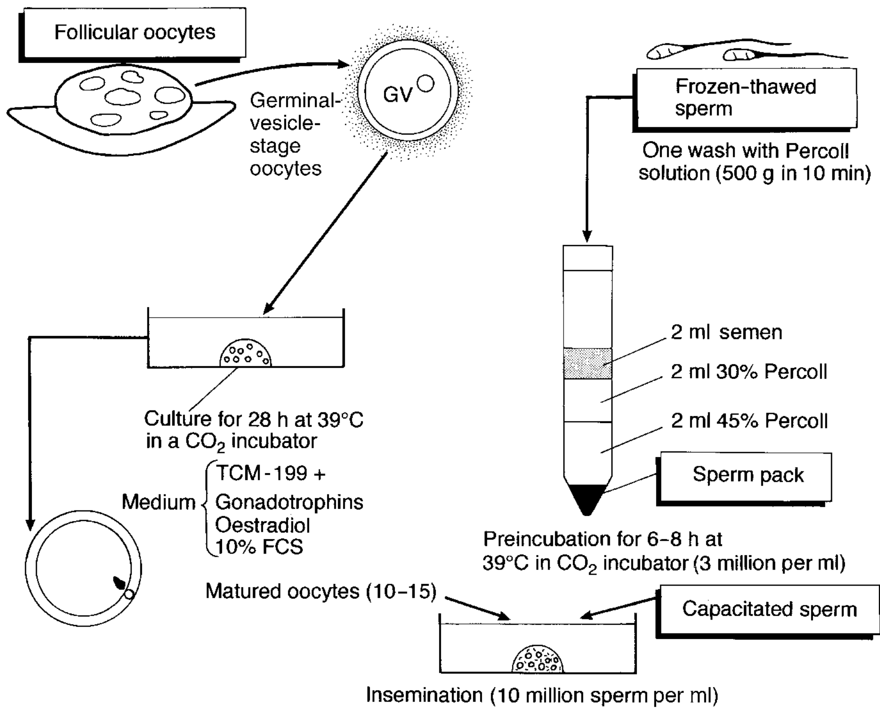

Fig. 6.1. *In vitro* fertilization system for cattle (Kyoto University). System as first used by Professor Iritani and colleagues.

Fig. 6.2. IVMF-derived calves born in Ireland in 1987; 18 calves were born alive and healthy to 13 recipient cows after non-surgical transfer of embryos, representing an embryo survival rate of 59%.

indications that the pH values employed in sperm capacitation (7.4) and in the Tyrode/albumin/ sodium lactate/sodium pyruvate (TALP) fertilization (7.8) medium may be important.

6.1.2. Chapter contents

Although there has been a remarkable increase in knowledge of the various events involved in

the fertilization process, information is still far from complete and many of the biochemical processes underlying the process are not fully understood (Berger, 1996). Research studies are increasingly concerned with events at the molecular level, particularly those involved in the binding of the sperm head to the zona pellucida (ZP). There are, for example, differing views as to how the fertilizing spermatozoon induces calcium oscillations and oocyte activation. The present chapter deals with events involved in the natural process of fertilization in the cow and subsequently with the different steps essential in preparing sperm and oocytes for fertilization *in vitro*. In looking for optimal methods to use in IVF, careful attention must be paid to the part played by the oviduct in providing for the needs of both sperm and oocyte.

6.2. Fertilization in the Cow

6.2.1. Oviductal environment

Fertilization in the cow is a complex process that normally occurs within the controlled and unseen environment of the oviduct. The role of oviductal fluid in modulating sperm physiology has been investigated in many studies (see Killian, 1996). A more complete understanding of sperm transport in the cow and factors affecting the number of sperm reaching the site of fertilization in the ampulla has been provided in several reports, many of them by Hunter and associates (Hunter, 1994, 1996; Nancarrow and Hill, 1995; Hunter *et al.*, 1998a). It is evident that there is a synchronization of sperm movement and ovulation in the cow, which has the effect of bringing relatively small numbers of sperm from a reservoir in the caudal isthmus to the ampullary region of the oviduct. In nature, the cow is served by the bull during the period of oestrus and sperm would normally be present in the reproductive tract for several hours prior to the occurrence of ovulation (which occurs about 10–12 h after the end of the heat period). The lifespan of sperm in the cow's oviduct is believed to be of the order of 24–48 h where freshly ejaculated semen is involved, but nearer to 12–24 h when frozen–thawed semen is employed in artificial insemination (AI).

Attempts have been made over the years to define the components of oviductal fluid and to understand the part that they may play in the function of the male and female gametes and in the early developing embryo. The identification of the molecules that enhance fertilization in the cow's oviduct may eventually lead to more efficient *in vitro* culture systems that improve embryo viability. Studies reported by Boatman *et al.* (1994) in hamsters were among those showing that the beneficial effects of the oviduct on embryonic development can begin prior to fertilization. The studies of Tervit and associates in the early 1970s in Cambridge were among the first to describe the successful culture of early-stage cattle embryos in a medium termed 'synthetic oviductal fluid' (SOF). This SOF medium was a formulation based on a biochemical analysis of sheep oviductal fluid and has been employed in many embryo culture studies reported in the last decade. Various modifications of the initial formulation have been suggested, usually on the basis of increasing knowledge of the factors in oviductal fluid; some workers have used protein-free, chemically defined forms of the medium to examine the action of a variety of agents, including growth factors and hormones.

In Belgium, Van Langendonckt *et al.* (1996) examined the *in vitro* kinetics of cattle embryos in serum-supplemented or serum-free modified SOF (mSOF); they found that the addition of fetal calf serum (FCS) before the five- to eight-cell stage accelerated the development of embryos into morulae. In the same year, Yoshioka and Kamomae (1996) employed a mSOF to show that the addition of recombinant human activin enhanced the development of cattle zygotes. In Argentina, Furnus *et al.* (1996) reported that certain glycosaminoglycans, such as hyaluronic acid (HA), may possess viability-promoting activity for bovine embryos when included in the SOF medium. It is clear from several reports that variations in the volume of oviductal fluid occur in different phases of the bovine oestrous cycle and that these are usually due to hormonal regulation of the secretory activity of oviductal epithelial cells, which increases under the influence of oestradiol and declines with rising progesterone concentrations (Menezo and Guerin, 1997).

In the USA, Elhassan *et al.* (2001) measured amino acids in bovine oviductal and uterine fluids (see Table 6.1) and compared them with

Table 6.1. Amino acids in bovine oviductal and uterine fluids (concentrations in μM) (from Elhassan *et al.*, 2001).

Amino acid	OF aa	UF aa
ARG	279 ± 23[b]	1,402 ± 15[a]
CYS	1,349 ± 193[a]	553 ± 67[b]
HIS	173 ± 12[b]	589 ± 53[a]
ILE	305 ± 15[a]	872 ± 62[a]
LEU	476 ± 44[b]	1,814 ± 130[a]
LYS	387 ± 34[b]	1,787 ± 162[a]
MET	100 ± 9[b]	551 ± 45[a]
PHE	217 ± 18[b]	696 ± 50[a]
THR	833 ± 60[b]	1,693 ± 146[a]
TRP	150 ± 6[b]	281 ± 10[a]
TYR	177 ± 13[b]	631 ± 61[a]
VAL	494 ± 51[b]	1,850 ± 140[a]
ALA	3,682 ± 332[a]	3,132 ± 238[a]
ASN	105 ± 10[b]	397 ± 59[a]
ASP	772 ± 72[b]	1,749 ± 155[a]
GLY	14,062 ± 1,215[a]	11,992 ± 1,126[a]
GLU	5,472 ± 476[a]	4,232 ± 176[a]
GLN	937 ± 135[b]	1,840 ± 135[a]
PRO	570 ± 72[b]	1,881 ± 111[a]
SER	625 ± 25[b]	2,675 ± 224[a]
β-ALA	136 ± 6[a]	83 ± 7[b]
CIT	92 ± 9[a]	93 ± 11[a]
ORN	61 ± 5[b]	149 ± 24[a]
TAU	461 ± 68[b]	3,429 ± 480[a]

Code	Name	Class[†]	Code	Name	Class	Code	Name	Class
ARG	Arginine	E	THR	Threonine	E	GLU	Glutamate	NE
CYS	Cysteine	E	TRP	Tryptophan	E	GLN	Glutamine	NE
HIS	Histidine	E	TYR	Tyrosine	E	PRO	Proline	NE
ILE	Isoleucine	E	VAL	Valine	E	SER	Serine	NE
LEU	Leucine	E	ALA	Alanine	NE	β-ALA	β-Alanine	NS
LYS	Lysine	E	ASN	Asparagine	NE	CIT	Citrulline	NS
MET	Methionine	E	ASP	Aspartate	NE	ORN	Ornithine	NS
PHE	Phenylalanine	E	GLY	Glycine	NE	TAU	Taurine	NS

[†]According to Sigma's MEM essential (E) and non-essential (NE) amino acid classification for cell cultures; NS, non-standard amino acids.
[a,b]OF aa and UF aa with different superscripts in the same row are different ($P < 0.05$)
OF, oviductal fluid; UF, uterine fluid; aa, amino acids.

those present in a modified simplex optimized medium (KSOM) supplemented with either FCS or minimum essential medium (MEM) amino acids in addition to bovine serum albumin (BSA), FCS or polyvinyl alcohol (PVA). The authors found that amino acids, particularly alanine, glutamate, glycine and taurine, were present in markedly high concentrations in both oviductal and uterine fluids, suggesting that they might have an important role in early embryo development; they concluded that the particular pattern of amino acid concentrations may be important in considering the improvement of embryo culture media.

In dealing with the oviductal environment, it is worth mentioning that in some mammalian species, events normally associated with the pre-ovulatory follicle occur in the Fallopian tube. In contrast to the cow and the other farm mammals, in which oocyte maturation occurs in the pre-ovulatory follicle, the domestic dog apparently ovulates immature oocytes, characterized by the

presence of a germinal vesicle, which subsequently mature to metaphase II over a period of 2–5 days within the oviduct (see review by Farstad, 2000). In view of the critical nature of many events in the cytoplasmic maturation of the mammalian oocyte, it may seem unusual that such events normally take place far from the microenvironment of the Graafian follicle. It may also be noted that as well as ovulating a primary rather than a secondary oocyte, the dog also deserves further comment because of the fact that canine spermatozoa can survive and maintain their motility in the bitch's reproductive tract for a prolonged period (4–6 days).

6.2.2. Lifespan of the secondary oocyte

Although not precisely defined, available evidence suggests that the unfertilized cow oocyte has a remarkably short viable lifespan (6–12 h) after leaving the ruptured follicle. It is thought possible that the microtubules of the meiotic spindle of the ovulated cow oocyte become disorganized within a matter of hours, with pairs of microtubules escaping laterally from the spindle apparatus and the consequent loss of chromosomes from the metaphase plate. A report by Hunter and Greve (1997) has shown that the age of the oocyte is inversely related to the estimated fertilization rate.

The cortical granules, membrane-bound organelles (0.2–0.6 μm in diameter) derived from the Golgi apparatus, which confer the block to polyspermy in the cow after releasing their contents into the perivitelline space of the activated oocyte, migrate from Golgi regions within the oocyte to take up their position just below the plasma membrane immediately prior to ovulation. The granules are believed to remain in position for several hours, until ageing of the oocyte sets in, when they begin to swell and wander away from the surface of the ooplasm. In this dispersed state, the release of the contents of the cortical granules into the perivitelline space cannot follow the activation of the oocyte and polyspermy is liable to occur. It seems clear that the bovine oocyte is designed to be fertilized shortly after ovulation and there is likely to be very little latitude in terms of its lifespan if sperm are not already in the reproductive tract at the time of ovulation. For cattle IVF, this probably means achieving fertilization of the oocyte as soon as possible after completion of nuclear and cytoplasmic maturation.

A study of fertilization *in vitro* of mature and ageing cattle oocytes was reported by Chung *et al.* (2001) in Canada; COCs were cultured for periods of 24, 36 or 48 h in tissue-culture medium 199 (TCM-199) supplemented with 10% fetal bovine serum, 0.2 mM pyruvate and gonadotrophins. The fertilization rate after 24, 36 and 48 h culture was similar but the incidence of polyspermy was 17%, 30% and 40%, respectively. The authors also found that the competence of early development was reduced following oocyte ageing; they concluded that mature and ageing oocytes have the same fertilizability but there is increased abnormal fertilization as cattle oocytes age. There is evidence that hardening of the ZP occurs *in vivo* as a consequence of the ageing of ovulating oocytes caused by the oviductal environment; it is also possible to induce hardening of the ZP *in vitro* by holding IVM cattle oocytes in oviductal fluid (Kania *et al.*, 1999, 2001).

6.2.3. Dispersion of cumulus cells

After leaving the ruptured follicle, the bovine oocyte is rapidly transported along the oviduct to the site of fertilization, this transport being facilitated by the action of cilia and smooth muscle contractions, which appear to be particularly active around the time of ovulation. In considering the interaction of sperm and oocyte in the cow's oviduct, it is known that the oocyte is ovulated with a covering of expanded and mucified cumulus cells; these cells are apparently dispersed in the ampullary region of the oviduct within a matter of hours (see Table 6.2; Greve and Callesen, 2001).

It is believed that most of the expanded cumulus cells are lost within 2 h but that the corona radiata cells immediately surrounding the oocyte may take a further 2 h before they disperse. The absence of a corona radiata on the bovine tubal oocyte was commented on by American and Cambridge workers in the 1930s and 1940s; Wisconsin and Danish researchers in the 1970s and later similarly observed that cattle

Table 6.2. Reports dealing with loss of cumulus cells from bovine oocyte shortly after ovulation.

Year	Details	Researcher(s)
1931	Reporting on tubal ova in the cow	Hartman *et al.*
1935	Reporting on an unfertilized bovine ovum	Evans and Miller
1946	Reporting on development of bovine ova	Hamilton and Laing
1979	Investigating factors involved in cumulus-cell dispersion	Lorton and First
2001	Unpublished information from IVF laboratory	Greve and Callesen

oocytes recovered from the oviducts within 2–3 h of ovulation were usually completely devoid of follicular cells. Such findings were also recorded in studies in Ireland and similar evidence came from sheep, where most of the cumulus cells were lost within a few hours of ovulation.

6.2.4. Fertilization rates in cattle

Estimates of fertilization rates in cattle have varied from 75 to 97% in heifers, with an overall mean of 88%; for cows, rates varied from 83 to 100%, with an overall mean of 90% (Sreenan and Diskin, 1986). Available evidence suggests that fertilization failure probably accounts for about 10–12% of conception failure, showing little difference between heifers and cows or between the use of frozen–thawed semen and the bull in natural service. Irish studies indicate that published fertilization rates probably overestimate the actual rates achieved at farm level. In practice, for example, it is likely that a proportion of cows put forward for insemination have not shown a genuine heat period; a study by White and Sheldon (2001) in Northern Ireland suggested that about 20% of cattle put forward in their study may not have been in oestrus. Clearly, fertilization is unlikely if the animal is not in genuine oestrus when inseminated.

While it is clear that a high fertilization rate is the norm in the cow, the causes of failure, when it does occur, are not well understood. It is unlikely to be a question of the individual cow or the timing of insemination alone; the quality of spermatozoa is probably an important factor. Research in cattle IVF may go some way towards explaining some forms of infertility in cattle, now that the oocyte–sperm interaction can be studied in the laboratory and novel forms of gamete manipulation (e.g. intracytoplasmic sperm injection) (ICSI) are available.

6.2.5. Fertilization rates in superovulated animals

It may be noted that the fertilization rates described for normally bred cattle may not always hold good for superovulated animals, where several oocytes are usually shed by the ovaries. The transport of such oocytes to the fertilization site, which is regulated by oestradiol and progesterone, may be disturbed by abnormalities in the levels of these hormones. It is not uncommon to recover embryos at widely different stages of development from superovulated donors; this may suggest that oocytes were fertilized over a period of several hours rather than at the one time. As a general rule, up to 50% of embryos recovered from superovulated cows may be unacceptable for subsequent transfer; part of the problem may lie in the different times at which ovulation and consequently penetration and fertilization by sperm occur. With IVF, where it can be arranged for insemination to be carried out at the optimal time, this is at least one problem that can be avoided.

As described in a review by Greve and Callesen (2001), the meeting and fusion of gametes in the cow's oviduct, which is normally a long and well-tuned process, may be adversely affected in several ways as a result of superovulatory treatment. Such disturbances are likely to include deviant oocyte maturation and impaired storage and transport of bull spermatozoa in the cow's reproductive tract. The Danish workers have done much to shed light on the structural, functional and chronological aspects of fertilization as it occurs in the oviduct of the superovulated animal. They recorded that some 25% of oocytes were unfertilized, possibly due to impaired sperm transport; limited numbers of sperm attached to the ZP (accessory sperm) also suggested defects in sperm transport.

Accessory spermatozoa

The sperm found firmly attached to the cow's ZP are termed accessory sperm; the number of such sperm is known to vary with the species (Saacke *et al.*, 2000). In cattle, the number of accessory sperm is also known to be influenced by superovulation (more sperm in single-ovulating animals) and embryo quality (more sperm in good-quality embryos than in poor). Presumably, the effect of superovulation is related to changes in the oviductal environment influencing the transport of sperm (see Table 6.3). The relationship between embryo quality and accessory sperm number is more difficult to understand but it appears that the number of such sperm should be around 10–20 per good-quality embryo; it is also noteworthy that abnormal sperm do not constitute part of the accessory sperm population (Saacke *et al.*, 1998).

6.3. Preparing Sperm for *in Vitro* Fertilization

Whether using semen freshly collected from the bull or semen frozen many years previously, certain steps must be taken before the sperm cells are ready for use in *in vitro* fertilization. It may be a matter of simply washing the sperm cells thoroughly or it may be a matter of putting them through one or other procedures which have been developed over the years to separate the highly motile and normal sperm from the rest of the population. The need for sperm separation techniques is likely to be greater when semen quality and sperm motility are poor.

6.3.1. Use of fresh bull semen

Although fresh semen is the norm in human IVF, it is likely to be the exception for those dealing with cattle, where frozen semen is widely and often exclusively used in commercial practice and in research. However, the needs of operators involved in small-scale embryo production after ovum pick-up (OPU) may sometimes be better met on occasion by semen freshly collected from the bull; there may even be a need to deal with sperm cells recovered from the epididymis of a valuable bull after an unexpected death. The washing of bull sperm to remove seminal plasma proteins rapidly and effectively prior to use of the male gametes in IVF is recognized as essential; this is usually achieved by washing the sperm by centrifugal sedimentation and resuspension in fresh medium. A paper by Verberckmoes *et al.* (2000) dealt with the influence of centrifugation on the motility and membrane integrity of fresh bull sperm; centrifugation may be required to obtain a sperm sample free of seminal or epididymal plasma. The authors concluded that an intermediate centrifugation speed ($3214 \times g$) was optimal for the separation of sperm and plasma

Table 6.3. Accessory sperm in superovulated and single-ovulating cows. Comparison of superovulated versus single-ovulating cows with respect to fertility and accessory sperm following AI with frozen semen characterized by high content of abnormal sperm heads (> 30%) and normal viability (> 50% motile). (From Saacke *et al.*, 1998.)

Characteristic	Superovulated	Single-ovulating
Number of cows	24	44
Number of ova/embryos recovered	155	31
% ova fertilized	64.5	83.8
% fertilized ova with accessory sperm cells	10	61*
Median (range) number of accessory spermatozoa per ovum/embryo recovered	0 (0–15)	2.5 (0–187)
Number of accessory sperm cells per ovum/embryo having accessory spermatozoa (mean ± SD)	1.1 ± 2.1	21.1 ± 30.5
Number of accessory sperm cells per cow having accessory spermatozoa (mean)	0.7	9.0*

*$P < 0.05$; other values not tested for significance.
SD; standard deviation.

without causing mechanical damage to the sperm membrane.

It may be noted that workers in the USA have examined the way in which seminal plasma may protect the viability of diluted bull semen (Garner *et al.*, 1999); it is well recognized in the cattle AI industry that dilution of semen below 20–30 million sperm/0.5 ml can lead to a loss of viability in frozen–thawed semen. Extreme dilution of bull sperm may result in what is known as the 'dilution effect', a phenomenon characterized by loss of motility, metabolic activity and fertilizing capacity (see Maxwell and Evans, 2000). Oxidative stress is a further consideration in dealing with semen; a review of reactive oxygen species (ROS) and sperm physiology has been provided by de Lamirande *et al.* (1997). Elsewhere, Kotaras and Seamark (1997) presented evidence to show that glutathione (GSH) can enhance the viability of frozen–thawed bull sperm.

6.3.2. Assessing the quality of frozen–thawed semen

Using semen obtained from cattle AI stations implies that the sperm must have attained a certain standard of motility and normality for them to be accepted for freezing in the first instance. There is still the need to check for satisfactory motility and other characteristics after the thawing of frozen semen. The increasing use of IVF by veterinary practitioners dealing with relatively small numbers of oocytes collected from donor cattle by OPU means that some bulls may be used which do not possess extensive fertility records. It is also relevant to note that the development of new techniques, such as sperm injection, means that spermatozoa may be employed in extremely small numbers and even when they are showing very poor motility; it is even possible, where necessary, to achieve fertilization of oocytes using sperm cells obtained from the epididymis or directly from the testis itself.

The diluents and cryoprotectants employed in the preservation of bull semen may also be factors to keep in mind. It has also to be appreciated that, although the freezing of bull spermatozoa has a long history, even with current techniques 50% or more sperm may fail to survive the process. It is known that altering the composition of the semen diluent or the sequence of adding diluent components may influence the post-thaw quality of frozen–thawed sperm. It is likely that some proportion of bull spermatozoa that do survive freeze–thawing may not necessarily be fully functional. A study by Coelho *et al.* (2000) in Brazil investigated the effect of three semen diluents (egg-yolk–lactose; egg-yolk–citrate; egg-yolk–TRIS) on IVF of cattle oocytes; although there were significant differences in cleavage rate, the yield and hatching rates of embryos were similar. It was concluded that these diluents had no effect on embryo production.

It is as well to remember that freezing bull sperm remains a relatively inefficent procedure. Advances in an understanding of the general principles of cryobiology, applicable to both bovine sperm and embryos, can be expected in the years ahead. It is possible that future times may see long-term storage of bovine sperm by way of freeze-drying. Already, the production of mice from freeze-dried sperm has been reported by Yanagimachi and Wakayama (1998); these researchers used low temperatures and pressures to dry the sperm and store them at 4°C for periods up to 3 months. In reconstituting the male gametes, it was a matter of replacing the water and injecting the sperm head into an oocyte; of 57 oocytes injected, 54 survived and 49 developed further. The transfer of 46 embryos into the uteri of three recipient mice resulted in the birth of 14 normal young. It should be noted that the freeze-dried sperm are motionless and not fertile in the conventional sense; however, that is not a serious obstacle when sperm injection is used to achieve fertilization (Yanagimachi, 1998). In the USA, a study reported by Keskintepe *et al.* (2002) was noteworthy in being the first to report the production of karyotypically normal blastocysts after the injection of bull sperm that had been selected, freeze-dried (lyophilized) and stored at 4°C until use.

6.3.3. Swim-up procedures

Motility is an essential feature of bull sperm if they are to progress through the cow's reproductive tract from the mouth of the cervix to the ampulla of the oviduct. Although their progress

through the uterus and oviduct is greatly aided by smooth muscle action, they still rely heavily on their own resources at certain stages of their journey. Penetration of the sperm head through the ZP presumably requires particularly vigorous propulsion (hyperactive motility) by the sperm tail. In the 1980s, the swim-up technique became a well-established step in many laboratories to obtain a highly motile sperm sample for use in the IVF of cattle oocytes (see Fig. 6.3); work in Madison by Parrish and associates showed significantly improved fertilization rates using the highly motile sperm cells obtained by way of swim-up.

In Ireland, Lu and associates used the technique, both in research and in attempts to commercialize the embryo production process. The swim-up procedure involved taking a thawed semen sample and overlaying it with an appropriate volume of a suitable medium, which was usually a calcium-free TALP preparation; the same medium was used both for swim-up and in the subsequent washing of spermatozoa. The semen sample was left in an incubator at 39°C for 30–60 min, during which time the progressively motile sperm in the semen sample swam up into the TALP medium. Although the swim-up procedure was capable of yielding a highly motile sperm sample, it suffered from the disadvantage of a relatively low yield of cells, an obvious practical consideration to those using very expensive semen straws. Angling the swim-up tubes (45° angle) rather than standing them vertically was one way of increasing the sperm yield, and French workers suggested that supplementing the TALP medium with 5 mM glycine could improve sperm quality and subsequent embryo yield. The relative merits of using swim-up, Percoll gradient and other separation techniques for the selection of bull sperm for IVF have been discussed in a number of reports (Cesari *et al.*, 1995; Gotz, 1995; Correa *et al.*, 1997b; Coscioni *et al.*, 2001).

Swim-up and hyaluronic acid

A swim-up procedure was developed by Shamsuddin and Rodriguez-Martinez (1994) in which frozen–thawed bull sperm were allowed to swim through a medium which contained HA (1 mg/ml) to a modified TALP solution; the HA-selected sperm showed significantly greater motility and resulted in a higher yield of embryos than those selected by the control medium.

Swim-up and caffeine

The function of bull spermatozoa and the production of embryos after swim-up in TALP containing different concentrations of calcium (1.8, 2.6 and 3.6 mM) and caffeine (2.5, 5.0 and 7.5 μM) was studied in Brazil by Coscioni *et al.* (2001); they found that the use of caffeine resulted in a greater percentage of sperm showing evidence of capacitation (based on chlortetracycline staining) in comparison with controls. The authors concluded that micromolar concentrations of caffeine in sperm TALP may stimulate sperm capacitation; the greatest frequency of capacitated sperm (53%) was found with the 7.5 μM dose level.

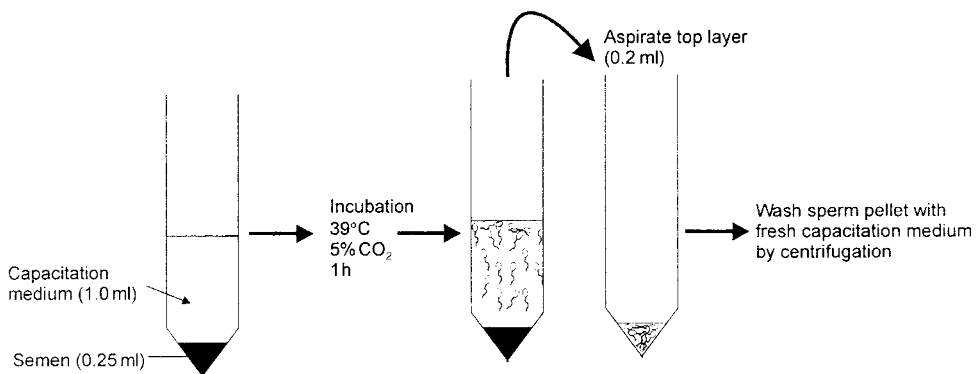

Fig. 6.3. Swim-up technique to obtain hypermotile sperm for IVF.

Swim-up and the sex ratio

Sex differences between bovine embryos have been attributed to various factors. A study by Lechniak *et al.* (2000) examined the effect of the duration of sperm–oocyte co-incubation and delayed insemination in their *in vitro* production (IVP) system, where sperm were selected by the swim-up method. They found that ageing of sperm for 6 h in TALP after swim-up and prior to sperm–oocyte co-incubation did not affect the rate of fertilization but did reduce the number of male embryos; ageing for 24 h significantly reduced fertilization rate and the number of male blastocysts.

6.3.3. Percoll density gradients

In human IVF clinics, the sperm separation method based on Percoll (a colloidal suspension of silica particles coated with polyvinyl pyrrolidone (PVP)) gained widespread acceptance in the 1980s and was successfully used in assisted human reproduction for several years. The agent was considered to be completely non-toxic to cells and to have essentially no free PVP. The Percoll separation method was seen to possess two advantages over alternative methods: (i) the most motile spermatozoa were effectively isolated; and (ii) motile sperm were quickly removed from seminal plasma, somatic cells and dead and morphologically abnormal spermatozoa. The latter consideration was thought important because motile sperm were protected from the potential stress of ROS, which would otherwise reduce their fertilizing capacity; it is known that superoxide radicals, hydrogen peroxide and the hydroxyl radical can result in peroxidative damage to the lipid membranes of sperm.

Although highly effective, the Percoll medium was withdrawn several years ago by its manufacturer (Pharmacia/Upjohn) for use in human sperm selection and for some time there was no obvious candidate to replace it. It was known that Percoll contained high levels of endotoxins, some 10–100 times the American Food and Drug Administration (FDA) cut-off level for human injectables; presumably in an age of high-cost medical litigation, the manufacturer felt there was little to gain in preparing it for use in the human field. There are now several alternatives to Percoll in use. One of these is the PureSperm density gradient, composed of silica-coated colloidal silica particles in a HEPES-buffered isotonic medium. A study reported by Sakkas *et al.* (2000) sought to determine the efficacy of swim-up and density-gradient centrifugation techniques in removing human spermatozoa with nuclear aberrations; their results indicated that both Percoll and the substitute (PureSperm) enriched a human sperm population by separating out male gametes with ‘nicked’ DNA and poorly condensed chromatin; in this regard, the same workers concluded that the swim-up technique may not be as efficient.

Work reported by Parrish *et al.* (1995) dealt with the Percoll gradient method to separate motile and non-motile frozen–thawed bovine sperm (see Fig. 6.4); they also compared the ability of Percoll- and swim-up separated spermatozoa to fertilize oocytes and produce embryos. They found that separation of sperm on a 45 and 90% discontinuous Percoll gradient required 15 min centrifugation ($700 \times g$) to obtain optimal recovery of motile sperm; motile sperm were present almost exclusively in the bottom 0.5 ml of the gradient after centrifugation. The authors recorded a much higher recovery of motile sperm with Percoll than with swim-up (see Table 6.4) and recommended the technique for routine use in cattle IVF. In Colorado, Seidel *et al.* (1995) compared the centrifugation of bull sperm through Percoll or BSA with a swim-up technique; they found centrifugation to be quicker and to give a much higher yield of sperm.

Although clearly successful in sperm separation, the use of Percoll was queried by some on the basis that the agent may bind calcium and for that reason interfere with the fertilization process. A paper by Keefer and Paprocki (1995) drew attention to the fact that a wash step to remove Percoll residues was sometimes omitted in published methods; they showed that this could seriously affect fertilization and subsequent embryo development. In Denmark, Avery and Greve (1995) identified Percoll treatment, using different batches of the agent, to be the cause of low cleavage rates in a cattle embryo production system; the authors suggested that the adverse effects of Percoll was not due to the Percoll particles *per se*, but to unbound PVP. In China, Chen *et al.* (1998) reported a recovery rate

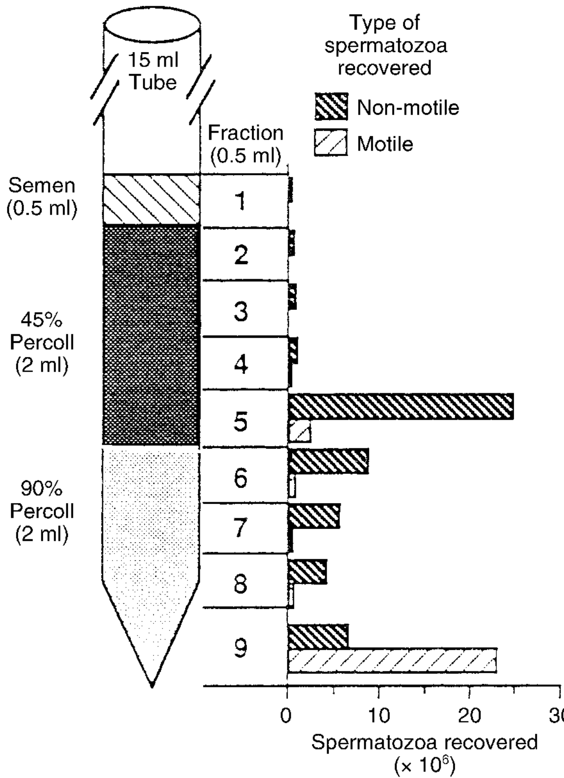

Fig. 6.4. Separation of bull sperm on a 45 and 90% Percoll gradient (from Parrish *et al.* 1995).

Table 6.4. Comparison of Percoll and swim-up techniques for sperm selection (from Seidel *et al.*, 1995).

Sample	Motile spermatozoa (%) (mean ± SEM)	Volume layered (ml)	Total no. spermatozoa layered ($\times 10^6$) (mean ± SEM)	No. of motile spermatozoa layered ($\times 10^6$) (mean ± SEM)	No. of spermatozoa recovered ($\times 10^6$) (mean ± SEM)	Motile spermatozoa recovered (%)[b] (mean ± SEM)
Initial	71 ± 4[c]	–	–	–	–	–
Swim-up	88 ± 1[d]	1.0	116 ± 5.0	80.7 ± 2.5	6.8 ± 0.8[c]	8.5 ± 1.0[c]
Percoll	91 ± 2[d]	0.5	58 ± 2.5	40.4 ± 1.3	15.9 ± 1.4[d]	39.8 ± 4.2[d]

[a]Replicated with semen from seven different bulls. For a replicate, three straws of frozen semen from a bull were thawed and pooled.
[b](Number spermatozoa recovered/number motile spermatozoa layered) × 100.
[c,d]Means with different superscripts within a column differ ($P < 0.05$).
SEM, standard error of the mean.

of motile bovine sperm after Percoll gradient treatment as 58% in comparison with 16% for sperm recovered by swim-up; there was also some evidence of a higher blastocyst yield with the Percoll treatment.

Sex ratio deviations

In the IVP of cattle embryos, it has been noted that a sex-ratio deviation may occur, resulting in the birth of a higher than usual proportion of males. The explanation of this effect is still

unclear but culture conditions and method of sperm preparation have both been implicated. A report by Rheingantz *et al.* (2000) compared Percoll separation with swim-up in regard to the sex ratio of blastocysts produced in their IVP system; they found that the swim-up method selected significantly more male embryos. A further paper by Rheingantz *et al.* (2002) dealt with the effect of glucose in the culture medium on the sex ratio of embryos produced with sperm selected by the swim-up or Percoll gradient methods; the sex ratio was similar for both sperm-selection methods. The authors concluded that the presence of glucose in the SOF with amino acids (SOFaa) culture medium resulted in a faster rate of development of the male embryos.

Other farm animals

The fertilizability and structural properties of boar sperm selected by Percoll gradient centrifugation were investigated by Grant *et al.* (1994) in Bristol; compared with a simple washing procedure, the Percoll method selected sperm with significantly higher motility and movement characteristics; such sperm resulted in significantly greater cleavage rates. In Korea, Rho *et al.* (2001) compared Percoll separation with swim-up and glass-wool filtration in their studies on the production of goat embryos *in vitro*; they concluded that the Percoll technique was superior to the other two methods for selecting motile goat sperm from frozen–thawed semen. Cleavage rate and blastocyst yield were significantly higher, as was the mean cell number.

6.3.5. Glass-wool filtration procedures

In the 1950s, it was shown that diluted bull semen, filtered through a layer of glass beads, resulted in dead sperm being retained by the filter while the live ones passed through. In human IVF, filtration of thawed human sperm samples through glass-bead columns has been shown to be more effective than the swim-up procedure for obtaining motile sperm. Work in human IVF has also shown that the use of glass wool rather than beads may also be used in obtaining a higher quality sperm population. Glass-wool filtration and swim-up separation of frozen–thawed bull sperm were compared by workers in Canada, who found comparable sperm quality but a much faster preparation time using the glass wool.

A paper by Miller *et al.* (1994) describes the selection of motile sperm by glass-wool column filtration. The filtration unit was prepared by loosely packing 90 mg of Pyrex glass wool into the barrel of a washed, 1 ml syringe. The syringe plunger was then replaced in the barrel so as not to pack the glass wool, and the unit was packaged and autoclaved. Immediately before use, the filtration unit was washed with 1 ml of TALP medium, the glass wool packed to a depth of 3–5 mm and the unit rewashed with 1 ml of TALP. Thawed semen from bulls was washed by suspension in 10 ml TALP and centrifuged at $200 \times g$ for 7 min. The resultant sperm pellet was suspended in 0.75 ml of fresh TALP, pipetted into the filtration unit and allowed to flow through the glass wool column; the column was then rinsed with an additional 0.75 ml of media to rinse through any residual sperm. Finally, motile sperm were concentrated by centrifugation at $200 \times g$ for 7 min and resuspended to the required concentration before being added to oocytes in 100 μl droplets under silicone oil.

6.3.6. Use of hyaluronic acid

Results from studies in Sweden in the early 1990s suggested that HA (a member of the proteoglycan family) could be usefully employed in isolating a motile fraction of bull sperm.

6.3.7. Cell-to-cell contact

In the cow, it is now well established that bull sperm are arrested in the caudal isthmus before ovulation to form an oviductal reservoir. When ovulation occurs, spermatozoa are released from the reservoir and ascend the oviduct to the site of fertilization in the ampulla. Cell-to-cell contact and adherence between the bull sperm and oviductal epithelial cells appears to be the main factor responsible for formation of the sperm reservoir. Increasing knowledge of events within the bovine oviduct may lead to improvements

in cattle embryo production and may have implications for human IVF. It seems likely that the sperm reservoir may act as a screening mechanism to select high-quality sperm for the fertilization process. In Italy, studies have produced evidence that: (i) bull sperm that have the ability to adhere to an oviductal monolayer *in vitro* are characterized by superior motility and lifespan and an intact acrosomal status; (ii) sulphated glycosaminoglycans, such as heparin, are powerful inducers of sperm release; (iii) adhesion to an oviductal monolayer may select highly fertilization-competent spermatozoa; and (iv) sperm selection may be due to exposure of adhesion molecules recognized by the oviductal cells (Gualtieri and Talevi, 2000a,b, 2001; Talevi and Gualtieri, 2000, 2001). The Italian workers suggest that further research to discover the nature of the molecular mechanisms involved may result in a separation technique that can be useful in IVF.

In the USA, a study by Ellington *et al.* (2000) compared fertilization and subsequent embryonic development by bull sperm that selectively attached to oviductal cells in co-culture; oocytes were added to oviductal cell monolayers that contained bull spermatozoa that remained attached to the oviductal cells after vigorous washing. They were able to demonstrate that such sperm supported significantly superior cleavage and embryo development rates; they believed this was due in part to superior chromatin quality in sperm selected by cell attachment.

Once attachment between bull spermatozoon and oviductal cell is established, it is difficult to induce detachment by normal washing procedures. For such reasons, Bosch *et al.* (2001) investigated the ability of heparin and Ca^{2+}-free medium to induce the detachment of sperm. They concluded from their studies that both calcium-free medium and heparin were capable of promoting displacement of the sperm and that such sperm can be used for IVF.

Cell-to-cell interactions with epididymal cells

Workers in Canada have shown that the co-incubation of frozen–thawed bovine spermatozoa with epididymal cell cultures could help in maintaining and enhancing their motility. A study reported by Reyes-Moreno *et al.* (1999) attempted to characterize the factors responsible for this beneficial effect; their work revealed the secretion of at least ten major proteins released by cauda epididymal cells. A further report from the Canadian laboratory dealt with a functional epididymal cell culture system that enhanced bull sperm motility; their epididymal-specific cell medium, with an osmolarity of 365 ± 5 mosmol (similar to the osmolarity of epididymal fluid) was prepared with RPMI-1640 medium, using HEPES as a buffer and adding myo-inositol, pyruvate, lactate, glycerol and carnitine (Sirard *et al.*, 1999b); the authors found that the motility and survival of cryopreserved sperm could be improved by the medium.

6.3.8. Sperm abnormalities

Sperm abnormalities are present in the semen of all mammalian species. Normally, in bull semen that has been accepted for freezing, the incidence of such abnormalities is such that it does not compromise the animal's fertility (see Table 6.5 and Correa *et al.*, 1997a,b). The sperm separation technique should clearly reduce rather than increase the incidence of defective sperm in the population used in IVF. A study of bull semen containing specific sperm abnormalities was made by Degelos *et al.* (1994) in the USA, using two-layer (45%/90%) and three-layer (50%/70%/90%) Percoll density gradients or swim-up separation. All the separation methods significantly improved the percentage of normal cells, primarily at the expense of distorted heads and protoplasmic droplets. Both Percoll methods increased sperm motility and decreased the percentage of abnormal cells.

Proximal droplets

A study in Colorado reported by Amann *et al.* (2000) reported on the fertilizing potential of semen containing a high percentage of sperm with a proximal droplet, using IVF. The workers concluded that semen from most yearling beef bulls with a high incidence of proximal droplets had severely compromised fertility; however, as these bulls matured, the incidence of proximal droplets decreased and IVF fertilizing potential increased. It appeared that semen containing > 30% spermatozoa showing the defect should

Table 6.5. Sperm abnormalities and fertility in bulls. Characteristics of frozen–thawed spermatozoa in low- vs. high-fertility Friesian bulls assessed by routine semen analysis, various other functional tests and fertility data (means ± SD). (From Correa *et al.*, 1997a.)

Characteristics	Fertility levels	
	Low	High
Concentration ($\times 10^6$ sperm per ml)	24.7 ± 5.1	24.3 ± 3.4
Motility (%)	67.6 ± 7.1	72.5 ± 6.0[a]
Grade (0 to 4)	3.6 ± 0.2	3.6 ± 0.7
SM1 units	285.9 ± 36.5	300.9 ± 57.3[a]
Morphology (% normal)	75.9 ± 4.9	82.2 ± 4.9[a]
Acrosomal status (% intact)	78.2 ± 5.0	83.7 ± 4.6[a]
Spermatozoa with coiled tails (%)	11.5 ± 2.7	9.8 ± 2.6
Swollen spermatozoa (%)	31.7 ± 9.9	41.9 ± 7.3[a]

[a]Significant differences between the low- and high-fertility Friesian bulls ($P < 0.05$).
SD, standard deviation.

be taken as strong evidence that the fertility of the bull is likely to be poor. In Canada, Thundathil *et al.* (1999, 2000a) conducted experiments to determine the effect of proximal protoplasmic droplets on sperm–oocyte binding, zona penetration, fertilization and developmental competence of resulting embryos, using IVF and *in vitro* culture. Some sperm with proximal droplets were selected out by swim-up and none were bound to ZPs; the fertilizing ability of normal sperm in semen containing gametes with proximal droplets was also impaired.

Nuclear vacuoles

The *in vitro*-fertilizing characteristics of bull sperm with multiple nuclear vacuoles were studied by Pilip *et al.* (1995) in Canada; they found that semen containing a high percentage of such sperm had poor fertilizing ability. Spermatozoa with this type of defect apparently gain access to the oocyte and bind to the ZP; however, vacuolated sperm cells penetrated the ZP at a reduced rate and apparently did not form normal male pronuclei *in vitro*. A report by Thundathil *et al.* (1998) dealt with *in vitro* embryo production with semen in which 60% of sperm had multiple nuclear vacuoles; significantly fewer vacuolated sperm bound to the ZP than in the control sample. The authors concluded that vacuolated sperm are defective in zona binding, but that after gaining access to the ooplasm they are capable of participating in fertilization and early embryonic development.

Knobbed acrosome defect

The effect of the knobbed acrosome defect in bull sperm on IVF and early embryo development was investigated by Thundathil *et al.* (2000b) in Canada. It was found that sperm with this defect had a reduced ability to bind to the ZP and did not penetrate it. The authors also showed that apparently normal sperm coexisting in the inseminate of bulls with a high percentage of knobbed sperm were also functionally defective; oocytes penetrated by such sperm had a reduced potential for fertilization and for normal embryonic development.

Robertsonian translocations

Translocations of the Robertsonian type (centric fusion) are associated with reduced fertility in cattle. IVF and *in vitro* embryo culture provide one approach to determining the impact of translocations on the ability of bull sperm to support early embryonic development and a possible method of predicting the fertility of the bull. A study in Norway reported by Lonergan *et al.* (1994b) dealt with the use of semen from a Norwegian Red bull heterozygous for the 1:29 translocation in an IVF programme; they found that sperm produced by the bull were capable of fertilizing IVM oocytes and were able to develop to the blastocyst stage, although the yield of blastocysts was significantly below that of the control bull (12% vs. 20%). The results of a study by Rubes *et al.* (1999) in the Czech

Republic indicated that sperm from a 16 : 20 translocation had reduced fertilizing ability and resulted in a decreased blastocyst yield; they also recorded similar results using a bull with a 14 : 20 translocation. It was concluded that early embryonic development was reduced after IVF with semen from these two bulls, probably due to genetically unbalanced spermatozoa.

Hypo-osmotic swelling (HOS) as a screening assay

Some workers have subjected sperm to the HOS test to evaluate sperm membrane integrity (Matkovic *et al.*, 2001) or to determine whether the results could be correlated to the fertilizing capacity *in vitro* (see Fig. 6.5; Rota *et al.*, 2000b). The HOS test has been suggested as a simple method of evaluating sperm fertilizing capacity by various workers in cattle fertility (Correa and Zavos, 1995) and human fertility. The ability of the sperm tail to swell in the presence of a hypo-osmotic solution can be taken as a measure of the transport of water molecules across the plasma membrane to attain osmotic equilibrium. As this osmotic phenomenon is dependent upon an intact semi-permeable plasma membrane, the sperm tail swelling test is believed to be an indicator of membrane integrity and functional ability.

6.3.8. Sperm doses

In the cow's oviduct, fertilization is likely to occur in conditions where the sperm–oocyte ratio may be close to 1 : 1 (Hunter, 1996). Cattle IVF is routinely performed in many laboratories at a fixed concentration of 1 million spermatozoa ml^{-1} (Rizos *et al.*, 2002a,b) and at a sperm–oocyte ratio of 5000 : 1. A study by Tanghe *et al.* (2000) in Belgium evaluated the effect on fertilization rates of increasing the sperm–oocyte ratio (from 5000 : 1 to 50,000 : 1) and confining sperm and oocytes in a small volume of TALP fertilization medium. IVF was performed either in a large (500 μl) or small (50 μl) volume and sperm concentration was increased by lowering the oocyte numbers. Their results showed that increasing the sperm–oocyte ratio above the normal 5000 : 1 value did not significantly affect fertilization rate or the incidence of polyspermy; varying the volume of the fertilization medium had no effect.

In Brazil, Camargo *et al.* (2000a) used four sperm concentrations (0.5 million–4 million per ml) in a study with Guzera bull semen and recorded a markedly lower fertilization rate after IVF with the 0.5 million dose level. In a further report, Camargo *et al.* (2000b) employed three different sperm concentrations (1 million, 2 million and 4 million per ml) and two co-incubation

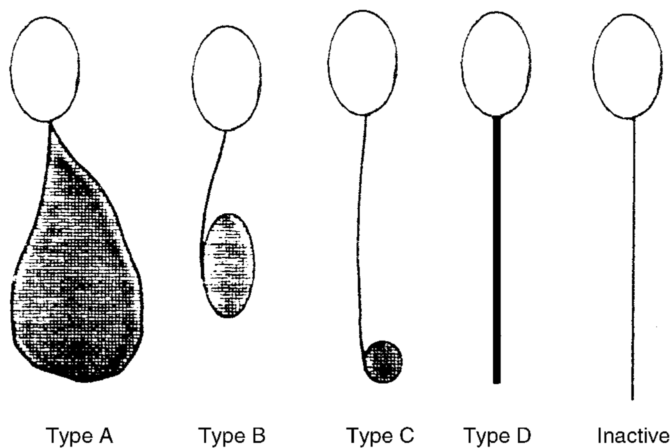

Fig. 6.5. Hypo-osmotic swelling to screen bull sperm. Diagrammatic illustration of sperm swelling patterns as measured by the hypo-osmotic swelling (HOS) test. Sperm swelling type A represents maximal sperm swelling; sperm swelling types B and C represent intermediate sperm swelling stages; and sperm swelling type D represents the initial swelling response in the HOS test. Non-swollen spermatozoa are considered to have a functionally inactive or damaged sperm membrane. (From Rota *et al.*, 2000b.)

periods (12 and 18 h) in their studies with zebu cattle; they found no effect on cleavage rate or blastocyst yield but there was a marked drop in cleavage rate when oocytes were incubated for 18 h at the highest sperm dose. It appeared that some embryos that cleaved after the 18 h period were unable to develop to the blastocyst stage, although this was not the result of polyspermy.

6.4. Enhancing Sperm Motility

It is clearly important to have highly motile bull sperm available for IVF; this may be achieved by applying various procedures for isolating motile samples (swim-up techniques, Percoll density gradients). There are also several pharmacological compounds that may be employed to stimulate and maintain the motility of bull sperm. In using such agents in IVF, due regard should be taken of possible effects on the oocytes that may come in contact with them. A paper dealing with the detrimental effects of human sperm motility-enhancing agents on mouse oocytes and embryos indicated the care that should be taken in this area (Scott and Smith, 1995); they found that pentoxifylline, caffeine, 2-deoxyadenosine and cyclic adenosine monophosphate (cAMP) may have adverse effects on mouse oocytes or embryos at concentrations commonly used to activate sperm in human IVF.

6.4.1. Penicillamine, hypotaurine, epinephrine (adrenalin) (PHE)

During the 1980s, researchers in various countries, including Ireland, demonstrated the beneficial effects of adding penicillamine (20 μM), hypotaurine (10 μM) and epinephrine (adrenalin) (1–2 μM) (PHE) to the IVF medium. It is known that penicillamine can increase the percentage of sperm that undergo the acrosome reaction (AR) when used in the presence of adrenaline. It is also known that hypotaurine increases sperm motility and that a combination of adrenalin and hypotaurine can increase oocyte penetration rate by bull sperm. Careful preparation and storage of PHE is essential. In the mixture, hypotaurine is reasonably stable, but in solution catecholamines, such as adrenalin, readily oxidize to cytoxic adenochromes; this change is accelerated by exposure to light, oxidizing agents or alkaline pH and is inhibited by penicillamine. The PHE stock solution is placed in vials, wrapped in aluminium foil and stored at −20°C until required.

Results reported by Miller *et al.* (1994) in the USA indicated that PHE or dispersed bovine oviductal epithelial cells (BOEC) added to their TALP fertilization medium were effective, either alone or in combination, in promoting earlier oocyte cleavage and in achieving a higher yield of developmentally competent embryos. The authors suggested that both PHE and BOEC may have served as antioxidants in their fertilization culture system, which used a gas atmosphere of 5% carbon dioxide in air; an adverse effect of atmospheric oxygen levels on early embryonic development has been attributed to the formation of oxygen free radicals, which accelerate the processes of lipid peroxidation and cell-damaging enzyme activation. It is known that two of the PHE components, hypotaurine and adrenalin, are able to limit superoxide formation, which may inhibit lipid peroxidation within the sperm cell. The PHE mixture is used by some commercial cattle embryo production laboratories. In the Netherlands, a report by Merton *et al.* (2000) dealt with various considerations in the preparation of sperm for IVF; they added PHE and heparin to their TALP fertilization medium either at the time of insemination or 6 h in advance. They found that timing of the PHE addition did affect embryo yield, recording a significant decrease when this was 6 h in advance rather than at the time of insemination (15.3% vs. 21.6%). In Brazil, working with zebu cattle semen, Peixer *et al.* (2002) used PHE and heparin in their TALP fertilization medium.

6.4.2. Caffeine, theophylline and pentoxifylline

More than 30 years ago, caffeine, a methylxanthine derivative and an inhibitor of cAMP, was shown to increase the motility and respiration of bull sperm; the stimulatory effect is mediated by cAMP, which accumulates after phosphodiesterase inhibition. The treatment of bull

sperm with a caffeine combination is known to improve sperm motility by promoting the uptake of calcium ions, suppressing phosphodiesterase and eliciting an increase in intracellular cAMP (see review by Lanzafame *et al.*, 1994).

Although caffeine will promote the ability of frozen–thawed bull sperm to penetrate oocytes, it may not be effective with all bulls; for such reasons, Hamawaki *et al.* (1995) in Japan investigated the effect of other methylated xanthines, including theophylline. They were able to show that sperm treated with 10 mM theophylline achieved a significantly higher penetration rate than those treated with caffeine. A study by Hori *et al.* (1997) in Japan reported significantly higher percentages of monospermic oocytes and of bovine embryos developing to the blastocyst stage when hypotaurine (10 mM) rather than caffeine (5 mM) was used in the IVF medium.

Pentoxifylline is another methylxanthine derivative, which has proved useful in improving the fertilization rate in human assisted reproduction. In Turkey, for example, Tasdemir *et al.* (1998) used the agent to initiate motility in testicular spermatozoa in an attempt to distinguish between live and dead sperm; the Turkish workers reported that sperm samples without pentoxifylline remain immotile even after 60–90 min of incubation, whereas addition of the agent initiated motility in all their samples. In Bulgaria, studies by Hinev *et al.* (2000) found that they could stimulate motility in surgically extracted sperm by the addition of pentoxifylline (3 μM/ml). In Japan, Numabe *et al.* (2001a) have reported on the effect of pentoxifylline on cattle IVF (see Table 6.6); they examined the effects on the fertilization rate of various concentrations of the agent (0–7.5 mM) in combination with heparin (10 iu/ml). The authors recorded a significantly higher fertilization rate with heparin plus 5 mM pentoxifylline than with heparin alone; the percentage of monospermy (81%) was significantly higher than with heparin alone (57%). The authors concluded that treating bull sperm with 5 mM pentoxifylline in combination with heparin is useful in cattle IVF and that this treatment is effective even for bulls that show low fertilization rates and embryo yields after sperm treatment with caffeine-with-heparin or heparin alone.

Table 6.6. Effect of pentoxifylline on cattle IVF. Effect of concentration of pentoxifylline in the heparin-containing* fertilization medium on the penetration rates of bovine oocytes.[†] Sum of four trials. (From Numabe *et al.*, 2001a.)

Concentration – pentoxifylline (mM)	Frequency and percentage[†]	
	Penetrated[‡]	Monospermy
0	30/50(60)[b]	17/30(57)[b]
2.5	38/50(76)[ab]	25/38(66)[ab]
5	43/50(86)[a]	35/43(81)[a]
7.5	32/50(64)[b]	20/32(63)[ab]

*10 IU/ml heparin.
[†]Spermatozoa obtained from bull A.
[‡]Percentage of oocytes penetrated at 16 h post-insemination.
[ab]Values within each column with different superscripts are significantly different ($P < 0.05$; chi-square test).

6.5. Preparing Oocytes for Fertilization

It is now clear that many factors associated with cattle oocytes influence the outcome of IVF in this species; certain of these factors relate to the source and quality of the oocyte and the steps that are taken in handling it prior to exposing it to sperm.

6.5.1. Beneficial effects of cumulus cells

IVF in the cow is routinely performed with cumulus-enclosed oocytes; when the cumulus oophorus is removed shortly before insemination, the IVF rate decreases markedly. In Canada, Chian *et al.* (1995a) presented results indicating that cumulus cells participated in the mechanisms of cattle sperm capacitation and the AR and that polyspermy was directly affected by the number of capacitated and acrosome-reacting sperm on the oocyte surface. Their results also suggested that the ideal sperm concentration for cattle IVF was 0.8 million–1.5 million sperm/ml and that removal of sperm–cumulus complexes from the oocyte surface 12 h after insemination may reduce the incidence of polyspermy, especially at high sperm concentrations. In a further paper, Chian *et al.* (1996) concluded that bovine cumulus cells

produce a chemical substance that either attracts spermatozoa or facilitates sperm penetration when very few sperm are around the oocyte.

A study by Tanghe *et al.* (2001) in Belgium sought to evaluate whether cumulus-conditioned medium was able to normalize the fertilization rates of cumulus-denuded oocytes. They found that the use of the conditioned medium went some way towards compensating for the loss of cumulus cells (39% vs. 57%) and was much above the rate found in denuded oocytes (16%). They suggest that the fertilization rate was not completely normalized by the conditioned medium because the causative factor was either unstable or diluted to below the effective dose or because direct or proximate contact of sperm cells with cumulus cells is necessary to improve the fertilizing ability of sperm.

In Germany, a study by Fontes *et al.* (2002) investigated the effect of different denudation methods on the fertilization of cattle oocytes and on their subsequent embryo development. The denudation methods included vortexing and pipetting (passing COCs in and out of a 100 μl pipette) in combination with either hyaluronidase or trypsin. Their results (see Table 6.7) demonstrated that, although the presence of cumulus cells is not absolutely necessary for fertilization, their denudation methods did result in significantly lower blastocyst yields. In the Netherlands, Fatehi *et al.* (2002) found that removal of cumulus cells prior to IVF significantly reduced cleavage rate (25% for denuded oocytes vs. 56% for COCs); they suggested, on the basis of experimental evidence, that this effect was due to the loss of a factor secreted by the cumulus cells, which they believed was progesterone.

A study by Saeki *et al.* (1994) examined the effects of cumulus cells on sperm capacitation, AR and penetration of bovine oocytes in a protein-free medium in which denuded oocytes failed to be fertilized. Although cumulus cells did not affect capacitation and the AR, the addition of cumulus cells was effective in facilitating sperm penetration of the denuded oocytes. The use of cumulus cells matured with gonadotrophin and oestradiol enhanced sperm penetration much more than when immature cells were employed. It was concluded from these and other data that direct or proximate contact of bull sperm with matured cumulus cells may be necessary for improving the fertilization rate.

The need for cumulus-enclosed oocytes in cattle IVF appears to be in contrast to what is thought to occur in the cow oviduct, where attached cumulus cells are rapidly eroded from the ovulated oocyte (see 6.2.3 above). Such dispersion of cumulus and corona cells after IVM does not appear to occur readily; follicular cells may be attached quite firmly to artificially matured oocytes even 22–24 h after being exposed to sperm in the IVF medium. Obviously, the factors operating in the bovine oviduct to free the cumulus cells are not normally simulated in the IVF dish. It may be that the particular method of denuding oocytes is of relevance. In Ireland, work in the late 1980s suggested that the use of a 3% sodium citrate solution over a 5 min period, with gentle agitation to disperse the cumulus, had no adverse effect on either cleavage rate or embryo yield (see Fig. 6.6).

Table 6.7. Effect of denudation method on IVF and embryo development in cattle. Effect of denudation protocol and oocyte selection on *in vitro* fertilization and embryo development (data shown as mean % ± SEM). (From Fontes *et al.*, 2002.)

Group	Treatment	COC (*n*)	Cleavage rate (day 3)	Blastocyst rate (day 7)	Hatched rate (day 7–day 12)
1	Control	228	$77.9 \pm 4.0^{a,d}$	$48.3 \pm 4.0^{a,d}$	$37.2 \pm 4.1^{a,d}$
2	Hyaluronidase + vortex	137	61.7 ± 4.9^{b}	19.3 ± 4.9^{e}	10.4 ± 5.0^{c}
3	Hyaluronidase + pipette	142	$67.5 \pm 4.9^{a,b}$	24.7 ± 4.9^{e}	10.4 ± 5.0^{c}
4	Hyaluronidase + pipette	111	$66.2 \pm 6.0^{a,b}$	27.7 ± 6.3^{b}	19.6 ± 6.1^{b}
5	Trypsin + vortex	140	60.0 ± 4.9^{e}	18.0 ± 4.9^{e}	4.5 ± 5.0^{e}
6	Trypsin + pipette + polar-body selection	92	$49.1 \pm 6.0^{c,e}$	25.3 ± 6.3^{b}	4.4 ± 6.1^{e}

Different superscripts within the same column differ significantly ($^{a,b,c}P < 0.05$; $^{d,e}P < 0.01$).
SEM, standard error of the mean.

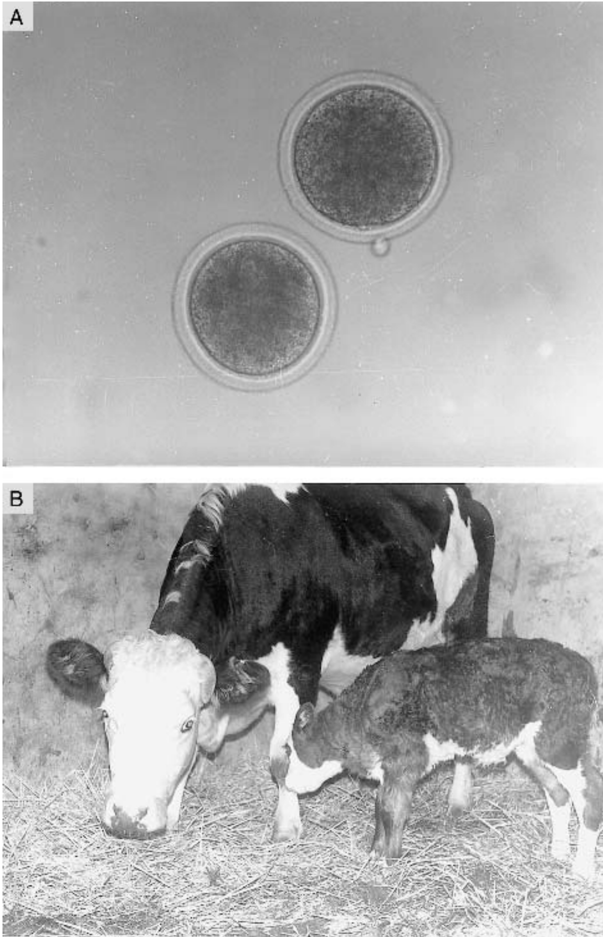

Fig. 6.6. Denuding the mature bovine oocyte with sodium citrate. A sodium citrate solution was used in Irish studies to clean bovine oocytes prior to fertilization (A). The use of this 'cleaning' process was examined at all stages through to embryo transfer and the birth of calves, as shown in (B).

Some of the potential advantages of using a simple, effective oocyte-cleaning procedure are detailed in Table 6.8; such cleaning may have particular relevance for those engaged in sperm injection or other forms of gamete micromanipulation.

Whether spermatozoa are faced with a cumulus-enclosed or a naked oocyte will vary from one mammalian species to the next. In the mouse, for example, the cumulus oophorus that surrounds the newly ovulated oocyte is believed to play an important part in fertilization, although its exact function remains uncertain. A study by Takahashi *et al.* (1994) in mice showed that cumulus-enclosed oocytes were more likely to be penetrated by sperm than cumulus-free oocytes; 30-fold more sperm were required to obtain the maximum level of fertilization in denuded cells. The authors concluded that soluble factor(s) sustained in the COC promoted fertilization and that the integrity of the three-dimensional structure of the cumulus oophorus was important for efficient sperm–oocyte interaction.

Cumulus-cell removal after fertilization

In routine cattle IVF, some hours after insemination presumptive zygotes are denuded by gentle vortexing or other means, before being washed and placed in the embryo culture medium. Looking towards the future, some workers have been engaged in designing and testing new ways of removing cumulus cells from zygotes. In Madison, Zeringue *et al.*

Table 6.8. Citrate cleaning of cattle oocytes.

Potential advantages of citrate cleaning of bovine oocytes	Examination of the cleaned oocyte permits easier assessment of quality
1. Potentially less risk of damage to the zona pellucida, which may have implications for freeze–thawing of embryos subsequently 2. Speed and reproducibility of oocyte preparation is enhanced 3. For research purposes, cumulus cell presence does not confound the interpretation of monolayer (IVC) studies 4. May result in a lower rejection rate of oocytes prior to insemination; may be 5% rejection rather than 10–15% with the partial stripping procedure 5. The citrate cleaning-technique is well suited for scaling-up procedures in cattle IVF, allowing less skilled personnel and possibly lower numbers of sperm per insemination. One operator can clean more than 500 oocytes in about 15 min	1. Permits the identification of polar bodies 2. Allows assessment of granulation of ooplasm to be made more easily 3. Can determine the extent of the perivitelline space 5. Can apply computer enhancement techniques which are capable of measuring oocyte size, volume, colour and other features 6. No interference from cumulus cells in carrying out enzyme analyses or other procedures with the zygote or embryo 7. For sperm-injection purposes (SUZI or ICSI) it is necessary to have a clean oocyte 8. Application of invasive procedures for embryo sexing is simplified by the absence of attached cumulus cells

IVC, *in vitro* culture; SUZI, subzonal sperm insertion; ICSI, intracytoplasmic sperm injection.

(2002) determined cleavage rates of bovine zygotes mechanically stripped of cumulus cells in microfluidic devices constructed by way of polydimethylsiloxane fabrication techniques. They reported that 90% of the zygotes underwent cleavage after cumulus-cell removal with their device and suggested that its use may decrease the handling necessary in dealing with single embryos in a commercial setting.

6.6. *In Vitro* Fertilization Culture Systems

Many factors are involved and contribute to the successful interaction of oocyte and spermatozoon in cattle IVF. In terms of the culture systems, there are several possibilities. The studies of Lu in Ireland in the late 1980s used microdroplets (50 μl) of modified TALP under mineral oil in a Petri dish; the TALP formulation, which has been widely used as a fertilization medium, was based on studies with hamsters by Bavister and colleagues in the late 1970s. Male-dependent variability in the outcome of embryo production in cattle has been described by various workers, as well as measures taken to lessen the extent of such variability (Kreysing *et al.*, 1997).

6.6.1. The fertilization medium

Fertilization in the cow normally occurs in a closely controlled environment within the confines of the ampulla of the Fallopian tube. In setting up an appropriate system for cattle IVF, it is clear that the medium employed must be capable of providing the mature oocyte and the capacitated sperm with the environment in which sperm penetration may readily occur.

TALP medium

The TALP medium is a modified Tyrode preparation containing 25 mM sodium bicarbonate and bovine serum albumin (0.6%). The Tyrode/albumin (TA) medium is further modified with varying amounts of the energy sources sodium lactate and sodium pyruvate (LP). Optimal concentrations of these energy sources appear to be 10 mM lactate and 1.0 mM pyruvate in the presence of 5.6 mM glucose; the osmolarity is held at 285–295 mosmol. When TALP is used as the fertilization medium, BSA is employed as a protein source. Various studies have shown that protein supplementation is not essential when oocytes are enclosed by cumulus cells but is essential when oocytes are cumulus-free. A study by Saeki *et al.* (1994) involved incubating cattle COCs and denuded oocytes with bull

sperm in medium with and without BSA; for medium with and without BSA, fertilization rates were 92 and 89%, respectively, for COCs and 57 and 6% for denuded oocytes. The same workers showed that 82% of denuded oocytes were fertilized when inseminated in the presence of isolated cumulus cells. A paper by Pavlok (2000) showed that granulosa cells were capable of extending the fertile lifespan of bovine frozen–thawed sperm, a finding that may help in improving cattle IVF when low-viability sperm are involved (see Table 6.9).

SOF medium

An alternative to TALP medium used for IVF in some laboratories has been an mSOF formulation. In Italy, Lazzari *et al.* (1999a) compared TALP medium with SOF and reported that the quality of embryos developed on day 7 was significantly superior when IVF was carried out in SOF supplemented with essential amino acids (EAA), non-essential amino acids (NEAA), glutamine and glycine. They concluded from their data that SOF devoid of glucose was a suitable medium for cattle IVF and that amino acid supplementation of the medium improved the quality of the developing embryos. In New Zealand, Tervit and Pugh (2000) examined the effects of sperm and pyruvate concentration in mSOF on the subsequent development and quality of bovine embryos after IVF in the medium; they found evidence of a significant interaction between sperm concentration and pyruvate. The authors showed that there was no effect of pyruvate on embryo quality at the lower sperm concentration (0.5 million/ml) whereas at the high concentration (2.0 million/ml) embryo quality increased as pyruvate concentration increased.

Fert-CDM medium

A study reported by Lu and Seidel (2002) examined the amino acid requirements of the bovine oocyte as it developed from fertilization to the blastocyst stage; oocytes were fertilized either in a chemically defined medium (Fert-CDM) containing NEAA or in a conventional TALP medium in the absence of NEAA. Sperm penetration rate at 5 h post-insemination (hpi) was significantly greater in Fert-CDM (69%) than in TALP (52%) and oocyte development to the two-cell stage at 25 hpi was much more advanced in the Fert-CDM (35% vs. 0%).

6.6.2. Protein supplementation

Although there have been reports of cattle oocytes being fertilized in a chemically defined, protein-free medium (Tajik *et al.*, 1994), it is usual for IVF media to be supplemented with cattle serum or BSA to achieve an acceptable fertilization outcome with bovine oocytes. There are reports showing that fertilization is possible in protein-free medium under some conditions. In Japan, Saeki *et al.* (1995) published results showing that protein supplementation was not necessary to augment the effects of heparin in cattle IVF; they showed that cumulus-enclosed oocytes were fertilized with and without BSA. The Japanese workers believed that cumulus cells may facilitate the penetration of the bovine oocyte by sperm in a medium without protein

Table 6.9. Effect of granulosa cells on bull sperm motility. The fertilizing capacity of spermatozoa for two sets of oocytes: the fertilization intervals were 0 to 8 h and 8 to 23 h for the first and second set of oocytes, respectively; the oocytes were either with or without cumulus cells. (From Pavlok, 2000.)

Cumulus cells	Fertilization interval		Total no. of oocytes	Penetrated oocytes		Polyspermic oocytes		Mean no. of spermatozoa per penetrated oocyte
	0 to 8 h	8 to 23 h		No.	%	No.	%	
+	+	–	67	65	97.0[b]	21	32.3[bc]	1.63
+	–	+	73	67	91.8[b]	18	26.9[bc]	1.48
–	+	–	73	73	100.0[b]	32	43.8[b]	2.10
–	–	+	76	19	25.0[c]	2	10.5[c]	1.00

[a]Percentage of penetrated oocytes.
[b,c]Data in the same column with different superscripts differ significantly ($P < 0.01$).

supplementation. In the absence of serum proteins, however, there have been reports that the formation of pronuclei may be retarded (Eckert and Niemann, 1995). In the USA, Martus *et al.* (1998a,b) studied the effect of a bovine oviductal-specific glycoprotein on the fertilization of oocytes; they showed that the fertilization rate was significantly higher than where albumin was used (62% vs. 31%). In Denmark, a study by Holm *et al.* (1999a) dealt with the effect of briefly exposing IVM oocytes to bovine serum or BSA on fertilization and subsequent embryonic development; they showed that exposure of oocytes matured and fertilized in defined conditions to serum for 2 min prior to IVF enhanced cleavage rate and blastocyst yield; this was not found with oocytes exposed to BSA.

Although it appears that very low levels of bovine serum will support fertilization, that does not apply to low concentrations of BSA. Studies by Holm *et al.* (2000b) attempted to identify the bovine serum fractions that most effectively support fertilization and early embryo development; they showed that supplementation of their IVF medium (TALP, with heparin, penicillamine, hypotaurine and adrenalin) with a high-molecular serum fraction (> 300 kDa) supported cleavage and blastocyst development as readily as complete serum. The authors concluded that substances of very high molecular weight, such as the larger globulins, and not albumin may be important for ensuring an acceptable outcome in cattle IVF.

6.6.3. Gas phase considerations

A report by Lazzari *et al.* (1998) dealt with the effect of lowering the oxygen level in their IVF culture system. While most of the bulls used in their large-scale IVP operations achieved a high fertilization rate using sperm concentrations varying from 0.1 million to 3 million sperm/ml in TALP supplemented with 10 μg heparin and a gas phase of 5% carbon dioxide in air, some bulls (1–2%) did not perform satisfactorily. In attempts to overcome this problem, the oxygen level was reduced from 20 to 5%, on the assumption that this might reduce oxidative damage to the sperm; as shown in Table 6.10, this reduction resulted in a dramatic improvement in fertilization rate. The same authors extended this low oxygen protocol to all bulls in their IVF programme and achieved results that led them to conclude that reducing the oxygen level had several advantages: (i) it usually improved the fertilizing ability of bull sperm and enabled those few bulls that did not fertilize at 20% oxygen to be used; (ii) it reduced the amount of semen required for IVF; and (iii) it had no adverse effect on the developmental capacity of the fertilized oocytes. In Japan, Takahashi and Kanagawa (1998) incubated IVM cattle oocytes with spermatozoa for 18 h under a gas atmosphere of 5% carbon dioxide with 5% or 20% oxygen; they recorded that the percentage of inseminated oocytes that developed to the blastocyst stage was higher under 5% oxygen compared with 20% (34.4% vs. 24.7%).

Table 6.10. Effect of oxygen level on cattle IVF (from Lazzari *et al.*, 1998).

Bull	IVF in 20% oxygen	IVF in 5% oxygen
A	23% (10/44)	79% (34/43)
B	34% (15/44)	90% (36/40)
C	19% (10/52)	69% (27/39)

6.6.4. Temperature, light and osmolarity

Temperature

It is well established that the fertilization of cattle oocytes is a temperature-dependent process; much evidence supports the view that a temperature of 39°C is optimal not only for the maturation of cattle oocytes but for sperm penetration as well. In studies with pigs in the 1980s, Cambridge workers found that the most important factor influencing successful penetration of oocytes by boar sperm was temperature; less than 1% of oocytes were fertilized at 37°C but a high penetration rate (89%) was achieved when sperm and oocytes were incubated at 39°C.

Light

In the course of cattle IVF, oocytes and sperm are inevitably exposed to either daylight or artificial light for variable periods; the general rule to

observe is to keep exposure of oocytes and sperm to light to the minimum. Work in Ireland in the late 1980s suggested that cattle oocytes, bull sperm and early cattle embryos are reasonably tolerant of the lighting conditions that normally prevail in an IVF laboratory.

Osmolarity

Little information is available on the effect of the osmolarity of the fertilization medium on the fertilization and development of cattle embryos. In Korea, Yoon *et al.* (2000) prepared TALP media of normal osmolarity (114 mM NaCl: 295–300 mosmol) or low osmolarity (96 mM NaCl: 260–265 mosmol) and incubated cattle oocytes for 18 h; each 50 μl droplet of medium contained heparin-treated sperm at a concentration of 2 million/ml. In contrast to what has been recorded in rats and rabbits, bovine oocytes fertilized in the hypotonic solution showed a much higher developmental competence to the blastocyst stage than those fertilized in the normal TALP medium (24.2% vs. 16.9%); the same authors found that the monospermic fertilization rate was significantly higher with the hypotonic solution than with normal TALP (91.5% vs. 71.7%).

6.6.5. Somatic cells in the fertilization medium

Workers in cattle IVF have exposed oocytes to sperm either singly or in groups of varying numbers (e.g. 5–50) It is generally held that IVF may be more effective when oocytes are exposed as a group rather than singly; the implication is that oocytes and their associated cells may produce and secrete substances promoting fertilization.

In the live cow, fertilization is believed to occur in the presence of relatively few sperm in the Fallopian tube, but these sperm would obviously be in contact with the live cells of the oviduct at all times. It should also be recognized that, in the live animal, the ovulated bovine oocyte is in constant contact with oviductal cells from the instant of ovulation; there are obviously endless opportunities for interactions between the oviductal epithelial cells and the oocyte.

Experiments reported by Schneider *et al.* (1999) dealt with cattle IVF systems in which sperm were exposed to somatic cell co-culture. It was thought possible that incubation of sperm with somatic cells could provide a fertilization environment that more closely simulated conditions *in vivo*. They were able to demonstrate that sperm cells co-cultured with buffalo–rat liver (BRL) cells fertilized cattle oocytes that cleaved at a higher rate and developed to the morula stage and beyond more readily than those co-cultured with bovine oviductal cells or cell-free controls (see Table 6.11). They speculated that this might be due to an antioxidant and/or sperm chromatin-stabilizing effect of factors from the BRL cells, similar to that recorded previously with bovine oviductal cells.

6.6.6. Activation of COCs with calcium ionophore (A23187)

Workers in Germany investigated the possibility that activation of cumulus-enclosed bovine

Table 6.11. Effect of co-culture of bull sperm with BRL cells (from Schneider *et al.*, 1999).

Sperm preparation protocol[a]	No. of oocytes inseminated	Embryo cleavage	Embryo development
BOEC	453	51 ± 22%	42 ± 33%[b]
BRLC	426	60 ± 20%	61 ± 28%[c]
Control	442	60 ± 17%	39 ± 24%[d]

[a]Bovine oviductal epithelial cells (BOEC), buffalo-rat liver cells (BRLC) or cell-free controls.
Percentage of embryo cleavage = embryos cleaved/oocytes inseminated (mean ± SEM).
Percentage of embryo development = embryos developing to morula or beyond/embryos cleaved (mean ± SEM).
[b]Data were pooled by treatment across all bulls.
[c,d]Different superscripts within column differ ($P < 0.01$).
SEM, standard error of the mean.

oocytes in the period between penetration of the spermatozoon and syngamy would influence the yield of blastocysts (Woehl *et al.*, 2002); they found that treatment of IVM COCs with calcium ionophore, in the presence of calcium and magnesium ions, at 9 and 12 h after insemination, increased blastocyst yield significantly (38.3% vs. 26.7%).

Table 6.12. Effect of glutathione (GSH) in fertilization medium on blastocyst yield.

Treatment	Blastocyst yield	Hatching rate
GSH in IVF medium	42%	20%
Control	23%	9%

Differences significant ($P < 0.05$).

6.6.7. Oxidative stress in the IVF culture system

In human assisted reproduction, interest has grown in the past decade in the role of oxygen toxicity and free-radical reactions in relation to fertility problems; it is evident that superoxide radicals, hydrogen peroxide and the hydroxyl radical can result in peroxide damage to the lipid membranes of the spermatozoon. In some infertile men, ROS have detrimental effects on sperm function, decreasing sperm motility and viability; this can impair the outcome of conventional IVF. A study of the effect of antioxidants on the fertilizing ability of ram sperm in an IVF system was reported by Stojanov *et al.* (1994b) in Australia; they found that the addition of either superoxide dismutase or catalase to the semen diluent improved the fertilization rate of ram semen that had been chilled–stored (5°C) for 7 days. The authors believed this effect might be attributable to a decrease in lipid peroxidation. In cattle, it is known that ROS may affect spermatozoa either beneficially or adversely, depending on the balance between the rate of their generation and removal.

In Japan, Miyamura *et al.* (1995) studied the kinetics of GSH concentration and the role of this molecule during maturation and in subsequent events; they concluded that GSH is an important cytoplasmic factor for regulating sperm nuclear decondensation and male pronucleus formation after sperm penetration. A report by Earl *et al.* (1997) presented evidence that GSH treatment of bovine sperm enhanced blastocyst yield. In the Netherlands, Van Soom *et al.* (1998) showed that the addition of GSH during fertilization doubled embryo production but had no effect on embryo quality (see Table 6.12). Studies by Kim *et al.* (1999a,b) in Belgium reported that the effect on embryo development of the addition of GSH to their IVF medium (modified TALP) varied according to the bull; the effect was positive with some and negative with others. The authors reported that GSH was stable in the fertilization medium even after 18 h of incubation and that there was a dose-dependent increase in GSH concentration in the oocytes.

6.6.8. Other factors influencing efficacy of IVF system

Glucose

Compounds such as glucose have been routinely included in fertilization media, such as TALP, as a source of energy for the gametes; this may not be essential. A study reported by Martins *et al.* (2002) assessed the effects of different concentrations (0–14 mM) of glucose added to the fertilization medium; they found that glucose was not required for cattle IVF under their culture conditions.

GH-RH

Clinical studies with subfertile bulls have shown that pulses of gonadotrophin-releasing hormone (GH-RH) often increase fertility within a few days, indicating that the effect may be on the sperm rather than on the sperm production process. In Colorado, Funston and Seidel (1995) added GH-RH to IVF media to determine the effect on cleavage rates of bovine oocytes; they found evidence that GH-RH and GH-RH agonists enhanced fertilization rates and they detected gene expression for the GH-RH receptor in COCs. The workers concluded that GH-RH may act directly on COCs to increase cleavage rates. In Italy, Rota *et al.* (2000a,b) also tested the effect of adding GH-RH to their cattle IVF medium (TALP); they found no beneficial effect on

cleavage rate and were unable to detect GH-RH receptors either on sperm or in COCs.

Methyl-β-cyclodextrin

It is known that cholesterol loss from the sperm plasma membrane is one of the events that occurs during capacitation of bull sperm. It is also known that this cholesterol can be removed by the agent methyl-β-cyclodextrin. Studies by Suh *et al.* (2000) in Colorado showed that adding the agent to a chemically defined IVF medium increased cleavage rate but not blastocyst yield.

Hyaluronic acid

There are some IVF culture conditions in which the use of HA as an additive may have a favourable effect. In Tehran, Tajik (1998) denuded cattle oocytes and inseminated them with thawed bull sperm in a protein-free Brackett–Oliphant (BO) medium containing various concentrations of HA; in the presence of PVP, significantly higher penetration rates were observed in the presence of HA.

Dimethylsulphoxide (DMSO)

The effect of DMSO added to the IVF medium on the motility and the AR of bull sperm and the subsequent development of bovine oocytes was examined by Tsuzuki *et al.* (2000a,b) in Japan; they reported that DMSO at micromolar levels in IVF might stimulate the development of embryos from some bulls.

Prostaglandins

It is known that prostaglandins are involved in many aspects of sperm function and may either stimulate or inhibit sperm motility. In Portugal, Baptista *et al.* (2000) evaluated the role of prostaglandins during swim-up and fertilization procedures; they confirmed that prostaglandins F and E (PGF and PGE) play a role in capacitation and fertilization and suggested that the very same prostaglandin may play different roles in each of these two processes.

Toxic factors

The residual toxicity of plastic ware sterilized by ethylene oxide (EO) gas was examined by Holyoak *et al.* (1995) in Utah; they concluded that, even after 12 days of aeration, the residues in plastic ware sterilized by EO may be more detrimental to sperm than to oocytes, resulting in a loss of fertilizing capability.

6.7. Interaction of Spermatozoon and Oocyte

Fertilization in most animal species involves a transient increase in the intracellular free Ca^{2+} concentration of the oocytes, which takes the form of a Ca^{2+} wave starting from the site of sperm attachment. The origin of this calcium is believed to be the inositol triphosphate (IP_3)-induced Ca^{2+} release from the endoplasmic reticulum. It is known that IP_3 is derived from the hydrolysis of phosphatidylinositol-4, 5-biphosphate (PIP_2), which is catalysed by the enzyme phospholipase C under regulation of a guanine nucleotide-binding protein (G protein). G proteins are a class of membrane proteins that couple membrane receptors to membrane effector enzymes.

6.7.1. Sperm–oocyte recognition mechanisms

For some time past, evidence has been accumulating to show that fertilization in the cow and other farm mammals is mainly controlled by interaction between the sperm and components of the oocyte. There is much evidence to show that oocyte and sperm surface adhesion molecules regulate the process of fertilization in mice and it is likely that similar regulation occurs in other mammals (see Wassarman, 1994; Brewis and Moore, 1997). Bull spermatozoa must undergo an exocytotic event (the AR) before they can fertilize the oocyte. It is believed that the mechanism involved in sperm–oocyte binding is based on the interaction of proteins and carbohydrates present on the surface of the gametes. A paper by Brandelli *et al.* (1995) suggested that fucosyl and *N*-acetyl-galactosaminyl (GalNAc)-binding sites on the bull sperm surface

were involved in the induction of the AR and ZP recognition. It appears that the carbohydrates (GalNAc) and galactose are part of the TEC-2 epitope located on the plasma membrane of the bovine oocyte. A study by Gougoulidis *et al.* (1999a,b) in Australia investigated the involvement of these carbohydrates in the fertilization process; their results led them to conclude that GalNAc has a specific role during sperm–oolemma fusion in cattle.

Fertilization marks the start of the transition from oocyte to embryo and the stimulus for oocyte activation at fertilization is an increase in the concentration of intracellular calcium; it is believed that calcium is released from intracellular stores in the endoplasmic reticulum. It is now well established that calcium is a second messenger employed by a variety of cells to convey signals generated by various factors. Calcium typically carries out its signalling role in the form of very short bursts (spikes) of increased free intracytoplasmic calcium concentration, which repeat periodically (calcium oscillations). In the mammalian oocyte, calcium oscillations occur during meiotic maturation and at fertilization, where they trigger oocyte activation; after fertilization, oscillations continue until around the time that pronuclei form (Mizuno *et al.*, 1994; Nakada and Mizuno, 1998).

There are differing views as to how the fertilizing spermatozoon induces calcium oscillations and activates the oocyte. There appear to be two main views about the type of messenger system used by the sperm cell to bring about calcium release (see Fig. 6.7). On the basis of the fact that the spermatozoon takes up calcium during its capacitation period, it is thought that the male gamete is responsible for either injecting calcium into the oocyte or mediating calcium influx into the cell. In the former view, the spermatozoon acts like a hormone molecule and binds to specific receptors on the oocyte plasma membrane; in the latter view, the spermatozoon triggers calcium release by first fusing with the oocyte and then introducing a soluble factor directly into the ooplasm.

A study by Okitsu *et al.* (2001) in Japan found evidence suggesting the presence of a soluble sperm factor in bull sperm capable of activating the bovine oocyte. In the USA, Knott *et al.* (2002a) noted that the inositol-triphosphate receptor (IP_3R) is implicated in mediating the release of Ca^{2+} at the time of fertilization; they presented results showing that a particular isoform, type 1 IP_3R, played an important part in the activation of cattle oocytes. They saw the need for further work to determine whether this is the only Ca^{2+} release channel responsible for generating oscillations in bovine oocytes during fertilization.

Changes in intracellular calcium concentrations in cattle oocytes after penetration by sperm in the normal fertilization process were recorded by Sun *et al.* (1994); activation induced multiple transient spikes at intervals of about 25 min. The Cambridge workers suggest that IP_3-independent Ca^{2+} stores are mobilized during fertilization of cattle oocytes. In Canada, Chung *et al.* (2000) examined a method of oocyte activation that mimics the calcium oscillations observed in the normal process of fertilization in the cow; oocytes were activated by three 5 min incubations with ionomycin at 30 min intervals.

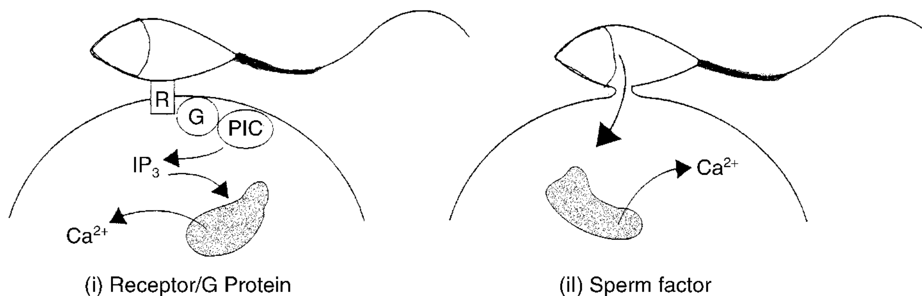

Fig. 6.7. Possible mechanisms whereby calcium oscillations may be generated in the fertilization process. R, receptor; G, G protein; PIC, phospholipase C. (From Homa *et al.*, 1993.)

Oviductal factors

Studies reported by Way *et al.* (1994) dealt with the effect of bovine oviductal fluid (ODF) on sperm binding and penetration of cattle oocytes in an IVF system. Their results indicated that the binding of bull sperm to the ZP was maximized by oocyte incubation in fluid taken from the isthmus in the non-luteal phase of the cycle; they also found that factors in ODF influenced the number of sperm binding to ODF-treated oocytes.

6.7.2. Early events in the fertilization process

Although the mechanisms of oocyte penetration have been reasonably well documented in laboratory mammals, the literature describing such events in cattle and other farm animals is more limited. Knowledge of events in the early hours after sperm penetration, including the time at which the male and female pronuclei form and when the first and subsequent cleavages of the embryo occur, is clearly essential for an understanding of the normality or otherwise of the zygotes and embryos produced by the IVF process. There have been several groups reporting on events at and after IVF in cattle in recent years (Choi *et al.*, 1994; Laurincik *et al.*, 1994a,b, 1998a,b; Navara *et al.*, 1994; Sun *et al.*, 1994; Wang *et al.*, 1997; Campbell *et al.*, 1998; Hunter *et al.*, 1998b; Kochhar and King, 1998; Kohler, 1998; Liu and Yang, 1999; Stephenson and Brackett, 1999; Fair *et al.*, 2000).

Fertilization involves activation of the oocyte by the spermatozoon; without this stimulus, the bovine oocyte would be unable to form pronuclei and become a zygote. A striking feature of activation in the cow is that the vitellus shrinks in volume, expelling fluid into the perivitelline space. At the same time, the sperm head in the vitellus swells and acquires the consistency of a gel, losing its characteristic shape. The final structure, which resembles the nucleus of a somatic cell much more closely than it does a sperm head, is termed the male pronucleus. A diagrammatic illustration of the cellular events and interactions that occur around the time of fertilization in the cow is provided in Fig. 6.8. The dramatic transformation of the sperm nucleus, in terms of morphology and synthetic activity,

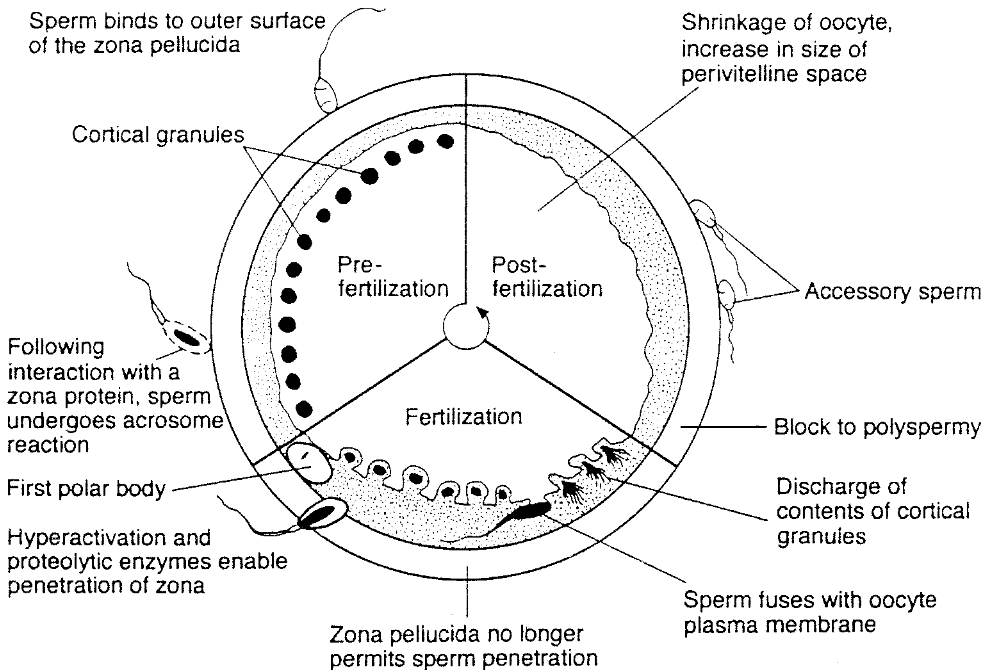

Fig. 6.8. Gamete interactions at fertilization in the cow.

involves the dissolution of the nuclear envelope and the decondensation of chromatin in the ooplasm, an event associated with a similar activation of the oocyte chromatin. Decompaction of the sperm nucleus involves a reduction in the disulphide bonds of protamine. The sperm-specific protamines are replaced by histone and non-histone proteins during formation of the male pronucleus in preparation for DNA synthesis.

In Ireland, an analysis of penetrated IVM bovine oocytes at various intervals after *in vitro* insemination led to the definition of six stages of early pronuclei development, set out in Table 6.13.

The time required for sperm penetration of IVM oocytes (with attached cumulus and corona cells) was less than 4 h; sperm-head decondensation occurred within 1–2 h of oocyte penetration and the male pronucleus developed after a further 3–5 h (see Figs 6.9 and 6.10).

A paper by Chian *et al*. (1999a) dealt with an evaluation of morphological events of sperm development during male pronucleus formation following IVF of cattle oocytes. The first evidence of sperm penetration was observed 4 hpi and reached its peak at 9 hpi. Male pronuclear formation was first recorded at 9 hpi and reached its maximum rate at 14 hpi. The morphological changes of the penetrated sperm during transformation into the male pronucleus were as follows: (i) sperm nucleus decondensation; (ii) recondensation of sperm nucleus; (iii) redecondensation of sperm nucleus; (iv) prepronucleus; and (v) male pronucleus. The authors suggest that the recondensation of the sperm following decondensation may be a necessary step for the bovine sperm nucleus to develop into a male pronucleus in order to replace protamines by histone and non-histone proteins. Further papers by Chian *et al*. (1999b,c,d) provided evidence that protein phosphorylation may be related to the formation of the male pronucleus in cattle oocytes.

Changes in zona pellucida

Workers in Japan studied the hardening of the ZP that occurs in the bovine oocyte after fertilization (Iwamoto *et al*., 1999); they concluded that formation of disulphide linkages together with specific proteolysis during fertilization induces the construction of a rigid structure that is responsible for the hardening of the ZP.

6.7.3. Crossing the interspecific sperm barrier

It is generally held that the mammalian oocyte is protected against penetration by foreign sperm by a barrier that operates at the level of the ZP, the vitelline membrane or both. An IVF culture system can be useful in examining events that are normally difficult or impossible to study under natural conditions in farm mammals.

Table 6.13. Developmental stages of male and female pronuclei.

	Male	Female
PN-1	The complete sperm in the ooplasm, head stains heavily, no sign of decondensation is seen, tail is still attached to head	Chromosomes are contracted to a dot or second meiotic division has just started, (anatelophase II)
PN-2	Sperm head starts to decondense, enlarges in size, stains less, and tail is detached	Second polar body is in the process of extrusion, or second meiotic division is completed
PN-3	Sperm head is further decondensed, nuclear envelope starts to form, midpiece is found in the vicinity of the head	Chromosome decondensation occurs, nuclear envelope starts to form, second polar body is located close to the PN
PN-4	Chromosome decondensation is completed, the more or less spherical PNs are surrounded by a complete envelope.	
PN-5	PNs enlarge in size, the PN contents stain evenly, second polar body and the sperm tail can still be located	
PN-6	PNs reach maximum size, the distance between the two is very small, sperm tail is still visible	

PN, pronucleus.

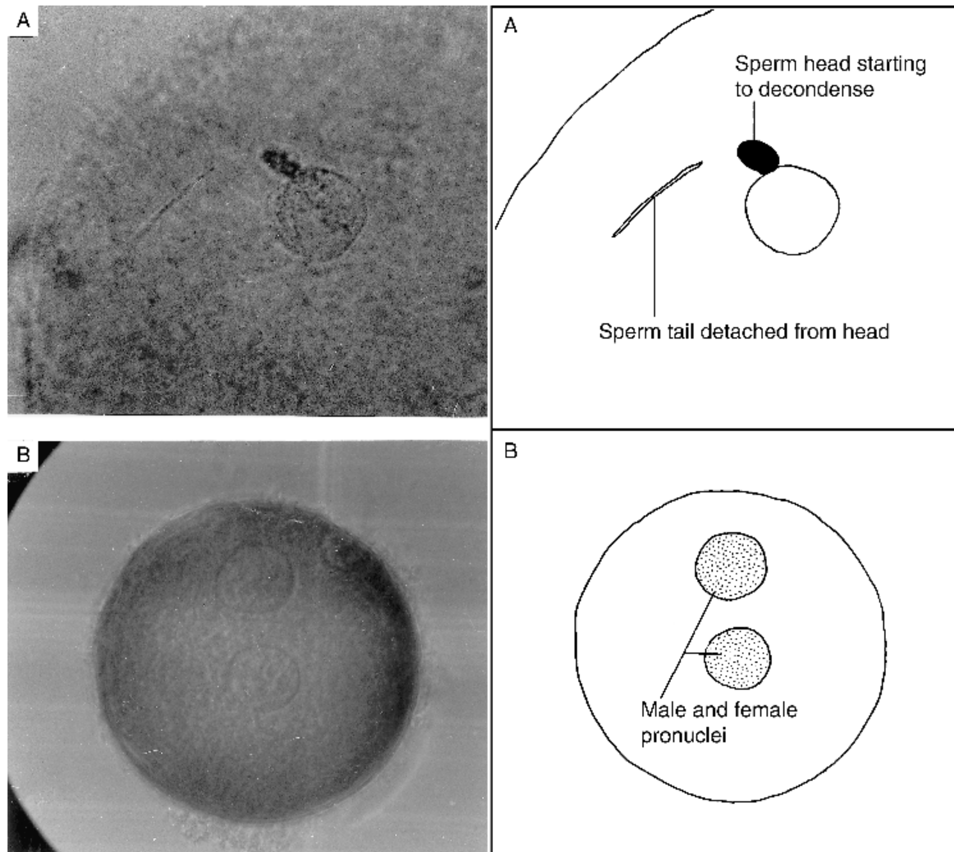

Fig. 6.9. Decondensation of sperm head and formation of pronuclei.

6.7.4. Factors with a negative effect on fertilization

Zona hardening

Various workers have dealt with some of the factors that may have a negative effect on the fertilization process due to their influence on the gametes. In Poland, Katska *et al.* (1999a,b) induced hardening of the ZPs of cattle oocytes by placing them in isolated bovine oviducts for periods of 20–40 min; the dissolution time of pronase-treated oocytes decreased in proportion to time spent in the oviducts. They recorded a decreasing cleavage rate with increasing time spent in the oviduct. They also recorded a normal sex ratio with epididymal sperm (50 : 50) but a markedly skewed ratio (69.2% males : 30.8% females) with ejaculated sperm.

6.8. Post-insemination Treatment of Oocytes

6.8.1. Effect of sperm exposure time

In many laboratories, mature bovine oocytes are co-incubated with spermatozoa for 18–20 h after *in vitro* insemination. In contrast to what occurs in the live cow, where the ovulated oocyte is believed to encounter very few free-swimming sperm at the site of fertilization, the oocyte in the IVF medium is likely to be surrounded by large numbers of sperm. In the cow's oviduct, there is usually ample time for blocks to polyspermy to be expressed before a second spermatozoon reaches the cell; in the Petri dish there is a chance that such blocks may not always operate in time to prevent polyspermy. At Washington State University,

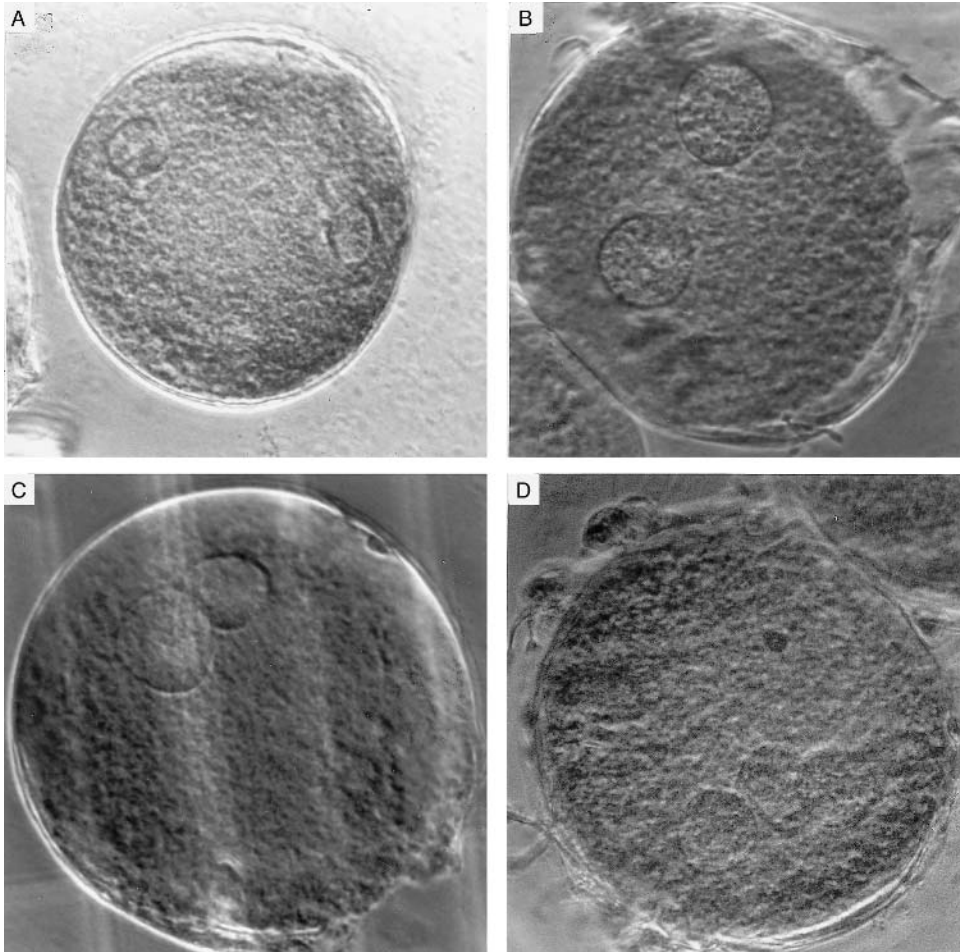

Fig. 6.10. Pronuclei in the bovine oocyte: normal and abnormal. (A) Pronuclei at stage PN-3; (B) pronuclei at stage PN-6; (C) pronuclei about to fuse; (D) abnormality – three pronuclei evident.

Rehman *et al.* (1994a,b) examined the effect of sperm exposure time on the outcome of IVF. They found that a 24 h period of co-incubation of cattle oocytes and frozen–thawed sperm yielded the highest fertilization rates (76%); rates were significantly lower (25–54%) for shorter co-incubation periods of 4–12 h. The authors found that the length of sperm exposure had no effect on the developmental potential of the fertilized oocytes.

A report by Gomez and Diez (2000), on the other hand, drew attention to the fact that prolonged oocyte and sperm co-culture during IVF may have adverse effects on subsequent embryo development. In addition to an excessive production of ROS, the polyspermy rate is likely to increase with prolonged co-culture (Sumantri *et al.*, 1996). The authors of the Spanish study removed bovine COCs about 2 h after co-culture with sperm and placed them in sperm-free TALP medium; their results, after removing cumulus cells at 18 hpi, showed no adverse effect on subsequent embryo development. It was concluded that those sperm playing a role in IVF are enclosed in the COC within 2 h of co-culture. In Brazil, Dode *et al.* (2002b), working with zebu cattle oocytes, reported that fertilization results were not improved when sperm and oocytes were co-incubated beyond 12 h; this was true regardless of the sperm preparation method used (swim-up, Percoll gradients, washing by centrifugation).

6.9. Micro-assisted Fertilization

There are four approaches to gamete micromanipulation that may assist fertilization under conditions of reduced sperm number or when sperm motility is severely compromised or even non-existent; these approaches are illustrated in Fig. 6.11 and involve the techniques of zona thinning, zona drilling, subzonal sperm insertion (SUZI) and ICSI. The first three of these are of relatively minor importance; the fourth is likely to be of much greater interest, for both research and practical application. It should be noted that these techniques are not necessarily directed towards influencing the fertilization process; some are directed towards enhancing the embryo hatching rate.

6.9.1. Zona thinning

There may be occasions when it would be useful to achieve fertilization of artificially matured cattle oocytes with lower than usual sperm doses. Zona thinning by enzymatic digestion has not been reported in cattle IVF. In human IVF, however, the technique has been one approach sometimes used in patients with a history of failed fertilization, using a mild trypsinization treatment. In mice, there have been studies demonstrating that partial digestion of the zona pellucida with the enzyme pronase was both safe and capable of enhancing fertilization at very low sperm concentrations. Zona thinning was attempted by Amano *et al.* (1999) to improve embryo hatching, but without any clear evidence of a useful effect.

6.9.2. Zona drilling and partial zona dissection

Zona drilling refers to the use of micromanipulation to introduce a gap(s) in the ZP either by laser ablation or chemically by localized application of a zona solvent. Partial zona dissection (PZD) is usually a mechanical technique that creates a breach in the zona. A report by Basovsky (1999) gave results for oocytes in which an incision was made in the zona (PZD); evidence was presented of a significant increase in the fertilization rate (28% vs. 8%) at the lowest sperm concentration employed (0.18 million sperm/ml) but not at the more usual sperm concentrations (1.55 and 0.54 million sperm/ml). Although the aim may be to facilitate fertilization of the oocyte, the same type of technique has been employed in an effort to assist the hatching of human and other embryos. There have been reports that no more than < 25% to 30% of human IVF embryos hatch in culture and the hope has been that

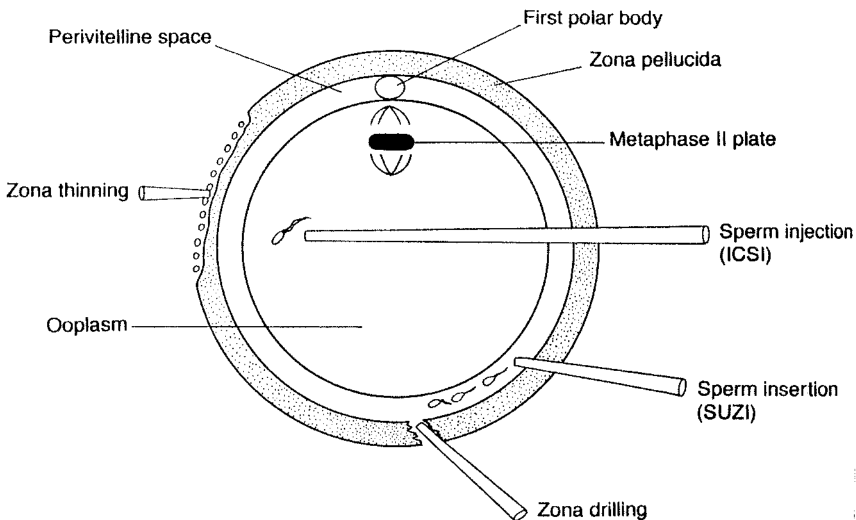

Fig. 6.11. Four approaches to micro-assisted fertilization in the cow.

making an opening in the zona to weaken it could enhance the hatching process. In Oxford, Dokras *et al.* (1994) made a slit in the ZP of 2–5-day-old human embryos and showed that this would significantly improve the partial and complete hatching rates; they concluded that this approach should be assessed in patients. In the USA, Gao *et al.* (1996) used a nitrogen-pulse cool laser to enhance hatching of cattle embryos; PZD has been shown to significantly improve the *in vitro* hatching rate of IVP cattle embryos in Austria. The effect of PZD on the hatching of cattle oocytes was dealt with in a report by Amano *et al.* (1999) in Japan; no clear effect on complete hatching was obtained. A paper by Stojkovic *et al.* (1998) dealt with the effect on the hatching rate of bovine embryos of laser microdissection of the ZP.

Zona drilling, first described in mouse oocytes, involved introducing a gap in the ZP through which sperm could gain direct access to the oocyte's vitelline membrane. There have also been reports in which acid Tyrode's solution has been employed to drill holes in the ZPs of rodent oocytes. Various workers have demonstrated the possibility of achieving higher fertilization rates using zona-drilled oocytes at very low sperm concentrations in mice; there are also those who have suggested that zona drilling may have a favourable influence on the subsequent hatching of the embryo. In this regard, it may be relevant to note a paper by Pokorny and Pokorny (1996) which claimed that puncturing the ZP of the bovine embryo may be a successful way of dealing with poor-quality cattle morulae/blastocysts; after such a puncture (using a small needle), the authors recorded evidence of a significant increase in pregnancy rate (65% vs. 34%).

A paper by Katska *et al.* (1999b) in Poland noted that the hatchability of zona-drilled cattle blastocysts was significantly greater than that of control embryos. In Korea, Park, S.P *et al.* (1999) used a 1.48 μm diode laser beam to drill cattle embryos in an effort to improve hatching; they reported a significant increase in the percentage of embryos that commenced and completed hatching as a result of the treatment. In Germany, Schmoll *et al.* (1999) showed that a single hole (40 μm in diameter) produced by a laser would significantly increase the hatching rate of IVP blastocysts; ZP thinning and expansion were not observed in the laser-treated group. In Boston, Eroglu *et al.* (2002) demonstrated that a non-contact, 1480 nm diode laser could be used for the immobilization of human sperm and for opening a hole in the ZP to facilitate ICSI, biopsy manipulation for preimplantation genetic diagnosis and assisted hatching.

6.9.3. Subzonal sperm insertion (SUZI)

SUZI is a technique in which one or more spermatozoa are placed under the ZP, on the understanding that one will be able to fuse with the vitelline membrane and effect fertilization. In human assisted reproduction, polyspermy has been a problem when SUZI was performed using more than ten sperm; other reports have suggested that the insertion of four spermatozoa was optimal in increasing the fertilization rate without the risk of polyspermy. A paper by Catt *et al.* (1994b) noted that, although the polyspermia rate decreased in their study when fewer than five sperm were inserted, the monospermic fertilization rate of 8% was unacceptably low; they favoured a method of inserting up to 20 sperm on the first attempt and adjusting sperm numbers as appropriate in subsequent cycles. Subzonal microinsemination bypasses the spontaneous process of sperm binding to the ZP, followed by the AR and penetration through the zona into the perivitelline space.

For such reasons, SUZI has occasionally been used to clarify some of the mechanisms involved in fertilization. Fujimoto *et al.* (1994) showed that the fertility of subzonally inseminated mice oocytes was enhanced when cultured in medium with 3.42 mM calcium, suggesting that the concentration of extracellular calcium exerted an important effect on the progress of events after sperm adherence to the vitelline membrane. In normal fertilization, only acrosome-reacted sperm are able to fuse with the oocyte's vitelline membrane under normal circumstances; for this reason, it has often been assumed that the efficacy of SUZI might be improved using sperm treated to induce the AR. Under normal physiological conditions, the AR is induced during sperm interaction with the oocyte investments, but Tesarik and Mendoza (1994) showed that the frequency with which sperm fused with the oocyte after SUZI was much

lower than the frequency of AR in the sperm sample used. It was concluded that a spontaneous AR does not necessarily predispose a spermatozoon to fuse with the oocyte after SUZI.

A limited number of reports dealing with SUZI in cattle first appeared more than a decade ago; although fertilization rates were low, the technique was regarded as useful in studying sperm–oocyte interactions in the cow. Studies by Pavasuthipaisit *et al.* (1994a,b) in Thailand compared fertilization and early embryonic development in cattle after the injection of capacitated sperm either directly into the ooplasm or under the ZP; the ICSI-treated oocytes showed the higher fertilization rate (66.7% vs. 50%). In Germany, Clement-Sengewald *et al.* (1996) achieved fertilization when three to five bull sperm were trapped with optical tweezers and inserted directly through a laser-drilled hole into the perivitelline space; although the fertilization rate was very low, it was believed that modifications of the technique would permit better results.

6.9.4. Intracytoplasmic sperm injection (ICSI)

Defective sperm function remains the single most important cause of human infertility (Hull *et al.*, 1985). At the same time, a survey of the medical literature suggests that traditional treatments of the human male have produced little or no improvement in sperm parameters (Devroey *et al.*, 1998). For such reasons, the most recent decade has seen ICSI applied increasingly around the world to alleviate problems in human patients with severe male infertility; many thousands of children have been born as evidence of the technical efficiency of the procedure since the first ICSI birth in January 1992. This has been the case despite the fact that, until relatively recently, well-regarded texts dealing with human IVF contained little in the way of encouraging information on the possibility of sperm injection being of relevance as a clinical procedure. That is a measure of the speed at which the ICSI procedure has been taken up in human assisted reproduction, with several books and hundreds of reports dealing with the technique being published by the year 2002.

In human assisted reproduction programmes, the first use of the ICSI technique was often as a follow-on to the use of the alternative procedure of SUZI. It became clear, however, from the many reports emanating from clinical practice, that sperm injection almost invariably achieved results, in terms of fertilization rates, considerably better than those found with SUZI (Van Steirteghem *et al.*, 1993, 1994; Fishel *et al.*, 1994; Catt *et al.*, 1994a,b,c,d). In fact, the ICSI procedure quickly established itself as superior to any other form of assisted reproduction in dealing with human male infertility. The mechanism of fertilization after ICSI in the human is dealt with in a paper by Dozortsev (1996) and detailed in Table 6.14. A paper by Joris *et al.* (1998) dealt

Table 6.14. Proposed sequence of events in human ICSI (after Dozortsev, 1996).

1. Immobilization of the sperm with the injection pipette induces damage of the sperm plasma membrane in such a way that the sperm nucleus decondensing factor (SNDF) of the oocyte is able to reach and decondense the sperm nucleus
2. Polyvinyl pyrrolidone (PVP) in the injection medium stabilizes the sperm plasma membrane or changes the chemical properties in its vicinity, thereby preventing sperm and oocyte interaction
3. As PVP becomes diluted after the placement of the sperm in the ooplasm, SNDF is able to reach the sperm chromatin, inducing initial, activation-independent, sperm nucleus swelling that ruptures the sperm plasma membrane
4. Such rupture allows sperm-associated oocyte activating factor to leave the spermatozoon and induce oocyte activation by binding to inositol-1,4,5-triphosphate, which in turn mobilizes intracellular calcium for oscillations
5. The release of sperm-associated activating factor takes place within 30 min of sperm injection
6. Resumption of meiosis (early anaphase of the second meiotic division) occurs some 2–3 h after ICSI. Two to three hours later, the late anaphase stage is reached and sperm and oocyte chromosomes become indistinguishable from each other; the second polar body is expelled at this time
7. Shortly afterwards, the male and female chromatin undergoes further decondensation to develop into male and female pronuclei

with the laboratory set-up and injection procedure employed in human ICSI.

However, as with any novel treatment, the early years of ICSI usage in human assisted reproduction has left several questions still to be satisfactorily resolved. The fact that sperm injection effectively circumvents the sperm-selection processes that have developed in the evolution of mammalian fertilization mechanisms has been regarded as a matter of serious concern (see Fraser, 1998; Hewitson *et al.*, 2000); many would agree with the view that short-term success in the use of ICSI should in no way reduce the urgent need for research to resolve many of the underlying problems of human male infertility. It has to be made clear, however, that the evidence from human ICSI births indicates that the malformation rate is no different from that in the general population or in other assisted-reproduction surveys.

It is evident that ICSI can be carried out with fresh and frozen–thawed epididymal sperm in human patients with obstructive azoospermia and with testicular sperm in most patients with obstructive azoospermia and in some patients with non-obstructive azoospermia. Despite current restrictions on embryo research in the European Union (permissible only in Italy, the UK and Belgium), considerable progress has been made in developing and refining the human ICSI technique as a result of the efforts of the formidable teams engaged in its clinical application.

Twelve thousand years into the past

Japanese researchers have been in the forefront of those working on ICSI in a range of mammalian species (see Table 6.15; Goto, 1997). A paper reviewing the achievements of workers in Japan in the application of ICSI in domestic and zoo animals was published by Iritani *et al.* (1998). A recent expedition to the Arctic wastes was made by Goto seeking to recover testicular tissue from the remains of the mammoth, a specialized elephant that died out some 12,000 years ago but whose remains periodically come to light as glaciers in the frozen north melt in the summer sun. Goto's hope is to use sperm cells from the mammoth to fertilize elephant oocytes, producing cross-bred offspring which may be graded up, using mammoth spermatozoa, until the cross-breds are almost pure mammoth.

Table 6.15. Factors affecting the success of ICSI (from Goto, 1997).

1. Sperm pretreatment (immobilization, acrosome reaction)
2. Sperm/semen parameters
3. Maternal age/oocyte quality
4. Characteristics of oocyte membrane
5. Site of sperm deposition
6. Stimulation protocols
7. Use of PVP
8. Type of micromanipulator (Piezo vs. conventional)
9. Experience

Although this sounds like the stuff of science fiction, it illustrates one of the more unusual potential uses of sperm injection. It is evident both in cattle and in humans that fertilization may be achieved using immature gametes (spermatids) recovered from testicular tissue (see Goto *et al.*, 1996; Tesarik and Mendoza, 1996; Tesarik *et al.*, 1998).

ICSI in cattle

Although there was one report in the early 1980s dealing with sperm injection in cattle, development went no further than pronucleus formation. Within a few years, however, a Japanese group at Kagoshima reported the fertilization of cattle oocytes with immobilized 'dead' sperm and the birth of several normal and healthy calves (Goto and Yanagita, 1995). In terms of fertilization and embryo production rates, it was evident from the Japanese ICSI results that they were far below those recorded in conventional cattle IVF. This was in marked contrast to the experiences of those engaged in human ICSI, where sperm injection apparently triggers normal fertilization and embryonic development. In cattle, however, there have been many workers who have believed that ICSI must be accompanied by artificial methods of oocyte activation in order to achieve normal fertilization (e.g. pronuclear formation). To that end, several methods of oocyte activation (e.g. 7% ethanol, calcium ionophore) have been tested in conjunction with sperm injection of IVM cattle oocytes (Jung *et al.*, 1996; Chen and Seidel, 1997a,b; Medvedev *et al.*, 1997; Rho *et al.*, 1997, 1998a,b; Wu, H. *et al.*, 1997;

Alberio *et al.*, 1999a,b; Campbell *et al.*, 1999; Greising and Torner, 1999; Horiuchi *et al.*, 1999, 2000, 2002; Ruddock *et al.*, 1999; Keskintepe *et al.*, 2002; Meo *et al.*, 2002); some of these methods are detailed in Table 6.16.

A study by Keskintepe and Brackett (2000) in the USA reported activating cattle oocytes 30 min after sperm injection by incubating them in a medium containing 50 μM calcium ionophore (A23187) for 5 min; bull sperm were capacitated before injection by incubating them for 1 h in a heparin-containing medium. These workers reported cleavage and blastocyst development rates of 52.4% and 24.4%, respectively. In a further report on ICSI, Keskintepe *et al.* (2002) activated bovine oocytes by treatment with ionomycin (15 μM) for 5 min plus dimethylamino-purine (DMAP) for 4 h; the same study was also noteworthy in being the first to report the production of karyotypically normal blastocysts after the injection of frozen–thawed bull sperm that were selected, freeze-dried (lyophilized) and stored at 4°C until use.

In Canada, Chung, J.T. *et al.* (2000) examined a method of oocyte activation which mimics the calcium oscillations observed in the normal process of fertilization in cattle; oocytes were activated by three 5 min incubations with ionomycin at 30 min intervals. Their study appeared to confirm the findings of others that the ICSI technique itself was not sufficient to activate cattle oocytes. They did find evidence of partial activation, which they termed metaphase III (an abnormal stage in which chromosomes remain condensed after telophase II due to insufficient ooplasmic activation). The same workers speculated that such partial activation could occasionally provide sufficient cytoplasmic factors to initiate formation of a female pronucleus but insufficient to process the highly stabilized bovine spermatozoon (bovine sperm have tightly packaged nuclei since they contain type 1 protamine, which is maximally cross-linked); they also speculated that partial activation may have occurred due to the sperm membrane remaining intact or that, during the injection procedure, the ooplasmic membrane was not penetrated but merely surrounded the spermatozoon, thereby protecting the sperm nucleus from the reducing environment of the ooplasm.

Table 6.16. Cattle ICSI in combination with oocyte activation

Oocyte activation treatment	Reference
Ionomycin Ethanol	Chen and Seidel (1997a,b)
Ionomycin (50 μM for 3 min) Ionomycin + DMAP	Keskintepe and Brackett (1999)
Multiple ionomycin doses	Chung, J.T. *et al.* (2000)
Ionomycin (50 μM for 5 min)	Keskintepe and Brackett (2000)

In Japan, Horiuchi *et al.* (1999, 2002) reported that the use of bull sperm immobilized by tail-scoring and a piezo-micromanipulator improved the outcome of ICSI, with near-normal cleavage rates and blastocyst yields recorded after injected oocytes were activated by exposure to ethanol for 5 min. Further results from the same laboratory published by Katayose *et al.* (1999) subsequently confirmed that the bovine oocyte can be effectively activated and fertilized when an immobilized spermatozoon is injected using a piezo-micromanipulator. Although the Japanese workers concluded that an activating procedure was not necessary to effect fertilization, additional stimulation was apparently required for the development of the embryo to the blastocyst stage. The same laboratory, in a further paper by Horiuchi *et al.* (2000, 2002), showed that exposure of oocytes (those with two polar bodies) to 7% ethanol for 5 min after ICSI resulted in cleavage rates and blastocyst yields comparable to those with conventional IVF; they were also able to report the birth of five normal calves after transfer of ten blastocysts to ten recipient animals. In Korea, Hwang, S.S. *et al.* (2000) treated cattle oocytes with an electric shock before, before and after and after sperm injection; although the exact mechanism involved was not understood, electric stimulation prior to and after ICSI was found to be effective in inducing oocyte activation and sustaining embryonic development to the blastocyst stage.

Successful cattle ICSI without artificial activation

Despite evidence suggesting that artificial activation was a necessary prerequisite for successful ICSI in cattle, some studies (Galli *et al.*, 1999a) and particularly those of Wei and Fukui (2000a, 2002) in Japan have gone some way to

demonstrate that such activation may not be a requirement. The Japanese workers took matured and denuded cattle oocytes (those showing the first polar body) and centrifuged them at $6000 \times g$ for 7 min to clarify the oocyte cytoplasm in preparation for sperm injection. After sperm selection by swim-up treatment, morphologically normal and motile sperm were selected and their tails were cut apart using a glass needle; part of the sperm midpiece, about 5 μm in length, was left with the sperm head. Using a piezo-micromanipulator and a low (2 mM) carbonate TALP medium supplemented with PHE, 4% PVP and 0.1% PVA, a spermatozoon was injected into the centre of each oocyte. Of the oocytes injected, 86.3% were activated, most cleaved and 23% developed to the blastocyst stage. The Japanese workers found that most blastocysts were cytologically normal (diploid) and when transferred into recipient cattle they gave rise to a normal pregnancy rate. The final confirmation of normality came with the birth of five healthy calves. It remains for such results to be confirmed by workers elsewhere; if they are, then it may be possible to make sperm injection the basis of a feasible commercial option.

The same two Japanese workers also reported on the possibility of using bovine oocytes for a heterologous fertility test (Wei and Fukui, 2000b); they did this by comparing pronuclear formation in ram, bull and whale sperm after ICSI. They observed male pronuclei with all three types of sperm, indicating that foreign sperm can participate in fertilization activities in cattle oocytes after sperm injection. They also showed, for the first time, that the male pronucleus plays a role in synchronizing the development of the female pronucleus.

Regardless of whether fertilization of cattle oocytes is achieved by IVF or ICSI, there still remains the possibility that marked differences between bulls may emerge. Although studies reported by Horiuchi *et al.* (2000) found no evidence of bull influence, with either IVF or ICSI, studies by Wei and Fukui (1999) did find evidence of bull differences using ICSI to achieve fertilization, even though problems of motility and zona penetration were avoided in this approach. The same workers showed that immobilization of the bovine spermatozoon by tail-scoring before ICSI significantly improved results; in this, their data were in agreement with those of Gordon and Carroll (1997), who recorded that total immobilization of the bull spermatozoon (by tail-scoring) was a significant factor in enhancing cleavage rate in their experiments.

Cattle ICSI in research

Studies in Australia by Gougoulidis *et al.* (1999b) examined the role of the TEC-2 epitope during fertilization in cattle. When cattle oocytes were incubated with the monoclonal antibody TEC-2 epitope before IVF, cleavage was inhibited in a dose-dependent manner; the block was overcome using ICSI as the method of fertilization. The authors suggest that TEC-2 specifically affects the fusion events of fertilization.

Gender preselection in cattle by ICSI

The sorting of bull sperm by flow cytometry is now well established and is currently being applied on a limited scale in commercial practice in the UK and Ireland; clearly, far fewer sperm are required for fertilization where IVF is employed rather than conventional AI. On the negative side, however, there are studies that show evidence of reduced viability and motility of sorted sperm. It is known that sperm membrane proteins undergo changes that may affect sperm function and sperm–oocyte interactions after staining and sorting by flow cytometry (McNutt and Johnson, 1996a,b); it is also known that certain bulls tolerate sperm sorting better than others (Doyle *et al.*, 1999). In Germany, Medvedev *et al.* (1997) found some evidence that frozen–thawed flow-sorted bull sperm were as effective as non-sorted cells in activating bovine oocytes after sperm injection; in Poland, Skrzyszowska *et al.* (2000) similarly reported that the yield of morula/blastocyst embryos did not differ between sorted and non-sorted sperm. Such results might suggest that sorting influences characteristics such as motility rather than the fertilizing ability of the spermatozoon.

As noted by Horiuchi *et al.* (2002), sperm injection in cattle could be very useful in producing calves from expensive semen; they instance frozen semen straws of certain bulls costing as much as US$1000 for one straw. The sperm from one such straw divided into hundreds of aliquots

would obviously appeal as a cost-cutting exercise to many, although not necessarily to the breeding company owning the bull. The ultimate in making the greatest use of a genetically superior bull would be the microinjection of a single sperm of the required sex. Already, in Japan, single sperm heads from a Y-chromosome-rich population have been injected into IVM oocytes in a study reported by Hamano *et al.* (1999a,b); 6.9% of the fertilized oocytes developed to the blastocyst stage and ten normal calves (eight males and two females) were born after their transfer to recipient cattle. Using the improved sperm injection technology of Wei and Fukui (2002) and semen aliquots with small numbers of sorted bull sperm, there may be commercial interest in providing a sexing service for those who are recovering oocytes by OPU. In human ICSI, it is possible to preserve small numbers (four to six) of spermatozoa or even just a single spermatozoon by cryopreservation (Cohen and Garrisi, 1997; Quintans *et al.*, 2000); such technology would enable sexed semen to fertilize many cattle oocytes. Quite apart from the technical efficiency of cattle ICSI, any talk of sperm injection obviously poses questions of equipment costs and skilled labour.

ICSI in the mare

IVF of IVM oocytes has proved to be much more difficult in horses than in cattle. In France, Guignot *et al.* (1995, 1996) were among the first to show that sperm injection may be a useful alternative to IVF in that species. In Italy, studies reported by Cho *et al.* (1995) suggested that, despite the very dark ooplasm (high lipid content) of the equine oocyte, sperm injection could be performed successfully. Further work in that country by Dell'Aquila *et al.* (1996b) showed ICSI to be significantly more effective than conventional IVF in fertilizing IVM oocytes, particularly when the oocytes had been matured in the presence of follicular fluid; similar views were expressed by Squires *et al.* (1996), who reported a pregnancy after transfer of an ICSI-derived embryo. In Cambridge, Li, X.H. *et al.* (2001) also reported the birth of several foals by way of the sperm-injection technique. In the Netherlands, Tremoleda *et al.* (2002), describing the nuclear and cytoplasmic events that occur during the development of the equine zygote, suggested that, until conventional IVF became more reliable in the mare, ICSI might be the best way to produce embryos. In Italy, Lazzari *et al.* (2002a) showed that the frozen–thawed semen of fertile and infertile stallions could be used to produce viable embryos by ICSI; embryo production was not correlated with the field fertility of the stallions (see Table 6.17).

Reports from those working with horses indicated that cleavage of equine oocytes might be achieved without recourse to artificial activation of the oocyte or capacitation treatment (e.g. calcium ionophore) of stallion spermatozoa. The morphology of the ooplasm of the sperm-injected oocyte was studied by Dell'Aquila *et al.* (1997a); a fertilization rate of 39% was recorded but there was no correlation between ooplasmic texture and oocyte fertilizability. It was concluded that even equine oocytes with heterogenous cytoplasmic appearance are capable of fertilization by sperm injection. Other papers appearing in that same year confirmed that ICSI could be applied successfully to IVM horse oocytes to increase fertilization rate (Dell'Aquila *et al.*, 1997b) and that follicular-fluid supplementation of the maturation medium improved the maturity of horse oocytes sufficiently for the use of sperm injection but not for IVF (Dell'Aquila *et al.*, 1997c). Elsewhere, in that same year, Danish studies, working with IVM oocytes, reported

Table 6.17. Use of sperm from fertile and infertile stallions by ICSI (from Lazzari *et al.*, 2002a).

Group of stallions	Number metaphase II injected	Number cleaved	Cleavage (%)	Number compacted morulae/blastocysts	% Compacted morulae/ blastocysts cleaved
A	117	88	75.2[a]	42	47.7[a]
B	78	49	62.8[a]	21	42.9[a]
C	91	72	79.1[a]	33	45.8[a]
D	46	4	8.7[b]	0	0.0[b]

Chi-square test. Numbers within columns with different superscripts are significantly different ($P < 0.05$).

recovering embryos up to the eight-cell stage at 72 h post-injection. In the following year, studies elsewhere produced evidence that progesterone may be useful in the maturation of the horse oocyte (Schmid *et al.*, 1998); it is known that progesterone is present in high concentrations in the preovulatory follicular fluid of the mare.

In considering the effectiveness of ICSI in the horse, it should be noted that in this species a large proportion of oocytes fail to complete meiosis during *in vitro* culture, although the biochemical and molecular basis of such failure remains unclear. In comparison with cattle, sheep and pigs, preovulatory follicular conditions differ in several ways; the ovulatory LH surge is progressive over a period of several days, with a maximum concentration of this gonadotrophin occurring 1 day after ovulation. IVM of the horse oocyte, no matter what culture conditions are employed, remains relatively inefficient, with < 70% of oocytes reaching metaphase II at the end of the IVM culture period. In France, Goudet *et al.* (1998c) suggested that the failure of equine oocytes to resume and complete meiosis may have its origins in a deficiency of regulators of maturation-promoting factor (MPF) and/or to an inability to phosphorylate mitogen-activated protein kinase (MAPK).

In Lousiana, Cochran *et al.* (1999) reported a procedure that they suggested showed promise of becoming a successful method of repeatedly producing equine embryos *in vitro*. Employing a protocol involving progestogen (altrenogest) treatment of donor mares, with oocyte collections at 10-day intervals, they reported cleavage rates of up to 58% using ICSI. In France, a similar approach by Guignot *et al.* (1999a) demonstrated that a weekly ultrasound-guided oocyte-aspiration routine enabled them to collect 2.1 oocytes per mare per week, which yielded about 1.5 embryos per cycle after sperm injection, in comparison with 0.6 embryos after *in vivo* fertilization.

A study by Dell'Aquila *et al.* (1999) showed that ZP hardening was unlikely to be a major factor underlying poor fertilization rates after IVF in the horse; other studies by the same group indicated that cumulus morphology was related to follicle apoptosis, although this did not affect maturation and fertilization of the horse oocyte by sperm injection. In Colorado, Maclellan *et al.* (2000a) found no evidence that adding equine oviductal cell-conditioned medium during IVM improved oocyte development after ICSI. In Cambridge, Li, X.H. *et al.* (2001) reported on the influence of co-culture during maturation on the developmental potential of horse oocytes fertilized by sperm injection.

ICSI and factors influencing oocyte activation

In France, Guignot *et al.* (2000) found that treatment of horse oocytes with ethanol and calcium ionophore resulted in an increased cleavage rate after ICSI in comparison with ethanol treatment alone. In Italy, Ledda *et al.* (2000) used ionomycin (10 μM) 30 min after ICSI as an activating agent. In Cambridge, studies by Li, X. *et al.* (2000) dealt with various activation treatments, demonstrating that thimerosal was more effective than ionomycin, ethanol or IP_3 in facilitating both meiotic activation and normal fertilization after sperm injection. Activation of the equine oocyte involves various events, including a series of repetitive Ca^{2+} transients; there are those who note that variable cleavage rates after ICSI may be due to suboptimal Ca^{2+} oscillations. In the USA, Bedford *et al.* (2002) recorded inconsistent Ca^{2+} responses after ICSI, which they considered might cause variation in cleavage rates; they speculated that the degree of membrane damage prior to sperm injection might influence the onset of Ca^{2+} oscillations and the persistence of these oscillations.

ICSI in mice

Although the earliest pioneering efforts in sperm injection used the mouse as the model (Markert, 1983), the first report of births in this species after ICSI was to take a further two decades (Ahmadi *et al.*, 1995). Part of the difficulty with mice lies in the size of their gametes; oocytes are much smaller than in humans and cows, whereas sperm, in terms of total length, may be twice the size. There have also been problems due to the poor wound-healing ability of the oocyte's plasma membrane, although the work of Kimura and Yanagimachi (1995) has shown that it is relatively easy to perform ICSI using a piezo-driven micropipette. The use of ICSI in mice is of considerable interest since it may be the means of improving methods of maintaining mouse breeding stocks in laboratories around

the world. There is also the need to preserve unique mouse genomes that have been produced for research purposes. Although mouse sperm are much more difficult to freeze than human and cattle male gametes and effective cryopreservation methods have only been developed in the past decade, Szezygiel *et al.* (2002) have shown that ICSI is a more efficient and effective technique than IVF for generating embryos from frozen murine sperm; the same workers have also demonstrated that ICSI may be particularly useful for mouse strains where IVF with fresh sperm produces few or no embryos.

6.10. Efficiency of IVF Procedures

In assessing the efficiency of IVF procedures and in evaluating the normality or otherwise of the cattle embryos that emerge from the production system, there is the obvious need to specify what is to be regarded as normal, as shown in the live cow; this provides the background against which to compare IVM and IVF zygotes/embryos with their naturally occurring counterparts.

6.10.1. Criteria for assessing fertilization

The penetration of the oocyte by the bull spermatozoon initiates a series of events involving the nuclear and cytoplasmic components of sperm and oocyte. Shortly after the sperm penetrates the ZP, the non-membrane-bound spermatozoon decondenses within the ooplasm; subsequently, a nuclear envelope forms and swelling of the male pronucleus occurs. Simultaneously, meiosis resumes in the bovine oocyte, with the extrusion of the second polar body and formation of a nuclear envelope around the decondensing female chromatin. Formation of the male and female pronuclei occurs simultaneously and within a few hours of sperm penetration. In contrast to what is possible with the oocytes of some species (e.g. human, mouse), in cattle, due to the presence of lipid vesicles and other pigments, the pronuclei are not visible, other than when special techniques are employed (differential interference contrast microscopy, staining, centrifugation). Data for events in the live animal have been provided by Laurincik *et al.* (1994b): at 10 h after the estimated time of ovulation, migrating pronuclei were predominant, at 12 h the first apposed pronuclei were observed and the first cleavage was observed at 20 h.

Chromosome preparation

An improved method of chromosome preparation for use with cattle oocytes and zygotes derived from IVF was described by Ocano Quero *et al.* (1998b) in Spain; the method involved the use of a trypsinized hypotonic solution, a vortex-agitation system and a two-step fixation process at −20°C to weaken the ZP and allow the swelling of the zygotes to give increased spreading of the chromosomes.

6.11. Fertilization Abnormalities

The two most common fertilization abnormalities that present problems in cattle IVF are polyspermy and parthenogenesis.

6.11.1. Polyspermy and parthenogenesis

Polyspermy

The phenomenon of polyspermy is not as well defined in cattle as it is in pigs, but none the less presents a problem that requires consideration by those engaged in embryo production. Although the cortical granules release their contents in response to fertilization in the artificially matured bovine oocyte, the efficiency of the zona reaction is generally held to be less than in the *in vivo*-matured oocyte. One study in Ireland showed that the bovine oviduct altered the ZP and may play a role in the block to polyspermy in cattle (Duby *et al.*, 1997). Information about the contents and function of cortical granules can be found in a review by Hoodbhoy and Talbot (1994).

Parthenogenesis

Parthenogenesis is defined as the development of the oocyte without the intervention of the male

gamete. The matured oocytes of most mammals, including cattle, are usually arrested at the second metaphase until fertilization or parthenogenetic activation occurs. Parthenogenetically produced embryos are of interest because they may reveal problems in the embryo culture system and in other aspects of quality control in the IVF laboratory. A study by Presicce and Yang (1994a,b) showed that their activation method (ethanol and cycloheximide) activated aged bovine oocytes more rapidly than young oocytes. They also recorded that spontaneous activation rates of ageing oocytes were also higher (6–57%) than those of the young ones (0–14%). A study by Lechniak *et al.* (1998a,b) in Poland led them to conclude that spontaneous parthenogenetic activation indicates suboptimal culture conditions and that a group of non-inseminated cattle oocytes should be routinely included by workers to check the efficiency of the medium used for *in vitro* embryo production.

Much information about oocyte activation and parthenogenetic development has been reported in recent years; the activation of recipient oocytes is a critical component of nuclear-transfer programmes and is one reason why some groups are interested in factors influencing parthenogenetic development (Alfonso and Hunter, 1994; Boediono and Suzuki, 1994; Fukui *et al.*, 1994; Presicce and Yang, 1994a,b; Chian and Sirard, 1995a,b; Landa and Kopecny, 1995; Winger *et al.*, 1995, 1997b; Liu *et al.*, 1998c; Ernst *et al.*, 1999; Gougoulidis *et al.*, 1999b; Jabbour and Kouzmina, 1999; Alberio *et al.*, 2000a,b,c; Campbell *et al.*, 2000; Fahrudin *et al.*, 2000).

Parthenogenetic embryos have usually been produced by simulating the conditions of fertilization, such as increasing the calcium concentration, applying an action potential to the oocyte or using sperm-associated factors to initiate activation and embryonic development. It is possible for the bovine oocyte to develop to a limited extent in the absence of the nucleus; in China, Shi *et al.* (2000) presented results indicating that cleavage of the first three cell cycles in cattle oocytes was able to proceed under the control of maternal mRNA stored in the ooplasm. In Japan, Aoyagi and Konishi (1994) demonstrated that treatment of bovine oocytes with an electric pulse in combination with calcium ionophore and cycloheximide was the optimal method for inducing development of oocytes to the blastocyst stage In Georgia, Gibbons *et al.* (2002a,b) found that an electrical pulse as a part of a bovine oocyte parthenogenetic activation protocol was beneficial to embryo development. The authors concluded that a study of parthenogenesis provides a useful insight into problems associated with nuclear transfer embryo production.

6.12. Variability in Bull Fertility

One problem in cattle reproduction is that of the variability in bull fertility. It is well recognized that bulls used in AI may differ in their fertility by as much as 10% or more (measured in terms of conceptions per 100 inseminations) and similar evidence can be produced by those working in cattle IVF. Having said that, it should also be mentioned that recent developments in the IVP of cattle embryos have opened the way to new approaches in the evaluation of bull fertility.

6.12.1. Effect of bull on IVF outcome

It is clear from many reports that the use of bulls producing sperm with a high fertilizing ability may be an important factor in determining the success of cattle IVF (Aurich and Hahn, 1994b; Yang, X. *et al.*, 1995). In Ireland, clear evidence of differences among bulls in cleavage rates and embryo yields emerged from studies by Lu; embryo yields in one study showed a threefold variation (from 10.6% to 30.5%) according to the bull employed in IVF. The general rule followed in early work in Ireland was to ensure that semen used in IVF was derived from several bulls with proved records of achievement in the field (high non-return rate % (NRR%)); obviously this approach cannot be used in normal cattle-breeding programmes where embryos need to be sired by specific bulls. What was true in 1989 was still true a decade later. In Brazil, working with zebu (Nellore) cattle, Lobo *et al.* (1999) compared the cleavage and blastocyst rates after IVF using sperm from 12 Nellore bulls; results obtained (see Table 6.18) indicated significant variation among these bulls as evaluated on the basis of cleavage rates and the development of zygotes to the blastocyst stage.

Table 6.18. Effect of bull on cleavage rate and blastocyst yield (from Lobo *et al.*, 1999).

Bulls	No. oocytes inseminated	Cleavage (%)	Blastocysts (%)[a]	Hatching (%)[b]
01	185	168 (91)	93 (50)	78 (84)
02	201	162 (81)	94 (47)	79 (84)
03	182	150 (82)	90 (49)	79 (88)
04	203	161 (79)	88 (43)	71 (81)
05	185	127 (69)	69 (37)	55 (80)
06	189	152 (80)	89 (47)	77 (87)
07	169	91 (54)	51 (30)	45 (88)
08	173	65 (38)	27 (16)	23 (85)
09	191	113 (59)	56 (29)	47 (84)
10	199	160 (80)	97 (49)	81 (84)
11	167	69 (41)	33 (20)	28 (85)
12	172	146 (84)	78 (45)	66 (85)
Overall	2216	1564 (70)	865 (39)	729 (84)

[a]Values within the same column are significantly different ($P < 0.0001$).
[b]Values within the same column are not significantly different ($P > 0.05$).

The authors concluded that bull testing is important in an IVF laboratory, particularly when working with high-quality donors.

An earlier report by Coelho *et al.* (1998a) had led to the conclusion that the selection of bulls for the IVP of embryos should use blastocyst production rate rather than cleavage rate as the criterion. In Uruguay, Roses *et al.* (1999) made a comparison between the IVF ability of frozen semen from three bulls and found evidence of significant differences in embryo production rate. In Germany, a study by Astiz Blanco *et al.* (1999) examined variation in cleavage and embryonic development due to bulls employed in their IVF system; although they recorded similar cleavage rates for the six Simmental bulls employed, there was a significant difference between bulls in blastocyst yield. The authors concluded that differences in embryo development can be related to individual bulls, which may be due to hereditary factors. In the USA, Wang *et al.* (1999a) concluded that the bull effect was an important factor influencing the results of IVP of cattle embryos.

Not all reports have shown clear differences between bulls. A study by Merighe *et al.* (2002) examined the bull effect in terms of embryo developmental kinetics, where embryos were evaluated in relation to the time taken to achieve the fourth cell cycle; embryos at the five- to eight-cell stage before 48 hpi were classified as fast developing, whereas those reaching that stage between 48 and 90 hpi were classified as slow developing. In Croatia, Matkovic *et al.* (2002) tested nine Simmental bulls and found great variation both between bulls and between samples from the same bull; they concluded that such variations need to be taken into account by those working in IVF. Such conclusions agreed with a previous report by Van Langendonckt *et al.* (1994) in Belgium, who showed evidence of considerable variation in fertilization rate according to the bull ejaculate employed; they concluded that the bull ejaculate is a parameter that must be controlled to optimize *in vitro* embryo production programmes. In Ireland, Ward *et al.* (2001a,b, 2002) showed that the bull can have a significant effect on the kinetics of early embryonic development as shown by the time of first cleavage; part of the explanation was due to a delay in sperm penetrating the oocyte. The authors found developmental competence to be significantly lower in the slow group, regardless of sire; they speculated that this effect may have originated from inferior oocyte competence.

6.12.2. *In vitro* fertilization tests in predicting bull fertility

Over the years, the usual assessment of sperm quality in AI bulls, either fresh or after processing, has involved examinations to determine semen density, sperm motility and morphology. Much information is now available on tests

based on IVF and related techniques, such as ZP-binding assays and oocyte penetration tests. Used either singly or in combination with other assays, such methods can provide valuable information about the likely performance of a particular bull when used in AI or IVF (see reviews by Lonergan, 1994; Rodriguez-Martinez and Larsson, 1999; Larsson and Rodriguez-Martinez, 2000). The ability to determine the fertility of a particular bull based on laboratory tests would be of considerable interest to the AI industry, which needs to determine the maximum number of sperm doses to prepare from a given ejaculate. Used in conjunction with the traditional measures of bull fertility (sperm motility and morphology), tests using novel methods of sperm assessment, including those associated with IVF, have obvious practical implications. Among reports dealing with various tests that might be employed in identifying high-fertility bulls are those dealing with penetration of zona-free cattle oocytes (Henault and Killian, 1995), *in vitro* embryo production (Schneider *et al.*, 1999), the heparin-binding properties of bull spermatozoa (Merkies *et al.*, 2000) and sperm binding to epithelial oviductal explants (De Pauw *et al.*, 2002b).

Sperm chromatin structure

A study reported by Bochenek *et al.* (2000) in Poland attempted to establish a possible relationship between bull fertility (on the basis of NRR%) and the percentage of sperm with abnormal chromatin in the ejaculate. Using a sperm chromatin structure assay, they found a significant correlation between fertility and the outcome of the assay; they concluded that bull fertility was significantly influenced by sperm chromatin damage.

The bovine centrosome (centriole)

Studies conducted in the early 1990s on the phenotypic expression of the bull centrosome are of fundamental interest as well as explaining certain differences in cattle fertility. The centrosome is contributed by the bull spermatozoon; immediately after the sperm nucleus is released into the ooplasm, it starts to increase in size as protamines are removed and the sperm head draws in fluid. As the nucleus swells, its attendant (proximal) centriole puts out long fibres into the ooplasm to produce a star-shaped structure, the sperm aster (see Fig. 6.12) The disassembly of the bovine sperm tail during fertilization has been described by Sutovsky *et al.* (1996); it occurs as a series of precisely orchestrated events involving the destruction (fibrous sheath and mitochondrial sheath) and transformation (DNA, sperm centriole) of particular sperm structures into zygotic and embryonic components.

In the normal fertilization process in the cow, as the highly compacted chromatin of the bull spermatozoon undergoes decondensation to form the male pronucleus, the last division of meiosis in the oocyte is completed, resulting in the formation of the female pronucleus. The two haploid pronuclei are brought together, to form the diploid zygote nucleus, by microtubule-mediated migration. Microtubules are complex polymers that are assembled from soluble tubulin subunits; although these tubulin subunits are present in the cytoplasm of all cells, they usually only form microtubules in the presence of a centrosome. The sperm astral microtubules (the sperm aster) are thought to play a crucial role in uniting the male and female pronuclei.

In conventional cattle IVF studies, fluorescent microscopy was used by Duby *et al.* (1995) in characterizing sperm aster formation in prepubertal bovine oocytes. In monospermic zygotes, they observed the sperm aster originating about 8 hpi between the decondensing sperm head and the midpiece. In the cow, as in other mammals, the completion of fertilization, as signalled by the intermingling of the parental genomes, is formally achieved at metaphase of the first mitotic division when the chromosomes are aligned on the metaphase plate.

Using bovine oocytes and spermatozoa from bulls proved in the field and assessed in IVF as being of excellent, good or poor fertility, it was shown that the organization and size of the sperm aster varied according to the sire used. The indications are that the quality or quantity of the sperm centrosome has a direct effect on the fertilization process and the calving rate. It may be useful to keep such evidence in mind in attempts to devise laboratory tests for predicting the fertility of bulls used in AI. Other work in the USA, reported by Long *et al.* (1994), suggested that sperm aster formation after IVF may be

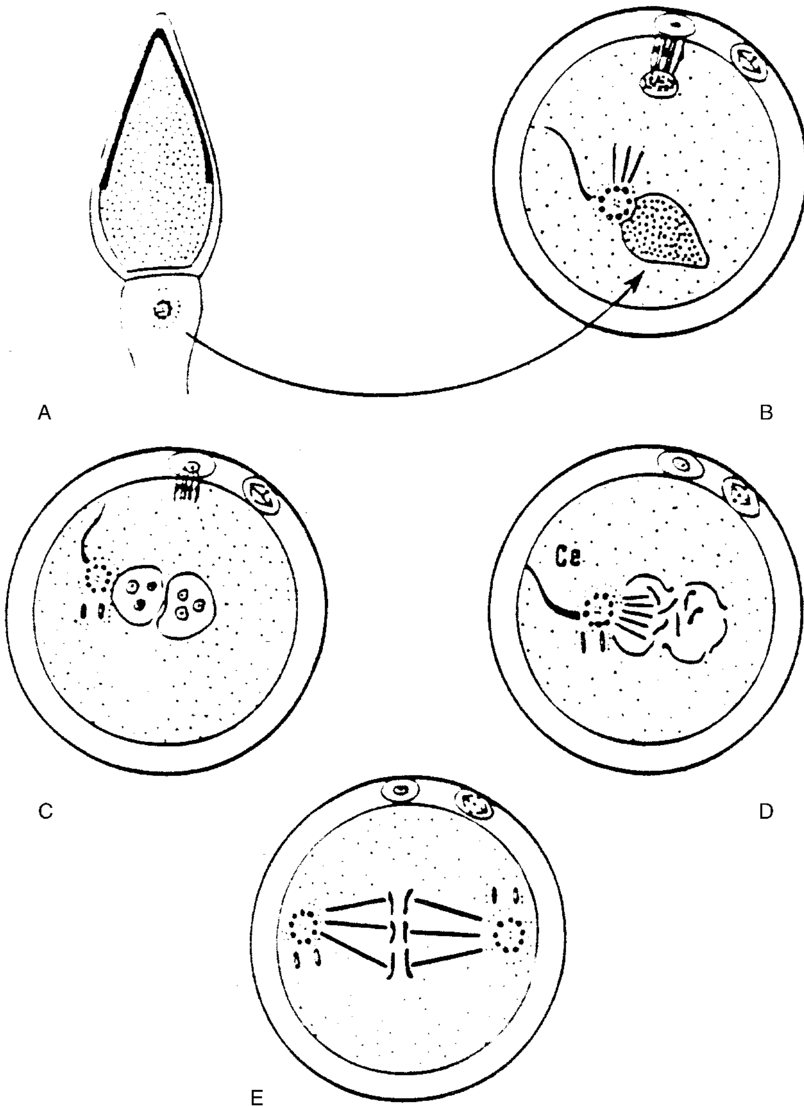

Fig. 6.12. Paternal centrosomal dynamics at fertilization. (A) Sperm proximal centriole in centrosome in sperm neck. (B) Incorporated sperm in ooplasm showing developing sperm aster. (C) Duplicating sperm centriole associated with the male pronucleus. Note the close association between male and female pronuclei. (D) Sperm aster (centrosome with duplicated centriole) organizing the first mitotic spindle at prometaphase. Pronuclear envelopes are disorganizing and the chromosomes are condensed. (E) The first bipolar mitotic spindle at syngamy showing duplicated centrioles (diplosomes) at either pole. Note the increase in pericentriolar material around centrioles and chromosomes at the equator (metaphase). (Based on Sathananthan, 1998.)

related to the maturity of bovine oocytes. The inability of less matured oocytes to respond to fertilization-induced intracellular signals was thought to account for the subsequent lower rate of development of these zygotes to blastocysts. The inheritance of sperm centrosomes in cattle embryos was studied by Sathananthan *et al.* (1997); the authors suggested that the bovine embryo may be an appropriate model for studying centriolar inheritance.

In a subsequent paper, the Australian group examined the behaviour of the centrosome

during fertilization and embryogenesis in cattle, showing that it displayed the same pattern of centriole behaviour that is common in most animals, including large mammals (Sathananthan *et al.*, 1999). The sperm centrosome was traced from fertilization to the hatching blastocyst stage and its presence was confirmed at every stage of cleavage. The authors concluded that most mammals obey Boveri's rule of paternal centrosomal inheritance and perpetuation; the mouse appears to be an exception to this rule, showing maternal inheritance.

6.12.3. Reducing bull fertility

As part of the scenario of increasing information about factors influencing bull fertility, there are those who have examined ways and means of reducing the ability of bovine spermatozoa to effect fertilization. In Wisconsin, Kim, C.A. *et al.* (1999) tested the hypothesis that antibodies against bull sperm can interfere with fertilization; they produced anti-sperm antibodies by autoimmunizing Friesian bulls. They showed that incubating bull sperm with the antibody serum significantly decreased fertilization rate in an IVF system. In human reproduction, it is well established that male infertility due to anti-sperm antibodies represents a small but none the less significant proportion of the infertile population, generally held to be about 5–10%. The presence of anti-sperm antibodies does not necessarily mean that fertilization cannot occur; with appropriate sperm preparation, the problem may be resolved by way of conventional IVF.

7

Culturing and Evaluating the Early Bovine Embryo

7.1. Introduction

The bovine embryo normally develops within the protected environment of the cow's reproductive tract. As with the many unseen events that take place during fertilization, information about the factors involved in early embryonic development has come from countless studies carried out under the microscope in the laboratory. Cattle embryos developing within the cow's reproductive tract are provided with all the necessary nutrients for early embryonic development and a system for removing waste materials. After the bovine oocyte is matured and fertilized *in vitro*, the final step is that of culturing the embryo to the blastocyst stage, at which it can either be transferred to a waiting recipient or cryopreserved for storage until required; such embryos must obtain all nutrients from the culture medium, once the initial store in the oocyte is depleted, and must also be protected from potential toxic effects.

7.1.1. Historical

Although well over a century has passed since the first report of *in vitro* culture (IVC) of a mammalian embryo (Schenk, 1880), most activity in this field has been in the last few decades, particularly since the emergence of effective tissue-culture techniques. Much of the early work was with mice and rabbits; Hammond Junior, working in Cambridge in 1949, was a pioneer in culturing the mouse embryo through its early cleavage stages *in vitro*, using egg-white in a physiological salt solution as his medium. The earliest report of a successful procedure permitting the continued cleavage of the early cattle embryo to the blastocyst stage outside the cow came from the work of Sreenan and associates in Ireland in 1968; in the same year, Adams in Cambridge confirmed that the bovine embryo could be cultured to the hatching stage in the rabbit oviduct.

7.1.2. *In vivo* culture systems

Since the late 1960s, workers have used a variety of animal systems, including mice, rabbits, sheep, cattle and even chicken eggs, for the *in vivo* culture of early cattle embryos. The use of the oviduct as an *in vivo* culture system served to focus attention on the unique properties of this part of the female reproductive system. It must clearly be regarded as much more than just a tube through which the early embryo must pass *en route* to the uterus. In Ireland, during the late 1980s, much work was done in using the sheep oviduct to culture cattle zygotes through to the morula/blastocyst stage. Elsewhere, that approach to culturing the cattle embryo to the blastocyst stage has been employed with considerable success in Italy (Galli and Lazzari, 1996).

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7.1.3. *In vitro* culture systems

The cells of the oviduct were used in the first successful co-culture systems in sheep and cattle; the use of the oviductal monolayer culture system in sheep marked an important milestone (Gandolfi *et al.*, 1986). In Ireland, work demonstrated that a high proportion of *in vivo*-produced cattle embryos could be brought to the blastocyst stage using the bovine oviductal epithelial cell (BOEC) monolayer co-culture system (see Table 7.1); not only that, but a high percentage of such embryos were of a high quality.

It also became apparent that not only oviductal cells but a wide variety of other somatic cells are capable of providing an environment in which the cattle embryo can develop. An analysis of some 72 reports on cattle IVC in the 1991–1995 period was made by Thompson and Duganzich (1996); they concluded that a better-than-average performance could be achieved (about 50% of cleaved embryos developing to the blastocyst stage) when embryos are cultured in Charles Rosenkranz-1 (CR1) medium in the presence of buffalo-rat liver (BRL) cells and serum.

The introduction of co-culture of embryos with somatic cells in the 1980s was an important step in overcoming the arrest of early embryonic development in the cow. However, such co-culture systems required the use of complex tissue-culture media (e.g. TCM-199). Subsequently, serum became implicated in serious abnormalities in calves, which occasionally became evident at birth (e.g. large-calf syndrome). The use of fetal calf serum (FCS) in an embryo culture medium remains a matter of contention; as an additive to the culture medium, it is implicated in decreased pregnancy and calving rates and calf abnormalities, and yet it may result in an increase in the yield of blastocysts and may be a factor influencing the formation of the blastocoel. Although an understanding of the requirements for the normal development of cattle embryos *in vitro* to the blastocyst stage from *in vitro* or *in vivo* zygotes improved greatly during the 1990s, a clear quality distinction remains between the embryos derived *in vivo* and those produced *in vitro*. It is known that subtle differences exist at the cellular level in terms of metabolic profiles and morphology as well as in gene expression between *in vivo*- and *in vitro*-derived embryos.

Synthetic oviductal fluid (SOF) is one of the media widely used for bovine embryo culture *in vitro*. The medium was originally based on the biochemical analysis of ovine oviductal fluid, but was subsequently modified by the addition of amino acids. During the 1990s, energy metabolism studies resulted in a fuller understanding of the energy substrate requirements for bovine embryo development. An increasing appreciation of the subtleties of the cow's reproductive tract environment has resulted in the use of more 'defined' culture systems, and subsequently in the development of 'sequential' media systems, where components change in accordance with the requirements of the embryos. Fully defined culture systems, although desirable on many grounds, have yet to prove capable of supporting similar levels of development to those with protein-containing media. On the horizon, there are novel embryo culture systems based on microfluidic technology, offering the advantage that the IVC medium can readily be changed to meet the precise requirements of the developing embryo (see

Table 7.1. Development of early bovine embryos on an oviductal-cell monolayer (from McCaffrey *et al.*, 1991).

	Control	BOEC-0	BOEC-3
n	32	46	37
Initial cell stage of ova (mean ± SE)	2.00 ± 0.16	2.19 ± 0.14	2.38 ± 0.18
No. ova ≥ 16 cells (%)	16 (50)	36 (78)	30 (81)
No. morulae/blastocysts (%)	0 (0)	36 (78)	30 (81)
No. of grade 1 and 2 ova (%)	0 (0)	31 (67)	26 (70)

BOEC-0, culture dishes seeded with bovine epithelial cells on day of ovum recovery; BOEC-3, culture dishes seeded 3 days prior to start of embryo culture.
SE, standard error.

Glasgow *et al.*, 2001); gradually changing the composition of the medium would avoid inducing the stresses that almost inevitably arise from the abrupt environmental changes that are usually part of conventional culture systems.

In looking towards optimal embryo culture systems, however, it is essential to think in terms of chemically defined media that are soundly based on a knowledge of embryo metabolism and on known embryo preferences for energy substrates and other essential nutrients. It is only by such means that it will be possible to provide for all those factors that are crucial for early embryonic development.

7.1.4. Chapter contents

The present chapter examines the use of the various *in vivo* and *in vitro* systems that have been employed in culturing the early embryo, from the time of fertilization to the stage at which it is ready for transfer to a recipient animal or for storage (i.e. from zygote to blastocyst). Of great practical importance is the need for the *in vitro*-produced (IVP) cattle embryo to be entirely comparable, quality-wise, to its *in vivo*-produced counterpart. It is also essential to have a full understanding of mechanisms involved in the development of aberrant young that sometimes arises from commonly applied *in vitro* procedures in cattle and other mammalian species (see Khosla *et al.*, 2001).

7.2. Early Embryo Development in the Cow

The cow's oviduct provides the optimal environment for the transport of sperm and oocyte, for the fertilization process and for the development of the early bovine embryo. Before fertilization, muscle contractions and ciliary action contribute to the upward transport of bull spermatozoa and the downward movement of the bovine oocyte to the site of fertilization in the ampulla. There then follows a period of several days before the early embryo finds itself in the uterus, a period during which many crucial changes occur.

7.2.1. The oviductal microenvironment

The cow's oviduct, which is known to be under cyclic endocrine control, is likely to have a dynamic relationship with the early developing embryo; the absence of assistance from the oviduct may compromise the developmental ability of the cattle embryo under IVC conditions. An understanding of events in the oviduct is likely to shed much light on what is required to provide an appropriate culture environment. Some reports have dealt with the activity of the bovine oviduct. In Switzerland, Rosselli *et al.* (1994) provided the first evidence that endothelin is produced by cultured BOEC; they suggest that this peptide may act as a paracrine factor influencing the underlying smooth muscle cells, which are involved in the rhythmic contractions of the oviduct. The authors speculate that endothelin may play an important role in the transport of bovine gametes and embryos. A review by Hunter (1994) dealt with oviduct glycoproteins and examined some of the subtle working relationships that may exist, and which may be of direct benefit to gametes and embryo, between the oviductal fluid constituents at the time of ovulation and shortly afterwards. In York, Cox and Leese (1994) presented evidence suggesting the presence of a functional purinergic receptor in the epithelium of the bovine oviduct, which may play a role in transepithelial ion movements and thereby influence oviductal fluid formation.

In sheep, evidence by Stevenson and Wathes (1994) suggested that a paracrine/autocrine system involving the insulin-like growth factor (IGF) family operated within the oviduct; this may be a factor in providing an environment favourable for early embryonic development. In the USA, Martus *et al.* (1997) noted that oviductal-specific glycoproteins are secreted by the oviductal epithelium and have been identified in several mammalian species; they studied the effects of a partially purified oviductal protein on embryo development and found that it increased the number of embryos developing to the morula/blastocyst stages on day 7. It was concluded that their protein had an important role in regulating embryo development, apparently mediating a more rapid progression of embryos. In the UK, Pushpakumara *et al.* (2002) presented data about the IGF system

within different regions of the bovine oviduct and showed the relevance of such data to fertilization and early embryonic development in the cow; they noted that the early bovine embryo expresses gene transcripts encoding the type 1 receptor throughout the first week of development and that IGF-I secreted into the oviductal fluid could act as a paracrine regulator of early embryonic development.

7.2.2. Cleavage of the bovine embryo

The various stages in the early development of the bovine embryo, as recorded *in vivo*, are illustrated diagrammatically in Fig. 7.1. Many reports have described the changes that occur as the early embryo develops. A paper by Schramm and Paprocki (2000) dealt with the expression of fibrillarin, a protein localized in the dense

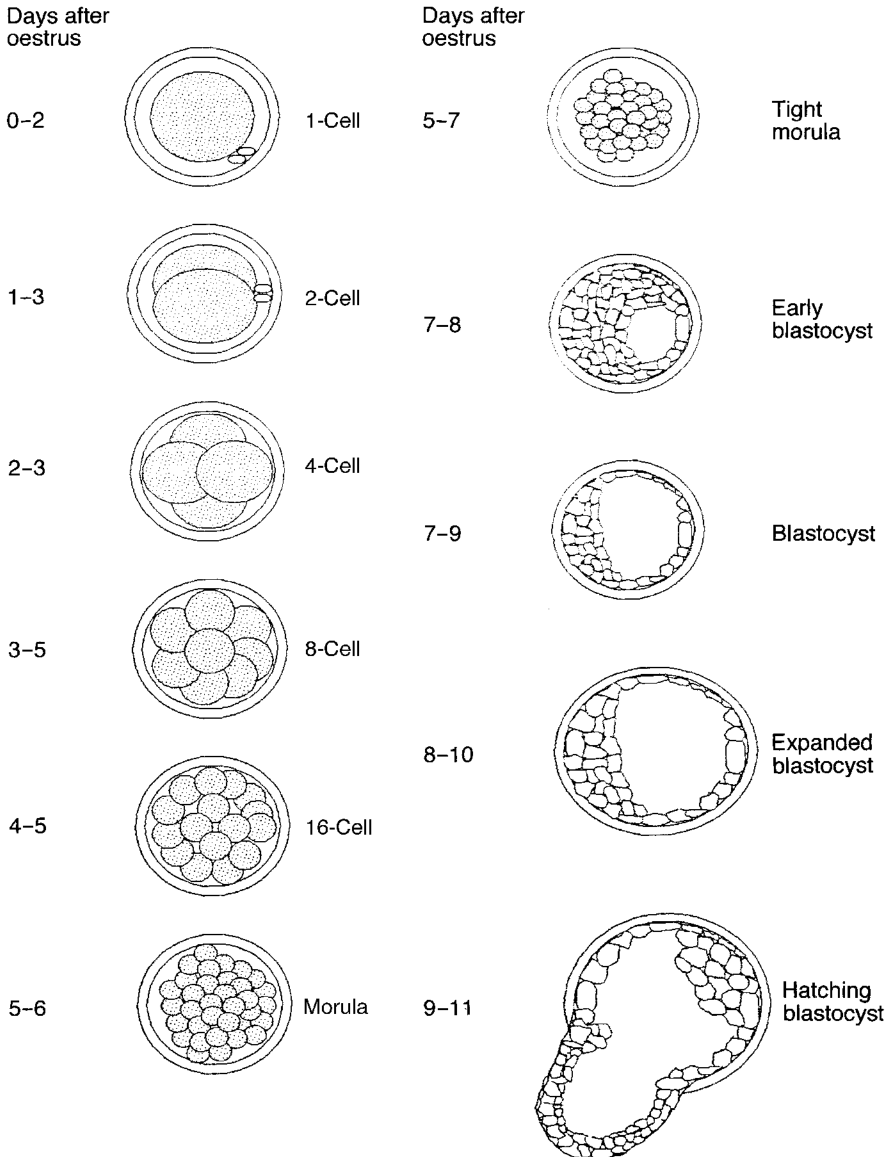


Fig. 7.1. Stages in the development of the bovine embryo.

fibrillar component of the nucleolus; their results provided a useful insight into the organization and development of the embryonic nucleolus during early embryo development.

Duration of cell cycles

The duration of the first, second, third and fourth cell cycles of cattle embryos developed *in vitro* have been estimated to be 34, 14, 11 and 54 h, respectively, in studies reported by Grisart *et al.* (1994); similar evidence (32, 9, 11 and 48 h for cycles 1–4, respectively) was presented by Holm *et al.* (1998a,b). Such data were in contrast to the values in the literature for bovine embryos developed *in vivo* (32, 13, 14 and 24 h for cycles 1–4, respectively). Such data clearly show that the fourth cell cycle is usually much prolonged in cattle embryos developed *in vitro*, resulting in a delay in the timing of development after the 8–16-cell stage, compared with embryos that develop in the cow's oviduct.

Steroidogenic activity of embryo

It is known that ovarian steroids play key roles in the establishment and maintenance of pregnancy in the cow; it is also known that mammalian embryos synthesize steroids, although their function remains uncertain. In Argentina, Baranao *et al.* (1999) studied the possible role of embryo-produced steroids on the development of the bovine embryo; they were able to demonstrate that early-cleavage cattle embryos have steroidogenic activity that is crucial for their development. Their findings provide evidence for the role of steroids as autocrine effectors in early embryo development.

Nucleoli and nucleolus organizer regions in the early embryo

Developmental changes in bovine embryos can be monitored using cellular or subcellular markers visible at the light- or electron-microscope level. One organelle that morphologically reflects changes in the metabolism or physiology of the cell is the nucleolus, which is unique in that its form is closely related to the transcription of multiple copies of certain genes. It is well known that activation of major portions of the embryonic genome occurs at a species-specific stage of development. In cattle, embryonic gene activation is generally held to take place during the fourth embryonic cell cycle; qualitative changes in bovine embryonic protein synthesis occur from the fourth to the fifth cell cycle. The major gene activation observed during the fourth cell cycle is paralleled by marked ultrastructural changes in the blastomere nucleoli. It is believed that nucleoli up to the fourth cell cycle occur in the form of spherical electron-dense nucleolus precursor bodies (NPB), that these structures became vacuolized during the fourth cell cycle and that they develop into fibrillogranular nucleoli at the end of this cycle; during the fifth cell cycle, the fibrillogranular nucleoli develop fibrillar centres as well.

Although activation of major portions of the bovine genome occurs during the fourth cell cycle, the genome is not completely silent in the preceding cycles; some embryonic transcriptions have been detected as early as the second cycle (Plante *et al.*, 1994; Viuff *et al.*, 1994, 1995). There is evidence for the belief that the activation of transcription may be triggered by a lengthening of the cell cycle; in comparison with the fourth cell cycle (21–30 h duration), the cycle in the cow may be too specialized and the second and third cycles too short (12–14 h) to allow for transcription. Cell-cycle chronology may play a role in determining embryonic transcription.

A study by Hyttel *et al.* (1996) showed that IVP cattle embryos displayed a certain rate of transcription during the second cell cycle without the presence of a well-defined transcriptional peak; such activity was paralleled by the cell-cycle-dependent appearance and complex interaction of intranuclear electron-dense bodies and granules. A study by Schramm and Paprocki (2000) in Wisconsin sought to characterize the expression of fibrillarin in the early cattle embryo. They showed that fibrillarin was initially expressed at the pronucleate stage and was recruited from maternal stores; it was subsequently transcribed by the embryonic genome starting at the eight-cell stage, coincident with the onset of nucleolar transcription. This work shed further light on the organization and development of the embryonic nucleolus during early embryo development.

In Slovakia, Kopecny *et al.* (2000) reported that an important morphological feature of the two-cell bovine oocyte is the vacuolization of the

NPB. Other studies in that country, reported by Laurincik *et al.* (1999, 2000a,b), dealt with nucleolar development in the nuclei of bovine embryos up to the fifth post-fertilization cell cycle; during the fifth cell cycle (16-cell stage), a spherical fibrillogranular nucleolus developed from the start of the cell cycle. In Denmark, studies reported by Hay-Schmidt *et al.* (2001) attempted to verify whether transcription is activated during the initial cell cycles of the bovine embryo in the live animal and to reveal the ultrastructural localization of this putative transcription; they found a low level of transcription as early as the one-cell stage (i.e. the zygote).

7.2.3. Compaction and cavitation

Two major events in the early life of the bovine embryo are compaction and cavitation. Compaction is the term used in referring to the tight intercellular junctions that develop in the late morula; cavitation is the formation of the blastocoel, the fluid-filled cavity found in the blastocyst. Prior to compaction, the blastomeres of the bovine embryo are loosely associated and they readily disperse if the zona pellucida is removed; compaction sees the formation of the first transporting epithelium of the developing organism. Compaction is seen as a prerequisite to the formation of the trophectoderm (TE) and a barrier against the extraembryonic environment. Compaction occurs around the 32-cell stage in both *in vitro* and *in vivo* cattle embryos (Van Soom *et al.*, 1997a,c). One factor affecting the viability of cattle IVP embryos is thought to be a failure to undergo compaction in the same way as *in vivo* embryos; the timing of compaction also coincides with the first period of embryonic loss recorded in the live cow. In Denmark, a paper by Hay-Schmidt *et al.* (1998) dealt with the development of cell adhesion and junctional complex proteins in IVP cattle embryos. Elsewhere, Carolan *et al.* (2000) showed the presence and localization of embryo compaction-associated molecules, E-cadherin (a cell-adhesion molecule) and ZO1-α + (a tight-junction protein) in cattle morulae and observed differences between those produced *in vitro* and *in vivo*. Work in Ireland by Morris *et al.* (2001) resulted in data showing that significant changes in the rate and pattern of protein synthesis and phosphorylation occurred in cattle embryos around the time of compaction; they also found evidence of differences in these activities between IVP and *in vivo*-produced embryos.

Both compaction and cavitation are dependent on transcription of appropriate embryonic genes and their sequential activation. Transformation of the morula to the blastocyst stage is regarded as a major step in early embryonic development; the process gives rise to two different cell types, those of the inner cell mass (ICM) and the trophoblast cells. The trophoblast cells (TE) give rise to the tissues of the fetal placenta and associated extraembryonic membranes, whereas the ICM develops into the three germ layers of the developing embryo (endoderm, mesoderm and ectoderm). The formation of the developmentally competent bovine embryo blastocyst involves the processes of cell division, differentiation and cell death. It is known that cell death in the early embryo occurs by apoptosis, which presumably serves to eliminate unwanted cells during the critical changes that take place in early embryonic development. The events of blastocyst formation are initiated during compaction, which is mediated by the appearance of cell-to-cell adhesion and the formation of tight junctions in the late morula; as shown in the studies of Watson *et al.* (1999a), blastocyst formation requires the coordinated expression of several gene products. In New Zealand, Thompson *et al.* (2000a,b) studied the effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastocyst formation of cattle embryos cultured *in vitro*; the authors concluded that ATP production via oxidative phosphorylation is essential for *in vitro* embryo production.

Cavitation (blastocyst formation) is mediated by fluid transfer across the outer blastomeres, eventually forming the fluid-filled cavity (the blastocoel). Cavitation accompanies the formation of the first two embryonic cell lines, including the outer epithelial TE and the undifferentitated ICM. For cavitation to proceed, a differentiated polarized epithelium must form to support the transport of water to form the blastocoelic fluid. A study by Offenberg *et al.* (2000) investigated the mechanisms underlying fluid movement across the TE by determining whether aquaporins (molecular water channels)

are expressed during early development of the bovine embryo; their studies led them to conclude that aquaporins may function as important conduits at the time of blastocyst formation. Expansion of the bovine blastocyst is an active process regulated by the activity of the Na^{+}–K^{+}-adenosine triphosphatase (ATPase) located on the basolateral membrane of the TE cells; a study of energy metabolism during blastocyst re-expansion was reported by Donnay *et al.* (1998a).

Time-lapse videography is a useful way of studying the kinetics of early development in cattle embryos without compromising their viability; as observed by Holm *et al.* (1998a,b), several treatment groups may be cultured at the same time without disturbing development. They observed that embryos capable of reaching the compact morula or blastocyst stage within 7 days in their culture system (supplemented TCM-199) passed through the first four cell cycles faster than embryos whose development was arrested at earlier stages. They found no sex-related differences in embryo development under their culture system.

Hatching

The mechanisms by which the bovine embryo hatches remain unclear. A study by Yuan *et al.* (2001) sought to identify the molecules involved in the hatching process, by comparing gene expression in blastocysts and hatched blastocysts culturing *in vitro*. A differentially expressed gene that was only expressed in hatched blastocysts was found to be 93% homologous to human calmodulin, a Ca^{2+}-binding regulatory protein. It remains unclear whether such proteins are necessary for the hatching process.

Apoptosis

It is known that cell death and developmental arrest occur in the early embryo, although information is limited about the mechanisms involved in blastomere death. Cell death may take the form of either necrosis or apoptosis. Necrosis usually occurs in a group of adjacent cells and is characterized by cellular swelling and membrane rupture; apoptosis in the blastocyst affects single cells and involves several well-characterized morphological features. A review by Hardy (1997) dealt with cell death in the mammalian blastocyst; further information, this time dealing with the developmental regulation of apoptosis in the early bovine embryo, was provided by Matwee *et al.* (2000a,b). Apoptosis was detected from the nine- to 16-cell stage in embryos produced *in vitro* (Byrne *et al.*, 1998, 1999a,b). The apoptotic process is characterized by chromatin condensation, DNA fragmentation and 'blebbing' of the plasma membrane and, in contrast to necrosis, it may often occur without damage to adjacent cells.

The involvement of apoptosis in bovine blastocysts was the subject of a report by Yang and Rajamahendran (1999); their results suggested that apoptosis is a mechanism involved in fragmented blastocysts produced *in vitro*. A study by Matwee *et al.* (1999) examined apoptosis in the early bovine embryo by direct staining for DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL); their results indicated developmental regulation of apoptosis in the *in vitro* cattle embryo. In a further report, Matwee *et al.* (2000a,b) concluded that cattle embryos become more capable of accommodating damaged or abnormal cells as development proceeds. In Oregon, a study by Neuber *et al.* (2002) showed that the majority of IVP bovine blastocysts contained one or more apoptotic cells; by way of the TUNEL assay, they found that apoptosis was proportionately higher in ICM than in TE cells. The authors speculated that the regulation of ICM is more sensitive than regulation of the TE.

In Belgium, Feugang *et al.* (2000a,b) deliberately induced apoptosis in cattle embryos by the use of pro-oxidant agents; they found that culture conditions associated with the accumulation of reactive oxygen species increased the incidence of apoptosis at day 7 post-insemination. Other work in that country reported by Donnay *et al.* (2000a,b) showed that apoptosis is a physiological process in cattle embryos at the morula and blastocyst stage; under their culture conditions (SOF + 5% FCS), the incidence of apoptotic nuclei appeared to be similar between *in vivo*- and *in vitro*-derived embryos, although the kinetics of development was different. Other work with IVP cattle embryos in Belgium reported by Van Soom *et al.* (2000) attempted to determine the mitotic, pycnotic and apoptotic cell index before and

during blastocyst formation; they found that blastocyst development was characterized by a high apoptotic/low mitotic cell index.

Papers by Brison and Schultz (1997) and Brison (2000) have dealt with evidence in several species that apoptosis in the embryo is regulated by soluble peptide growth factors acting as survival factors in an autocrine or paracrine manner; such factors included transforming growth factor alpha (TGF-α) and members of the IGF family. It is also apparent that apoptosis may be affected by environmental factors, including culture conditions and the composition of the media. The authors concluded that regulation of apoptosis in the early embryo is likely to be of critical importance both for embryo viability and for later development, since the cells of the ICM give rise to the fetus and carry the germ line. An article by Allan *et al.* (2001) suggests that one possible mechanism unifying the IGF system in embryonic development is programmed cell death, which may be important in the sculpting of embryonic tissues. In Brazil, Watanabe *et al.* (2002) presented results suggesting a possible role of apoptosis in regard to the developmental competence of early cattle embryos; they showed that slow-developing embryos are more likely to cease development at the eight-cell stage, possibly by activating programmed cell-death pathways.

In Spain, Gutierrez-Adan *et al.* (2001a,b) sought to clarify the role of various mechanisms that may be involved in the adverse effect of high concentrations of glucose on bovine embryo development. They showed that the expression of BAX, a death-promoting member of the Bcl-2 family of proteins, is increased at the blastocyst stage in the presence of high concentrations of glucose. These findings provided evidence that a metabolite such as glucose, which may be influenced by many environmental factors in the cow, may be related to embryo mortality. In Belgium, a study by Feugang *et al.* (2002a) sought to evaluate the kinetics of apoptosis in both *in vivo*-produced and IVP cattle embryos, using the TUNEL technique; no significant difference in apoptosis was detected between the two sources of embryos. The authors found that the rate of apoptosis increased with time, regardless of embryo origin.

It is known that both oxygen tension and the presence of antioxidants can influence bovine embryo development. With that in mind, Van Soom *et al.* (2002) evaluated the prevalence of apoptosis in cattle embryos cultured in media supplemented with L-cysteine in modified SOF (mSOF) under two different oxygen tensions; apoptosis was displayed during blastocyst formation but under 5% oxygen in the presence of cysteine embryos possessed a significantly higher number of ICM cells.

7.2.4. Post-hatching progress

The bovine embryo undergoes a rapid and considerable expansion of its outer TE cells and inner endoderm cells after hatching from the zona pellucida. Hatching prepares the embryo for attachment to the uterine epithelium and results in the production of embryonic factors necessary for maternal recognition of pregnancy. It is now well established that the elongated cattle embryo produces (from the TE) a family of interferon molecules that mediate recognition of pregnancy (Stojkovic *et al.*, 1999b); there is also a family of TE-derived glycoproteins that has formed the basis of a pregnancy test. Various research groups have examined the needs of the elongating bovine embryo. In Ireland, Grealy and Sreenan (1995) measured cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and the protein content of 13–16-day-old hatched *in vivo*-produced bovine embryos; protein concentration increased 60-fold between days 13 and 16. Later, in the same country, Morris, D.G. *et al.* (2000) reported on amino acid utilization by the 14–16-day-old *in vivo*-produced embryo; the most consistent finding was the efflux of alanine, indicating a possible regulatory function for this amino acid in normal embryo development. In New Zealand, Thompson *et al.* (1998) reported on the total protein content and protein synthesis within pre-elongation stage cattle embryos.

The establishment of a hypoblast layer in IVP cattle embryos was the subject of a study reported by Alexopoulos *et al.* (2002); the hypoblast is a thin cell layer lining the TE in the hatched embryo and its presence is considered to be the first important criterion for the formation of extraembryonic structures to support further development. The results of the study showed

that a hypoblast layer was established in a higher proportion of embryos cultured with cow serum than in those cultured in FCS or bovine serum albumin (BSA). The variability observed in the outcome of the experiment may explain some of the differences in pregnancy rates found in reports on protein supplementation of culture media.

7.2.5. Embryo mortality

After ovulation and breeding, the cattle oocyte is fertilized in the ampulla of the oviduct and enters the uterus 72–84 h later (see Fig. 7.2). The blastocyst is formed after a further 3–4 days; this hatches at about day 9 of gestation and starts attaching to the uterine wall from about day 22. Before it reaches the blastocyst stage, and despite cell division, the embryo shows no increase in volume or protein content. At the blastocyst stage true growth commences, with rapid cell division and differentiation. Embryo size and protein content increase markedly between hatching at day 8 or 9 and day 16 (Grealy and Sreenan, 1995, 1996; Grealy *et al*., 1996).

Even in normal healthy cattle, some proportion of embryos (25% or more) that have passed through the Fallopian tubes into the uterus fail to continue development, generally during the first 3 weeks of pregnancy. Embryo mortality has long been recognized as a major source of loss in breeding cows and numerous studies have reported on it. As described by Sreenan and Diskin (1994), the fertilization rate after the cow has been bred can be taken as about 90%, whereas the average calving rate to a single service may be below 50% (Humblot, 2001); much of this loss is the result of embryo mortality occurring between days 8 and 18 after breeding (see Sreenan *et al*., 2001). Between days 18 and 50, a further 10–15% of embryos die. When embryo death occurs before days 6–17, the cow can be expected to repeat after a normal oestrous-cycle interval (i.e. 18–24 days); when embryo mortality occurs after days 16–17, the cow repeats at long and irregular intervals. Between day 50 and full term, the incidence of fetal death is usually given as 5–8%. Data from beef cross-bred heifers presented by Dunne *et al.* (2000) in Ireland dealt with embryo and fetal loss between day 14 of gestation and full term; most embryo losses occurred before day 14, similar embryo survival rates being recorded at days 14, 30 and full term. The occurrence of embryo mortality in a conventional cattle embryo transfer programme was reported by Rodrigues (1995).

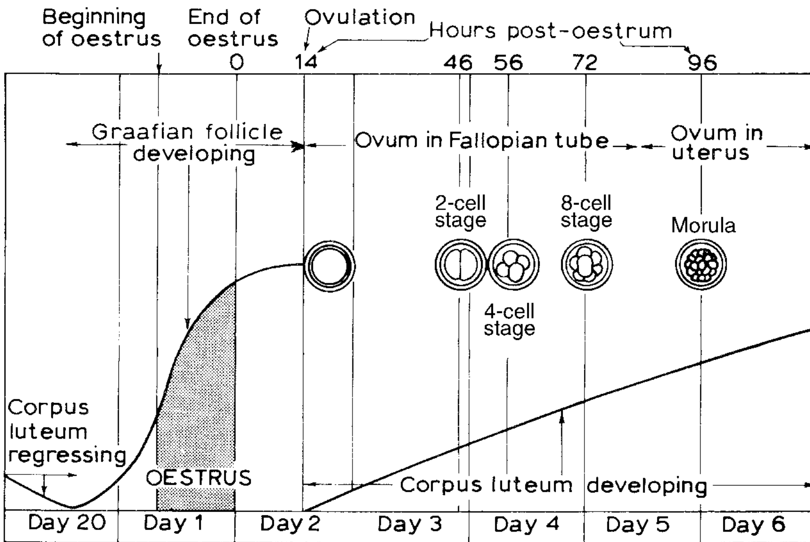


Fig. 7.2. Events in the early life of the bovine embryo: diagrammatic representation of events at and about oestrus in the cow (from Laing, J.A., 1949).

In the cow, attachment of the embryo to the uterine wall does not occur until about 3 weeks after conception, by which time considerable growth and development of the embryo have taken place. Embryo recovery at specific intervals after breeding has been employed to determine the time at which embryo mortality occurs. From the published evidence on the extent and timing of embryo mortality, it appears that the greatest single increase in the rate of embryo loss occurs between days 15 and 18. In practical terms, if only half of those embryos lost in the first 3 weeks of pregnancy could be saved, the financial and welfare benefits would be considerable. As it is, in the dairy cow of the Western world, calving rates after a single insemination are only about 50% and have been steadily decreasing over the past two decades (Lamming *et al.*, 1998; Humblot, 2001).

Factors in embryo mortality

Some of the factors currently recognized as involved in embryo mortality in the cow are set out in Fig. 7.3. Much of the embryo loss appears to be due to environmental factors, either internal to the cow itself or arising from external influences. Studies have shown that the rate of genetic abnormalities in lost embryos is about 8%. Studies with cattle embryos produced *in vitro* looked at the influence of chromosomal constitution on the growth rate of embryos (Kawarsky *et al.*, 1994); they found that slow development in chromosomally abnormal embryos could be a cause of embryonic loss. A review by McEvoy (1999) of cattle fertility has dealt with the many factors affecting the intrinsic competence of oocytes and embryos. A review by Sreenan *et al.* (2001) points to the urgent need to establish fertilization rates and the pattern of embryo loss in high-producing cows.

Embryo–pathogen interactions

The causes of embryo mortality in the cow can be divided into infectious and non-infectious categories. Specific uterine infections are caused by viruses, bacteria and protozoa that enter the uterus by way of the blood circulation or via the vagina; non-specific pathogens are mainly bacteria that enter the uterus by ascending infection. Uterine pathogens may cause embryo mortality by altering the uterine environment (endometritis) or by a direct cytolytic effect on the embryo. Although attention has often been directed towards infectious agents, the evidence is that non-infectious causes probably account for 70% or more of the causes of embryo death (Vanroose *et al.*, 2000b). In dairy cows, the more intensive production systems and associated higher milk yields generally involve a decreased dependence on grass and an increase in the energy content of the diet. It is obviously

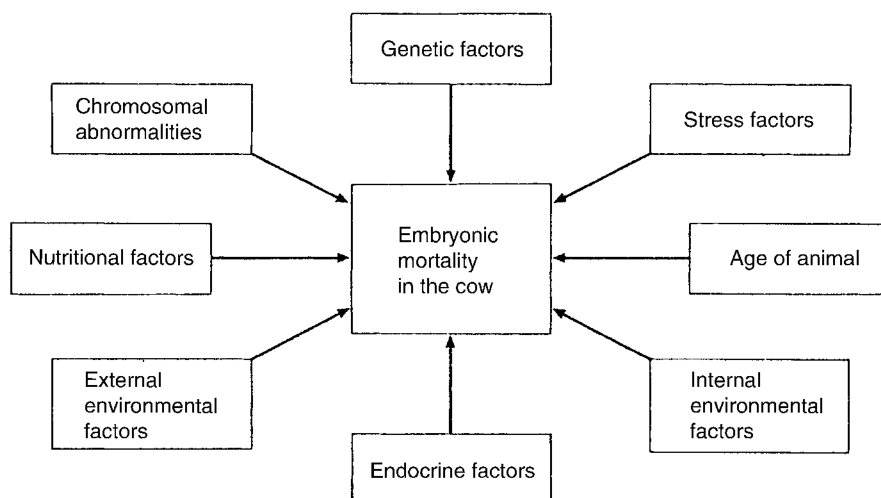


Fig. 7.3. Factors in embryonic mortality in the cow.

important to understand how the changing composition of the dairy cow's diet affects fertility. Among recent reports is one from workers in the UK who have shown that the polyunsaturated fatty acid content of the dairy cow's diet may affect ovarian and uterine function (Robinson *et al.*, 2002); it remains to be seen whether fertility can be influenced positively by this means. In Ireland, Kenny *et al.* (2002b) found no effect on embryo survival in beef heifer cattle when they employed a diet containing rumen-degradable protein, with or without fermentable carbohydrate supplementation, Elsewhere, Laven *et al.* (2002) investigated the effects on embryo growth and survival of feeding heavily fertilized spring grass, containing high levels of quickly degradable nitrogen to pregnant cows; they found no evidence of adverse effects from 20 days of pregnancy onwards. It was concluded that the impact of spring turnout on fertility mainly affected ovulation, fertilization and/or the early embryo.

Fate of the conceptus

Transrectal ultrasound examinations have been used in studying the relationship between the time of spontaneous embryo death (cessation of heartbeat) and luteal regression and determining the fate of the conceptus after embryo death. It appears that the conceptus and its breakdown products are eliminated by expulsion through the cervix rather than by resorption.

Losses after embryo transfer

There have been suggestions that embryo mortality may be greater after embryo transfer than after natural service or artificial insemination. It would not be altogether surprising if this were true, given the inevitable insults to which embryos are exposed; they clearly have to contend with a microenvironment quite alien to the oviduct and uterus of the cow. Efforts to understand factors responsible for embryo mortality should certainly include a study of the mechanisms involved in the maternal recognition of pregnancy (see Bazer *et al.*, 1994). Although most embryo losses after artificial insemination occur in the first 3 weeks of pregnancy, after embryo transfer there are some worrying indications of continuing loss in the period of placental establishment, which probably require evaluation.

Fetal losses

Embryonic development covers the period of prenatal life up to about 50 days of age in cattle; beyond that, fetal growth and development continue through to term. There is still a scarcity of information on the factors affecting the development of the embryo and the fetus; the influence of maternal factors needs to be clearly separated from those arising from the quality of the embryo itself. The rate of bovine embryo/fetal deaths between 30 and 60 days of gestation in Friesian heifers has been recorded as about 5% (Alexander *et al.*, 1995). Estimates of endemic fetal loss, based on studies in dairy herds over five decades, have given an overall figure of 6.5% (Forar *et al.*, 1995). The use of ultrasound scanning to confirm losses in dairy cows indicated by milk progesterone profiles is dealt with by Ball *et al.* (1995).

7.3. *In Vivo* Culture Systems

It is now clear, from numerous reports, that the oviducts of several mammalian species (including rabbits, sheep and mice) can sustain early cattle embryos *in vivo* and yield morulae and blastocysts capable of establishing normal pregnancy rates in recipient animals. Although the practical employment of such *in vivo* culture systems is likely to be of historical interest only in the years ahead, they can be regarded as having contributed substantially to knowledge of factors influencing early embryo development.

7.3.1. The rabbit oviduct

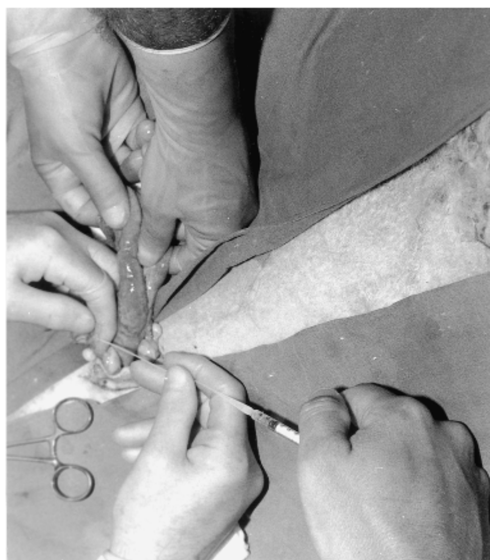
Although early attempts to store cattle embryos in the rabbit proved unsuccessful (Hafez and Sugie, 1963), later work in Ireland had a more positive outcome (Sreenan and Scanlon, 1968). It became clear in Cambridge and Ireland that, in contrast to experiences with sheep, homologous blood serum was an unsatisfactory medium to use when transferring cattle embryos to the rabbit oviduct. Although Cambridge work

suggested that it was inadvisable to store cattle embryos in the rabbit for more than 3 days or in the sheep oviduct for more than 4 days, the Irish experience was that early-cleavage cattle embryos would continue developing satisfactorily for several days. This was shown by the high percentage that developed into a normal conceptus on transfer to the recipient cow (see Boland, 1984).

The number of cattle embryos transferred per oviduct has usually been in the range of five to 20; the expected rate of embryo recovery from the ligated Fallopian tube has been about 70% (Boland, 1984). The doe may be treated or not treated with human chorionic gonadotrophin (hCG) to induce ovulation prior to the transfer of embryos; however, there is no clear evidence that the rabbit's reproductive status is crucial in determining the effectiveness of the *in vivo* culture period. In using the rabbit, the Fallopian tube is usually ligated at the uterotubal junction (UTJ) to prevent embryos passing into the uterus; some evidence has suggested that cleaved cattle embryos may be better retained in the oviduct. In the USA, workers at Beltsville found that leaving the rabbit oviduct open rather than ligating it resulted in the loss of most embryos; it was believed that the embryos were lost via the cervix. In recollecting Irish experiences with cattle embryos exposed to the rabbit oviduct, where hormonal and other factors were clearly different from those found in the bovine oviduct, it is of interest to note that no instance of fetal abnormality or health problem in offspring appears to have been recorded after transfer of rabbit-incubated bovine embryos to recipient cattle.

7.3.2. The sheep oviduct

Sheep oviducts have been employed for many years as an *in vivo* culture system (see Fig. 7.4). In work reported almost a half-century ago, it was shown that sheep embryos retained in the oviducts of the ewe for up to 8 days were capable of developing into normal healthy lambs on transfer to recipient animals (Wintenberger-Torres, 1956). In Ireland, work dates back to the 1960s and the studies of Sreenan, who attempted to fertilize artificially matured cattle oocytes in the oviducts of sheep previously inseminated with bull semen. In providing for the needs of cattle oocytes/embryos, it is not unreasonable to assume that the closely related sheep may have a possible advantage over the rabbit; in practice, differences between the two species have not always been evident. For



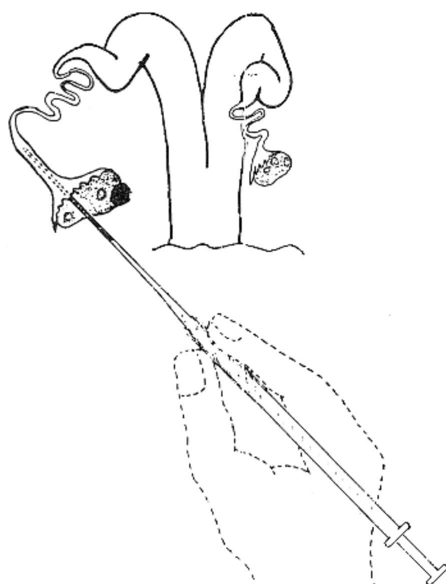


Fig. 7.4. Transferrring bovine zygotes to the sheep oviduct for *in vitro* culture.

transfer purposes, however, the ewe's greater size and consequent oviductal capacity have enabled it to accept many more embryos than the rabbit. In Ireland, Lu found that several hundred presumptive cattle zygotes could be transferred to the oviduct without adversely affecting the subsequent embryo recovery rate. In contrast to the rabbit, non-ligation of the sheep oviduct did not necessarily have an adverse effect on subsequent embryo recovery rate. It should be mentioned that the sheep has been the subject of much research in the control of oestrus and ovulation (see Gordon, 1997); this makes it possible, with relative ease, to have animals available in all seasons of the year to take embryos at a clearly defined stage of their oestrous cycle.

The appropriate technique for the artificial control of oestrus and ovulation in the sheep (cyclic or anoestrous) involves the intravaginal administration of an appropriate progestogen (e.g. fluorogestone acetate (FGA); methyacetoxyprogesterone (MAP)) over a period of 12 days in conjunction with an intramuscular injection of gonadotrophin (500–750 iu pregnant mare serum gonadotrophin (PMSG)) at the time of pessary removal. The ewe can be expected to come into oestrus 36 h after pessary withdrawal and to exhibit a heat period with a duration of a further 36 h; ovulation can be expected to occur some 60–70 h after pessary withdrawal. In Ireland, Lu introduced presumptive cattle zygotes into the ligated oviduct shortly after the occurrence of a controlled ovulation; this was on the assumption that this timing would provide a tubal environment corresponding closely to *in vivo* events in the cow. Elsewhere, however, reports led to the general conclusion that ovarian activity in the ewe was not essential for the normal development of cattle embryos in the oviduct.

A study by McCracken *et al.* (2000) at the Roslin Institute examined the effect of temporary *in vivo* culture of sheep embryos, recovered from superovulated ewes, on their subsequent fetal size and development. Day 2 embryos were recovered from donor sheep and cultured in the ligated oviducts of synchronous ewes; there was no evidence that the temporary (1 week) *in vivo* culture of sheep embryos compromised their subsequent development. Such evidence would be in agreement with that accumulated over many years in Ireland and elsewhere that apparently failed to record adverse sequelae in ruminant embryos (*in vivo*-produced) temporarily incubated in the sheep oviduct. Some data on recovery rates and embryo yields from the sheep oviduct are provided in Table 7.2.

Despite improvements in cattle *in vitro* embryo production systems, IVP embryos still display several differences in comparison with their *in vivo*-derived counterparts, including significant aberrations in mRNA expression patterns. It is known, however, that the ligated sheep oviduct can be used to produce high-quality blastocysts from *in vitro*-matured (IVM) and *in vitro*-fertilized (IVF) zygotes (Enright *et al.*, 2000b; Galli *et al.*, 2001; Rizos *et al.*, 2002a,b). A report by Herrmann *et al.* (2002) compared the mRNA expression pattern of *in vivo*-cultured cattle blastocysts compared with that of IVP embryos; they found that culture in the sheep oviduct provided an environment similar to that in the cow and superior to that provided by culture in SOF. The authors saw the need to further optimize the existing culture systems employed in embryo production.

Elongation-stage bovine embryos

A feature of ruminant embryonic development is the amazing growth that occurs in the space of a few days in the dimensions of the organism (see Fig. 7.5). The investigation of factors involved in bovine blastocyst elongation and attachment has been difficult because of the costs involved in doing this with cattle. However, it is now clear from studies at Beltsville that the sheep uterus can maintain the early development of the bovine embryo (Powell *et al.*, 1996; Rexroad and Powell, 1999; Talbot *et al.*, 2000),

Table 7.2. Summary of data on cattle embryo yields after culture in the sheep oviduct.

Year	Oocytes to ewe	Recovered from ewe	Embryo yield	Researcher(s)
1987	543	370 (68%)	113 (30.1%)	Lu *et al.*
1988	668	420 (63%)	191 (46.0%)	Lu *et al.*
1990	55,797	31,469 (56%)	7,401 (23.5%)	Lu *et al.*

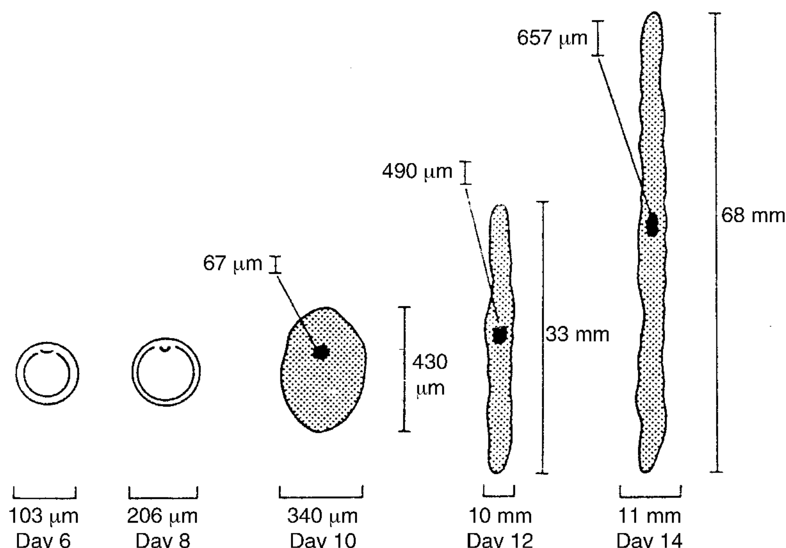


Fig. 7.5. Diagrammatic representation of the growth and development of the sheep embryo.

thereby providing an economical source of material for research.

Beltsville workers demonstrated that *in vitro* cattle embryos continued development in the sheep's reproductive tract when transferred either as four-cell embryos or as expanded or hatched blastocysts; development continued with extreme elongation (filamentous) when the ewe's oestrous cycle was extended by progestogen treatment. IVP embryos transferred into cattle were similar in size to those transferred into sheep. Such findings indicate that the uterine environment of the sheep is capable of providing most of the needs of the post-hatching cattle embryo. Such *in vivo* incubation may be useful in assessing the developmental competence of cattle blastocysts. As noted elsewhere, there are those who believe that undue reliance is placed on the blastocyst as the end-point of studies on cattle embryo production. It is, after all, known that most embryonic loss (70–80%) in cattle occurs between days 8 and 16, which is the period during which the roughly spherical embryo elongates to its characteristic filamentous form.

7.3.3. The isolated mouse oviduct

Until there is a more complete understanding of what the mammalian oviduct provides for the early developing embryo, an isolated organ culture system may have an advantage over some other methods (oviductal/granulosa cell monolayers), which may not always maintain cells in the same state of differentiation as in the live animal. In this regard, the isolated mouse oviduct culture system (IMOS) has been used successfully in the culture of mouse, rat, hamster and pig embryos from the one-cell to the morula/blastocyst stage. The method used in Ireland a decade ago with mice is outlined in Fig. 7.6; using such a system, it was concluded that the mouse oviduct was capable of giving embryo yields comparable to those found with the other *in vivo* culture systems (rabbit/sheep oviducts).

In Japan, Minami *et al.* (1994) also demonstrated that cattle embryos could develop to the blastocyst stage when co-cultured with isolated mouse ampullae; they also showed that the absence of serum from their TCM-199 culture medium had no effect on bovine embryo development. Although serum supplementation is usually required to maintain somatic cell co-culture, the beneficial effects of mouse ampullae in their culture system could be maintained for at least 8 days in the absence of serum.

Using oviducts of live mice

A study to investigate the viability and development of the early ovine embryo in the oviducts of

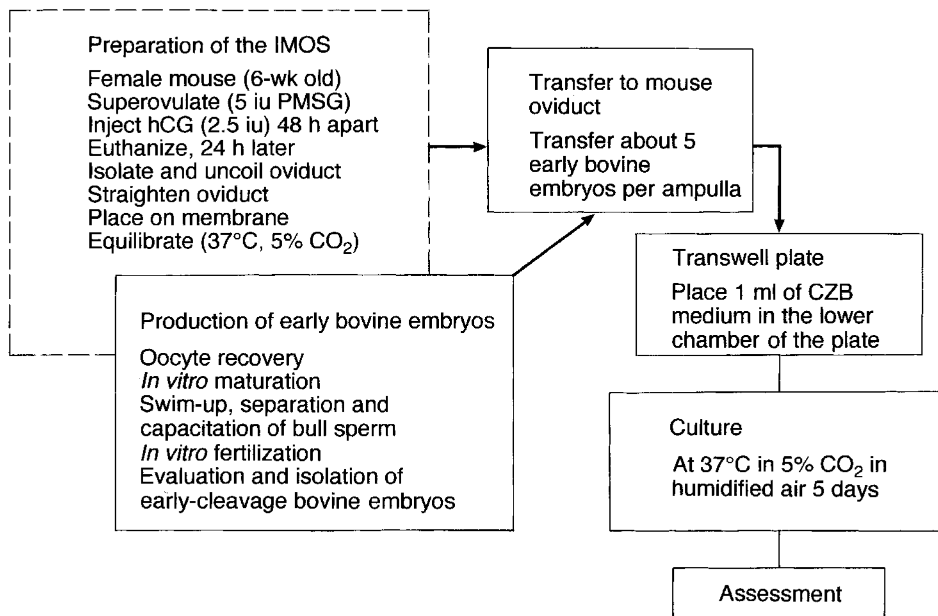


Fig. 7.6. Culturing cattle embryos in the isolated mouse oviduct (from Sharif *et al.*, 1991).

live mice was reported by Capitan *et al.* (1994) in London; sheep embryos were transferred to the oviducts of mice for 48–72 h, recovered, assessed and retransferred until developmental arrest. The authors concluded that such embryos were able to develop to stages suitable for transfer or cryopreservation. Some years later, Kim, C.H. *et al.* (2000) in Japan described the use of mouse oviducts without ligation as a culture system for IVP cattle embryos; two-cell cattle embryos were transferred to the oviducts of pseudopregnant mice and recovered by flushing oviducts and uterus 5–7 days later. Of the embryos recovered from mice, 71% had developed to the morula/blastocyst stage, in contrast to a figure of 34% for embryos in culture *in vitro*. The authors concluded that the mouse culture system could be useful for obtaining late-stage embryos from farm animals.

7.4. Metabolism of the Early Embryo

A paper by Barnett and Bavister (1996) critically reviewed comparative data on the metabolism of the early mammalian embryo and the relationship between metabolism and developmental competence. They reviewed the many methods that may be employed in obtaining information about embryo metabolism and concluded that, despite considerable advances in culture technology, embryo development *in vitro* may still be suboptimal; many advances have resulted from empirical adjustments to the culture environment. They note that undue emphasis has been placed on the proportion of embryos reaching the blastocyst stage and that relatively few studies have demonstrated the normality of cultured embryos by transfer to recipients and the birth of young.

7.4.1. Monitoring embryo metabolism

Adenosine triphosphate (ATP) is the major energy currency of the oocyte and its content reflects the balance between energy production and consumption. Preliminary observations on the ATP content of bovine oocytes and embryos were reported by Rieger (1997); ATP content increased during oocyte maturation and reached a maximum at the eight-cell stage before declining to a minimum at the hatched blastocyst stage. A report by Stojkovic and Zakhartchenko (2000) showed that the later developmental stages of cattle embryos contained

more ATP than earlier stages, possibly due to their higher metabolic activity or to an increase in the number of cells/mitochondria (see Table 7.3). Studies reported by Tamassia *et al.* (2002) failed to establish any clear-cut relationship between oocyte ATP content and blastocyst yield in their embryo production system.

As shown by Pinyopummintr and Bavister (1995), few cattle embryos are able to complete the first cleavage stage without exogenous energy sources; they also showed that amino acids cannot support cleavage but significantly enhance blastocyst development in combination with lactate. Pyruvate is known to be the preferred energy substrate for bovine pre-elongation embryos during *in vitro* development (Thompson, 1996; Thompson *et al.*, 1996a,b); it has been shown that as embryos begin compaction, a greater dependence on glycolysis occurs for ATP generation, to coincide with the transition from the relatively well-oxygenated oviduct lumen to the poorly oxygenated uterine lumen. A study by Gardner *et al.* (1996) showed that frozen–thawed cattle blastocysts that expanded their blastocoels during the 5 h period after thawing had significantly greater glucose and pyruvate uptake and lactate production compared with those that failed to expand after a 14 h incubation period. It has been suggested that pyruvate utilization may be a useful indicator in identifying suitable constituents of an embryo culture medium for cattle embryos (Donnison *et al.*, 1996); in sheep, Thompson *et al.* (1996a,b) recorded marked differences in pyruvate utilization between *in vitro* and *in vivo* embryos. The utilization of various energy substrates by bovine embryos has been the subject of studies by several groups (Gomez, 1997; Gomez and Diez, 1997, 1998; Lee, S.H. *et al.*, 1998; Gomez *et al.*, 2000, 2001).

Table 7.3. ATP content of IVP cattle embryos at different stages of development (from Stojkovic and Zakhartchenko, 2000).

Day of culture	Embryo stage (*n*)	Mean ATP content (pmol ± SEM)
7	Morula (11)	0.70 ± 0.04[a]
7	Blastocyst (13)	0.88 ± 0.09[a]
7	Expanded blastocyst (13)	1.13 ± 0.78[b]
8	Expanded blastocyst (11)	2.33 ± 0.25[c]
8	Hatching blastocyst (10)	2.37 ± 0.42[c]
8	Hatched blastocyst (11)	4.67 ± 0.44[d]

[a-d]Different superscripts indicate significant differences ($P < 0.05$).
SEM, standard error of the mean.

Oxidative metabolism is the main source of energy for supporting the development of the cattle embryo up to the blastocyst stage, although during blastocyst expansion both oxidative metabolism and glycolysis are used. A study by Donnay and Leese (1999) examined the relationship between energy metabolism and the activity of Na^+–K^+-ATPase during expansion of the IVP bovine blastocyst; the authors suggested that oxidative metabolism has a role in providing the energy necessary for blastocoel expansion. Further studies in cattle reported by Donnay *et al.* (2000a,b) suggested that inhibiting oxidative metabolism is detrimental to embryonic development, particularly during early cleavage stages (days 1–3) and during blastocyst formation (days 6–8).

A review by Thompson (2000) notes that a new concept in embryo culture *in vitro* is that of the regulation of energy metabolism; compounds such as ethylenediamine tetra-acetic acid (EDTA), NaN_3 and 2,4-dinitrophenol have been shown to increase embryo development and embryo quality (Thompson *et al.*, 2000a,b). Such evidence supports the view that the process of ATP production is a key regulator of *in vitro* embryo development. A study by Gardner *et al.* (2000) showed that EDTA inhibited glycolytic activity of the cleavage-stage bovine embryo, thereby preventing the premature stimulation of glycolysis and enhancing development; the authors emphasize that EDTA should not be used for the later-stage embryo as the inhibition of glycolysis reduces energy production at the blastocyst stage and significantly inhibits ICM development.

Oxygen consumption

Observations on the uptake of oxygen by day 7 *in vivo*-produced cattle embryos were reported by Thompson *et al.* (1995); they measured oxygen uptake on groups of two to four blastocysts, using a novel, non-invasive fluorescent technique. The authors found that the cattle

blastocyst has a low oxidative capacity compared with other body tissues. The technique of scanning electrochemical microscopy was used by Shiku *et al.* (2001) in Japan to quantify non-invasively the oxygen consumption of individual cattle embryos; such measurements appeared to be strongly related to morphological embryo quality as assessed by optical microscopic observation.

Glucose utilization

Glucose utilization is known to increase during bovine blastocyst formation and expansion, but high concentrations of the hexose sugar are believed to be detrimental to blastocyst development and may induce apoptosis. In Belgium, Donnay *et al.* (1999a) found that the addition of 5.5 mM glucose to their SOF medium when cattle embryos were at the morula stage increased blastocyst yield without affecting the rate of apoptosis; there was decreased pyruvate uptake in the presence of glucose. In a study reported by Martins *et al.* (2001), development was faster and bovine embryo quality improved in a SOF-based medium without glucose as compared with results using TCM-199 with 5.5 mM glucose added.

Energy metabolism-related gene expression

Work reported from Japan by Konishi *et al.* (1994) investigated the effect of glucose on the development of bovine embryos fertilized *in vitro* up to the blastocyst stage using CR1 with amino acids (CR1aa) medium; they found that glucose concentrations of 0.1 mM–1.0 mM in the medium plus 2% calf serum from day 3 to day 10 in co-culture with cumulus cells were beneficial. Energy metabolism-related gene expression has been examined by various workers. It is known that maternal glucose is the predominant exogenous energy substrate in the blastocyst and that glucose uptake is undertaken by specific glucose transporters (GLUT), one isoform of which (GLUT1) was described in bovine embryos up to the 7-day-old blastocyst stage (Wrenzycki *et al.*, 1998a,b,c) and a second isoform (GLUT4) was reported by Augustin *et al.* (1998) in day 14 and day 16 elongating bovine blastocysts.

Myo-inositol, adenylyl cyclase

Investigations by Hynes and Kane (1994) and Hynes *et al.* (2000) examined the uptake and incorporation of myo-inositol by cattle embryos ranging from the two-cell stage to elongated blastocysts (days 2–17); they found that uptake of inositol did not vary significantly from the two-cell to the early blastocyst stage. Elsewhere in Galway, Grealy and Sreenan (1994) measured activatable adenylyl cyclase levels in early bovine embryos; the enzyme was present in one- to two-cell embryos but its level had not increased by the early hatched blastocyst stage.

Ultrastructural autoradiography of RNA synthesis

A study by Pivko *et al.* (1999) in the Czech Republic assessed the morphology and autoradiographic detection of RNA synthesis in embryos from superovulated cows; ultrastructural analysis and synthesis of RNA confirmed that embryos had a high metabolic activity. They concluded that RNA synthesis was greater in ICM cells than in trophoblastic cells.

7.4.2. The development block

A very real obstacle to progress in developing embryo technology in cattle in the 1960s and 1970s was the fact that cattle embryos at the one- to four-cell stages rarely developed beyond the eight- to 16-cell stage; in contrast, embryos cultured at the eight- to 16-cell stage could usually be expected to undergo compaction, blastulation and hatching. It was this difficulty in early embryo cleavage that led to the development of the different *in vivo* culture systems described earlier (see Section 7.3). Stages in the development of IVP cattle embryos produced by Lu and associates, from the two-cell stage to late morula, are shown in Fig. 7.7.

7.4.3. Activation of the bovine embryonic genome

Prior to the maternal to zygotic transcription transition in early embryonic development in

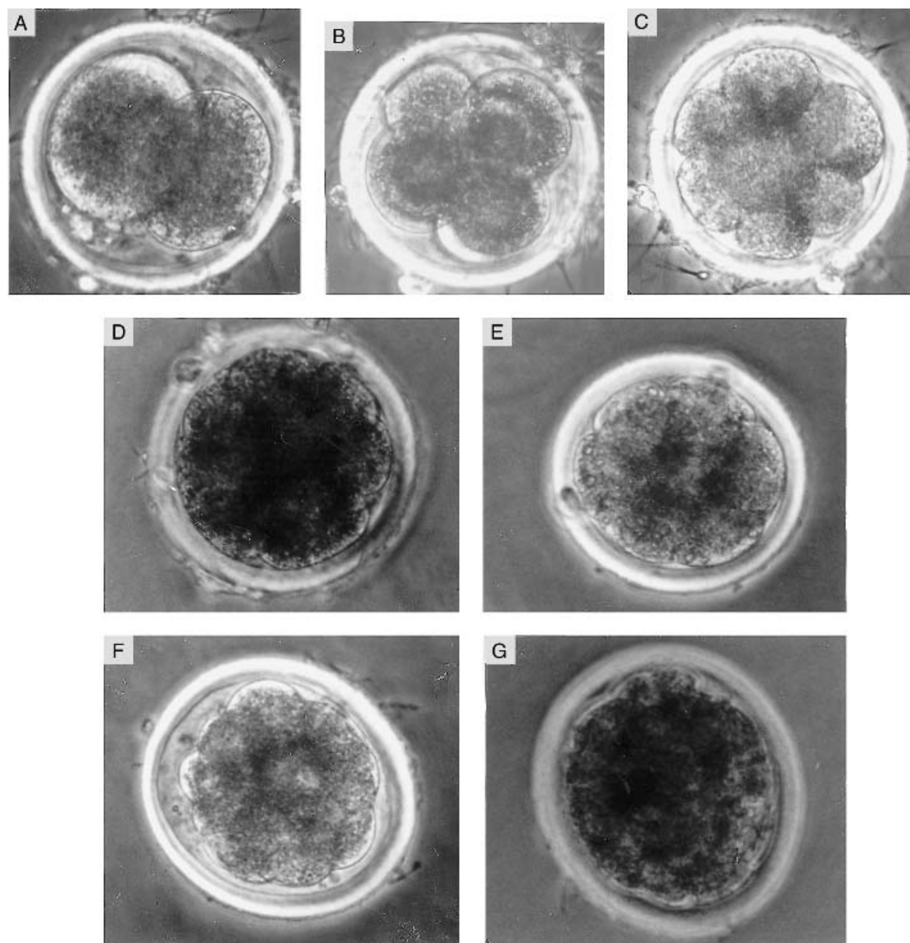


Fig. 7.7. From two-cell embryo to late morula in 6 days. The bovine embryo reaches the two-cell stage at about 30 h after IVF (A); four-cell (B) and eight-cell (C) stages are found at 48 h after IVF; by day 3 the embryo should have reached the 16-cell stage (D) and by day 4 it should be at the 16–32-cell stage (E); by day 5 it would be forming a compact morula (F) and by day 6 it can be expected to have reached the late morula stage (G).

cattle, little RNA is synthesized and exported from the nucleus. After activation of the bovine embryonic genome, the embryo becomes dependent on new transcripts produced by the nucleus to continue development. Although many lines of evidence point to a major turn-on of the bovine embryonic genome at the eight- to 16-cell stage (Cabot *et al.*, 1999), it is also apparent that the genome is not completely silent during earlier cell cycles (Plante *et al.*, 1994; Iwasaki *et al.*, 1995; Marcucio *et al.*, 1995; Hyttel *et al.*, 1996; Saeki *et al.*, 1998; Campion *et al.*, 2000; Lim and Hansel, 2000). Reports by Memili *et al.* (1998a,b) and Memili and First (1999) indicated that the change from maternal to embryonic control starts as early as the two-cell stage in cattle. Studies by Laurincik *et al.* (1998b) led them to conclude that the protein requirements for ribosomal RNA (rRNA) transcription and ribosome production are built up gradually over the first four cell cycles in IVP bovine embryos.

Evidence for transcription in early bovine embryos (two-cell) was reported by Chandolia *et al.* (1999a,b) on the basis of synthesis of heat-shock protein 70. In Japan, Saeki *et al.* (1999) reported results indicating that gene transcription in cattle embryos produced *in vitro* may be already active in one-cell embryos at 24 h

post-insemination (hpi). A review by Memili and First (2000) drew attention to studies suggesting that cattle zygotes and two-cell embryos are both transcriptionally and translationally active. A low level of transcription was detected by Hay-Schmidt *et al.* (2001) in cattle embryos produced *in vivo* as early as the one-cell stage.

7.5. *In Vitro* Culture Systems

A review by Bavister (1995) described early milestones in embryo culture technology and surveyed the large body of published literature available at that time. Among the many useful suggestions made for advancing the efficiency of embryo culture were as follows: (i) the need to place emphasis on discovering the embryo's needs and on designing media that supply them; (ii) if possible, avoid using serum in the medium entirely, at least in the early cleavage stages; (iii) where use of serum seems useful (for blastocyst development), attempt to replace serum with defined components, such as growth factors; (iv) if BSA is used in the culture medium, use fatty acid-free preparations, but even so be aware that contaminants may be present; (v) examine the replacement of protein in the culture medium with an inert polymer, such as polyvinyl alcohol (PVA); (vi) evaluate the usefulness of multi-step media formulations to improve embryo development; and (vii) ensure that embryo transfer is used regularly to validate the normality of cultured embryos. A later paper by the same author (Bavister, 2000) dealt with interactions between embryos and the components of the IVC medium and about the nature of the support provided for early embryos by the female reproductive tract. The author suggested that further elucidation of these events and their underlying regulation will assist in improving culture media formulations to support normal embryo development.

7.5.1. Embryo culture systems: past and present

Although much early work in the culture of cattle embryos employed TCM-199, later research made increasing use of much simpler media, such as mSOF, CR1 medium, hamster embryo culture medium with 11 amino acids (HECM-6), modified simplex optimized medium (KSOM) and complete defined medium (CDM); CDM is a simple chemically defined medium with minimum essential medium (MEM) non-essential amino acids (NEAA), 0.5% BSA and heparin. Complex tissue-culture media such as TCM-199 were designed specifically for sustaining somatic cells *in vitro* rather than for the culture of the early mammalian embryo. An analysis of some 72 reports on cattle IVC by Thompson and Duganzich (1996) led them to conclude that a better-than-average performance (about 50% of cleaved embryos developing to the blastocyst stage) could be achieved by culturing embryos in CR1 medium in the presence of BRL cells and serum; however, as noted elsewhere, in terms of fetal and subsequent development, the possible limitations to the inclusion of serum in culture medium (disease risk, adverse fetal effects) must be kept in mind. It has been generally accepted that serum and substances secreted by embryos and/or by somatic cells contain a number of unknown embryotrophins; it is well established that bovine serum contains a variety of potent bioactive substances, including growth factors (see Table 7.4).

A great many factors influence the effectiveness of an IVC medium; some workers concentrate attention on a single variable while others attempt to cover a multiplicity of factors. Fully defined media were employed in studies reported by Keskintepe and Brackett (1996), who found that SOF supplemented with NEAA and citrate was as effective as previously reported media containing serum, BSA and/or somatic cells. Several reports have dealt with the effect of growth factors. In Germany, Eckert (1994) found evidence that platelet-derived growth factor (PDGF) improved embryo development. The influence of various factors, including growth factors, was examined in IVC medium by Brackett *et al.* (1997); using an SOF with amino acids (SOFaa) formulation, they found that epidermal growth factor (EGF) and PDGF enhanced expanded blastocyst development.

Numerous reports have appeared during the past decade that may be consulted on a range of factors dealing with bovine embryo culture, including the merits of different media (Brackett and Keskintepe, 1994; Konishi and Aoyagi, 1994; Lu and Lu, 1994; Avery *et al.*, 1995a; Keskintepe *et al.*, 1995; Blume *et al.*, 1998;

Table 7.4. Growth factors related to reproduction in the cow.

Growth factor	Principal functions
Epidermal growth factor (EGF)	EGF is a polypeptide with potent mitogenic activity in various types of cells
Fibroblast growth factor (FGF)	Stimulates the growth of blood vessels as well as being mitogenic
Platelet-derived growth factor (PDGF)	Is a polypeptide with potent mitogenic activity
Platelet-activating factor (PAF)	A potent phospholipid mediator that induces a wide range of physiological responses
Insulin-like growth factors (IGFs)	Are polypeptide growth factors that mediate most of the growth-promoting actions of growth hormone
Transforming growth factor (TGF-α and TGF-β)	Is closely related to EGF; produced by theca cells
Tumour necrosis factor (TNF)	Traditionally associated with inflammation

Sagirkaya, 1998; Abe *et al.*, 1999; Gao *et al.*, 1999; Kajihara *et al.*, 1999; Sata *et al.*, 1999; Watanabe *et al.*, 1999b; Weniger *et al.*, 1999; Long *et al.*, 2000; De la Torre-Sanchez and Seidel, 2002), serum sources (Palma *et al.*, 1995b; Satoh *et al.*, 1995; Thibodeaux *et al.*, 1995b; Pereira *et al.*, 1997a,b; Tsuzuki *et al.*, 1998a; Northey *et al.*, 1999; Duque *et al.*, 2000), energy sources (Ferguson and Leese, 1999a; Gomez and Diez, 1999; Avelino *et al.*, 2000; Diez *et al.*, 2000), somatic cells (Fancsovits *et al.*, 1998a; Camargo *et al.*, 2001), amino acids (Liu and Foote, 1994; Elhassan *et al.*, 1999a; Lu, 1999; McEvoy *et al.*, 2000b), fatty acids (Farrar *et al.*, 1999), protein sources (Holm *et al.*, 1995; Choi *et al.*, 1998a,b), hyaluronic acid (Furnus *et al.*, 1998a), metal chelators (Olson and Seidel, 2000a), selenium additives (Lee *et al.*, 2001), allopurinol (Iwata *et al.*, 1999), gas phases (O'Kearney-Flynn *et al.*, 1998b; Geshi *et al.*, 1999), surfactants (Osada *et al.*, 1999), water quality (Nagao *et al.*, 1995a,b) and antioxidants (Olson and Seidel, 1994; Liu, Z. *et al.*, 1995).

Serum-restricted culture systems

During the past decade, the efforts of many researchers have focused on formulating defined or semi-defined culture systems capable of supporting embryo development and viability. Such work has allowed an examination of the requirements of bovine embryos for specific energy substrates, such as glucose, pyruvate, lactate, glutamine and amino acids, and their role in stimulating embryo development. The addition of amino acids to culture media has proved to be a major step forward; one-cell ovine embryos can be successfully cultured to the blastocyst stage by way of a chemical semi-defined medium without glucose but containing amino acids, pyruvate, lactate and BSA (Gardner *et al.*, 1994; Pinyopummintr and Bavister, 1996; Olson and Seidel, 2000a,b). Similarly, in sheep, the addition of amino acids has stimulated the development of blastocysts in a way normally only found with a complex medium supplemented with serum (Walker *et al.*, 1995).

Evidence from various studies has suggested that the bovine embryo changes its amino acid requirements during development (Partridge and Leese, 1997; Steeves and Gardner, 1999b). When added to SOF culture medium during the first 72 h, NEAA and glutamine significantly increased development to the eight- to 16-cell stage and subsequent blastocyst development. Culture beyond 72 hpi with 20 amino acids (non-essential + essential) and glutamine increased blastocyst development, total cell number and the number of cells in the ICM and the TE. The indications are that a two-step culture system is required to meet the needs of the embryo. A further study by Lu and Seidel (2002) demonstrated that, after bovine oocytyes are fertilized in Fert chemically defined medium (Fert-CDM) containing NEAA, an optimal blastocyst yield may be achieved when presumptive zygotes are cultured in CDM-1 containing NEAA without essential amino acids (EAA) for 72 h, and then cultured in CDM-2 containing both NEAA and EAA in combination with insulin (0.12 iu/ml) from day 5 of culture.

Serum-supplemented complex media (e.g. TCM-199) conditioned by somatic cells (oviductal, BRL, Vero) are known to support early bovine

embryo development; so also are much simpler formulations, such as Fert-CDM and SOF. However, the use of serum-restricted media for the culture of bovine zygotes to the blastocyst stage is usually regarded as essential in providing a firm basis for *in vitro* embryo production. Many components of serum may be eliminated by using BSA as the protein source; the macromolecular requirement for cattle embryo culture is often satisfied by BSA, although it is well recognized that this is a relatively impure compound. Using B2 medium, which contains BSA, as the serum-free medium with bovine oviductal cells, Semple *et al.* (1995) found that serum is not essential for embryo production at any time after fertilization; they suggested that the elimination of serum lipids from the IVC medium might permit IVP embryos to better tolerate freeze–thaw procedures.

Using a serum-restricted culture system, Farin *et al.* (1997a,b) reported the development of fetuses and placentas at 63 days after embryo transfer; no difference was evident between embryos produced *in vivo* or *in vitro* under serum restriction. Using B2 medium in co-culture with BRL cells, Damiani *et al.* (1997) found that blastocyst yield was comparable to that with 10% FCS; such serum restriction during early development (first 4 days) was detrimental neither to embryonic development nor to pregnancy rate after embryo transfer.

In terms of viability of cattle embryos cultured in serum- and protein-free media, Lee *et al.* (1997) in Japan cultured zygotes in SOFaa containing either BSA or PVA; although there was a significantly greater yield of blastocysts with SOFaa + BSA (22% vs. 11%), pregnancy rates after cryopreservation were similar (50% vs. 53%). Studies in Japan by Sata *et al.* (1999) examined the fatty acid composition of cattle embryos cultured in serum-free medium; such embryos had different morphologies and fatty acid compositions compared with those coming from a serum-supplemented medium. A report by Krisher *et al.* (1999) noted that cattle embryos cultured in the presence of serum appeared dark and granular and had higher glycolytic rates than embryos developed in chemically defined medium.

Use of commercial media

The media used in cattle embryo production are usually prepared in the laboratory, which may be labour-intensive and costly, in terms of both equipment and personnel. In research, where comparisons are often made between data from different laboratories, confounding problems may arise due to variations in chemicals, water quality and other factors related to a particular laboratory. There are those who suggest that liquid media purchased from commercial vendors may be a solution to such problems. A report by Monson *et al.* (1999) used a vendor-supplied medium (KSOM, Speciality Media, Lavellette, New Jersey) and found it as effective as their in-laboratory-produced medium. The authors drew attention to the convenience of using such media in the management of an embryo production laboratory and in reproducing the culture system in other laboratories. It is worth mentioning that the bovine embryo is extemely sensitive to water quality in the preparation of medium and that early embryos may be seriously affected by the purification method and the storage period of that water (Nagao *et al.*, 1995a).

Towards defined culture systems

At the start of the 1990s, co-culture of cattle embryos with oviductal or other types of cells was the usual method employed to support embryo development; however, as observed by Thompson (2000), use of this culture system probably retarded rather than advanced progress towards efficient embryo production systems. During the 1990s, increased understanding of energy-substrate requirements and a greater appreciation of the role of the reproductive tract environment led to the development of more ‘defined’ systems and eventually to the ‘sequential’ media systems that are currently utilized in some human and cattle IVF laboratories.

Sequential media

In the light of the changing physiology and metabolism of the early developing bovine embryo, increasing attention has been directed towards the use of sequential media for culture, each medium reflecting the changing requirements during development. According to Gardner (1998, 1999a,b), although there are considerable species differences, certain aspects of nutrient utilization appear to be common to

all, especially the low glucose utilization and the beneficial effects of NEAA and glutamine prior to compaction. The author notes that during the past three decades, the role of amino acids in embryo development in culture has tended to be overlooked; it is now clear that those amino acids initially classified by Eagle in 1959 as non-essential are those found in the highest concentrations in the mammalian oviduct and have been shown to stimulate embryo development before compaction. Amino acids appear to be fundamental regulators of cell function, including energy metabolism, in the early life of the embryo; metabolism may be used to monitor the degree of stress imposed on the embryo in culture and to determine its viability before transfer (Gardner, 1998).

Microfluidic embryo manipulation

Currently appearing on the horizon are embryo culture systems based on microfluidic technology, offering the advantage that medium can be readily changed to meet the precise requirements of the developing embryo (Glasgow *et al.*, 2001; Beebe, D. *et al.*, 2002; Hester *et al.*, 2002); gradually changing the composition of the medium would avoid inducing stresses arising from the abrupt environmental changes that are usually a part of conventional culture systems. Some of the results from studies with mouse embryos are illustrated in Fig. 7.8. For large-scale cattle embryo production, it may eventually be possible to think in terms of maturation, fertilization and culture to the blastocyst stage being under computer control. Such a possibility may be facilitated when sperm injection rather than IVF can be used to effect fertilization. As noted in an earlier section (see Section 6.9.4), it may now be feasible to think in terms of achieving fertilization by intracytoplasmic sperm injection (ICSI), using very low sperm numbers and with gametes carrying the requisite sex chromosome.

7.5.2. TCM-199 and SOF culture media

Two contrasting embryo culture systems have been widely used in studies during the past decade: on the one hand, TCM-199, an example of a complex and undefined medium, and, on the other, SOF, an example of a simple and relatively well-defined medium. Many studies designed to optimize the use of the SOF medium for cattle embryo culture have been reported during the recent decade (Rorie *et al.*, 1994a,b; Carolan *et al.*, 1995a,b, 1996b). The complex TCM-199 medium was designed specifically for sustaining somatic cells *in vitro* rather than for culturing the early mammalian embryo. There are those who believe that cattle embryos generated by the SOF system show greater similarities to their *in vivo* counterparts, on the basis of gene expression patterns (Wrenzychi *et al.*, 2001b; Yaseen *et al.*, 2001), than those generated by the TCM-199 system. There is also the fact that the reduced oxygen tension in the SOF system, which may reduce the adverse effects of reactive oxygen species, is more in line with the oviductal microenvironment.

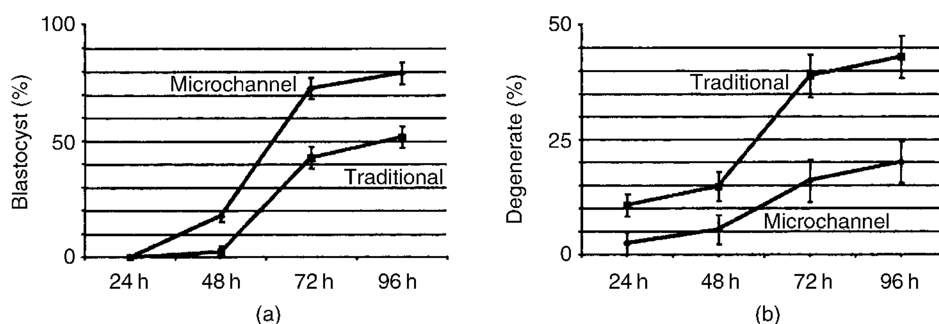


Fig. 7.8. Mouse blastocysts produced in traditional and microchannel culture systems. Developmental efficiencies in microchannels. Microchannels significantly (a) improve blastocyst rate and (b) decrease the number of degenerate embryos. (From Beebe, D. *et al.*, 2002.)

It was Tervit and associates in the early 1970s in Cambridge who pioneered the SOF formulation; the medium was based on a biochemical analysis of sheep oviductal fluid. The Cambridge workers reported the successful culture of one- to eight-cell cattle embryos to the morula/blastocyst stage in a medium they termed ‘synthetic oviductal fluid’, using a 5% rather than a 20% oxygen-in-air gas atmosphere. In comparison with the TCM-199 and granulosa cell co-culture system, the SOF system was found by Holm *et al.* (1997, 1999c) to increase the yield of blastocysts; these workers replaced their TCM-199 + granulosa cells culture system with SOF supplemented with amino acids, citrate and myo-inositol (see Table 7.5). In the Netherlands, Merton and Mullaart (1999) showed that the yield of transferable cattle embryos was higher in a semi-defined culture system (SOFaa/BSA) than in a more complex system (TCM-199/FCS/BRL co-culture).

In Ireland, Lonergan *et al.* (1999b) demonstrated that better results were obtained with SOF when used with a gas atmosphere of 5% oxygen rather than 20%; they also showed that both BSA and FCS improved embryo development when added to SOF. In the same laboratory, Rizos *et al.* (2000) tested the possibility that culture in SOF (to maximize blastocyst yield) in combination with granulosa cell co-culture (to optimize blastocyst quality) would provide a high yield of good-quality embryos; they found that the presence of granulosa cells in SOF overcame the detrimental effect of culture in a high-oxygen atmosphere in terms of blastocyst yield and quality. In further studies, Rizos *et al.* (2001a,b,c,d) showed that the addition of FCS to SOF or an increased concentration of BSA (16 mg vs. 3 mg/ml) significantly increased the rate of bovine embryo development. Blastocyst quality, however, as measured by ability to withstand cryopreservation, was significantly decreased by FCS or the increased BSA level. In Spain, Diaz *et al.* (2000) found blastocyst yield to be significantly higher in mSOF than in B2 medium + Vero cells.

It may not always be possible, for practical reasons, to use low oxygen levels in embryo culture. In Brazil, Dode *et al.* (2002a) examined various cattle embryo production systems in SOF medium under high atmospheric oxygen tension; they reported that the transfer of zygotes

Table 7.5. Composition of synthetic oviductal fluid (original formulation and a modified SOF formulation) (from Holm *et al.*, 1999c).

Original		Modified[a]		
Constituent	Concentration	Component	Product, Company	Concentration
NaCl	107.70 mM	NaCl	S 5886, Sigma	107.63 mM
KCl	7.16 mM	KCl	P 5405, Sigma	7.16 mM
KH_2PO_4	1.71 mM	KH_2PO_4	P 5655, Sigma	1.19 mM
$MgCl_2$	0.49 mM	$MgSO_4$	M 2643, Sigma	1.51 mM
$CaCl_2$	1.71 mM	$CaCl_2$-$2H_2O$	C 7902, Sigma	1.78 mM
$NaHCO_3$	25.07 mM	Sodium lactate	L 4263, Sigma	5.35 mM
Na lactate	3.30 mM	$NaHCO_3$	S 4019, Sigma	25.00 mM
Na pyruvate	0.33 mM	Na – pyruvate	P 3662, Sigma	7.27 mM
Glucose	1.50 mM	L-Glutamine[b]	G 6392, Sigma	0.20 mM
BSA	32 mg/ml	BME amino acids[b]	B 6766, Sigma	45.0 μl/ml
Penicillin	100 iu/ml	MEM amino acids[b]	M 7145, Sigma	5.0 μl/ml
Streptomycin	50 μg/ml	Trisodium citrate[c]	1,06448, Merck	0.34 mM
pH: 7.2–7.4		Myoinositol[d]	I 7508, Sigma	2.77 mM
270 osmol		Gentamycin	G 1264, Sigma	50.0 μg/ml
		Phenol red	P 5530, Sigma	10.0 μg/ml
		H_2O	W 1503, Sigma	

[a]The osmolarity of SOFaaci with or without supplements is adjusted to 280 mosmol.
[b]SOF including amino acids (but excluding citrate and myoinositol) is designated SOFaa.
[c]SOFaa including citrate is designated SOFaac.
[d]SOFaac including myoinositol is designated SOFaaci.

to SOF immediately after IVF accelerated embryo development, with a high percentage of hatched blastocysts on day 7. The authors suggest that, under a gas atmosphere of 20% oxygen, zygotes can be transferred immediately after IVF to SOF with no need for denudation or exposure to cumulus cells during culture. In Denmark, Holm *et al.* (2002) cultured *in vivo*-produced and IVP embryos in SOF with amino acids, citrate and myoinositol (SOFaaci), with or without serum supplementation; the presence of serum decreased the duration of the fourth cell cycle and triggered premature blastulation. It is believed that impaired compaction and premature blastulation were the result of decreasing the time between the early morula, compact morula and early blastocyst stages without an appropriate increase in cell numbers.

7.5.3. Co-culture with bovine oviductal cells

The introduction of co-culture of cattle and sheep embryos with somatic cells a quarter-century ago was regarded at the time as being an important milestone in overcoming the arrest of development at the eight to 16-cell stage. Cambridge workers did much to establish the ruminant oviductal co-culture system as equal or superior to alternative culture procedures available at that time (Gandolfi and Moor, 1987). Unfortunately, the co-culture system was eventually to be shown to be markedly inferior to the *in vivo* (rabbit or sheep oviduct) systems that it replaced, particularly in terms of producing embryos that could be successfully cryopreserved. None the less, the fact that early embryonic development takes place in the oviduct means that a more appropriate culture system, modelled on greater understanding of the oviductal environment, could be valuable in improving bovine embryo production systems. In Germany, Rief *et al.* (2002b) described a system based on the culture of BOEC in SOF plus 5% oestrous cow serum (OCS), which they suggest may be useful in establishing an *in vitro* model to study early embryonic development; the system was sensitive to biological indicators of normal embryo development, such as energy metabolism and expression of selected genes of developmental capacity.

Bovine oviductal cell monolayer

The bovine oviduct provides the microenvironment for the transfer and final maturation of gametes, for fertilization and for the early development of the embryo; in setting up the BOEC monolayer, the hope was to provide the same microenvironment in the laboratory (see Fig. 7.9). Numerous papers were published on the normality or otherwise of the BOEC monolayer. There were those who noted that oviductal cells grown in a monolayer rapidly undergo dedifferentiation; they flatten on the culture surface, losing the characteristic columnar shape and cilia. In culture, the oviductal cells begin to divide regularly, in contrast to their activity *in vivo*, where mitotic figures are rare; such changes may result in the loss of steroidogenic activity, possibly through inactivation or loss of receptors. Clearly, such changes provide an environment that may differ in several respects from that in the oviduct itself. In North America, Xia *et al.* (1995) noted that oviductal-monolayer cultures could support early bovine embryo development, but, if high rates of development to the blastocyst stage were to be maintained, embryos must be moved every 2–3 days to fresh 48 h monolayer cultures. On the other hand, non-attached bovine epithelial cells were capable of supporting development to the blastocyst stage without the need for such movements. The workers examined the two culture systems for the presence of an oestrus-associated glycoprotein, the outcome of such studies being much more positive for non-attached epithelial cells.

None the less, there were reports showing that the BOEC monolayer culture system can be highly effective in promoting the development of cattle embryos. In Ireland, Sreenan and colleagues examined the development of cattle embryos from the one-cell to the blastocyst stage according to whether they had been produced *in vitro* or *in vivo* (recovered from the oviducts). As shown in Table 7.6, the co-culture was successful with the *in vivo* embryos but less so with those produced by IVM/IVF. The indications at that time were that IVM and IVF oocytes were of inferior quality; if cattle zygotes and embryos were of the quality normally associated with the live cow, they were capable of developing to the blastocyst stage in this particular culture system. Whether that quality included ability to

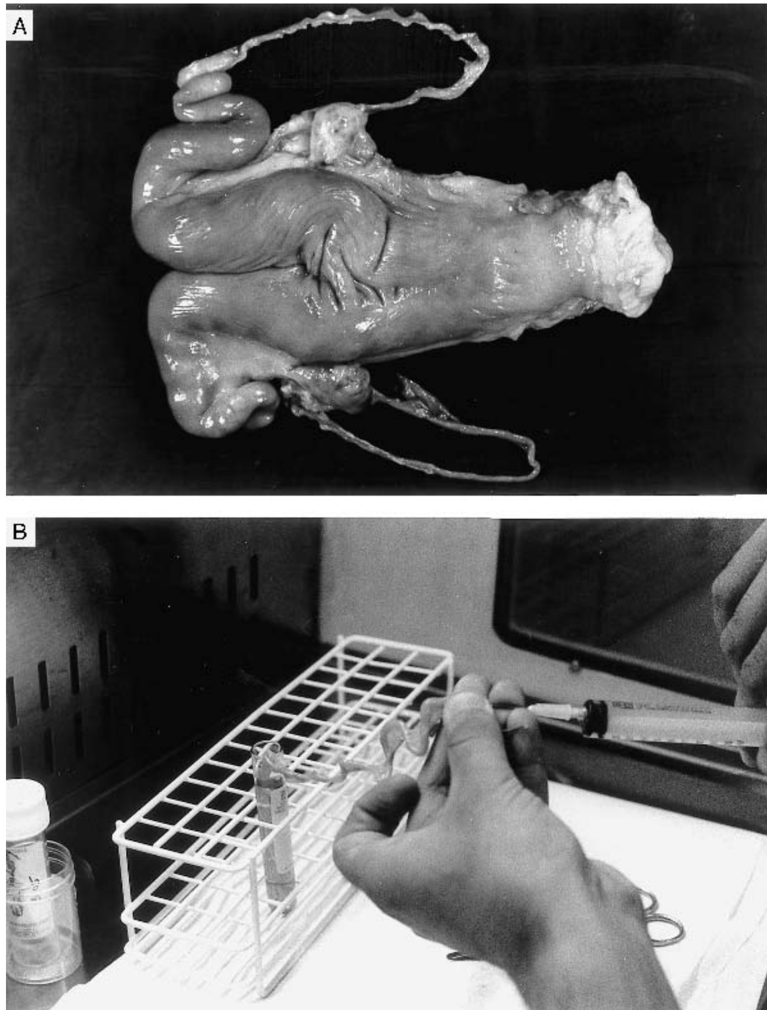


Fig. 7.9. Recovering bovine oviductal epithelial cells. (A) Cattle tracts recovered at the abattoir have been used as a source of oviductal cells for use in co-culture studies or other research purposes. (B) Flushing the oviduct to recover epithelial cells.

withstand freeze–thawing or other forms of cryopreservation was not clear. More recent work in Ireland strongly suggests that, regardless of the origin of the zygote (*in vivo*-produced or IVP), the culture system employed in bringing the zygote to the blastocyst stage (*in vitro* or *in vivo*) is the major determinant of blastocyst quality (Lonergan *et al.*, 2001a,b,c).

Among the reports appearing in the recent decade is one dealing with the use of porcine oviductal cells in cattle embryo development (Pavasuthipaisit *et al.*, 1994b); oviductal cells apparently produce a soluble component that enhances embryo development but is not species-specific. Results presented by Satoh *et al.* (1994) suggested that the tissue inhibitor of metalloproteinase-1 produced by bovine oviductal cells possesses embryogenesis-stimulating activity. At Beltsville, Hawk and Wall (1994a,b) reported experiments which indicated that the best combination for supporting cattle embryos *in vitro* was B2 medium and BOEC for co-culture. A report from China by Feng *et al.* (1994) recorded the establishment of an efficient and reliable culture system, using BOEC. In Texas, Applewhite and Westhusin (1995) showed that

Table 7.6. Development of *in vitro* or *in vivo*-produced zygotes in a bovine oviductal cell co-culture system (from McCaffrey *et al.*, 1991).

	In vivo	Day 1 IVF	Day 2 IVF
No. of ova	41	77	52
Initial ovum cell no. (mean ± SEM)	2.47 ± 0.52	1.04 ± 0.03	4.9 ± 0.35
No. of morulae/blastocysts (%)	32 (78)[a]	28 (36)[b]	13 (25)[b]
No. of grade 1 and 2 mor./blast. (%)	20 (62)[a]	9 (32)[a]	3 (23)[b]

Values within rows with different superscripts are different, $P < 0.01$.
SEM, standard error of the mean.

more embryos developed to the morula/blastocyst stages when oviductal cells were added to culture drops. In Utah, Wang *et al.* (1995) concluded that oviductal epithelial cells had to be combined with FCS to take full advantage of their co-culture system. In Poland, Katska *et al.* (1995) showed that the source of oviductal cells and their previous freezing had no effect on their effectiveness in bovine embryo development. A paper by Edwards, L.J. *et al.* (1997) dealt with some of the changes produced in the embryo culture medium by bovine oviductal cells. In Spain, Diez *et al.* (1999) showed that different BOEC batches exhibited differing levels of embryotrophic activity, which was reflected in similar patterns of embryo development; some batches were thought to lack factors needed to reach the later morula/blastocyst stages.

Many of the studies dealing with BOEC co-culture today are research-orientated rather than for embryo production purposes. In Japan, for example, Kamishita *et al.* (1999) studied the effect of BOEC collected from oviducts ipsilateral to cystic follicles on the development of IVP cattle embryos; they showed that blastocyst yield from oestradiol-dominant oviductal cells was significantly higher than that from progesterone-dominated cells (61.6% vs. 39.5%). In the USA, Mishra *et al.* (1999) noted that BOEC contain luteinizing hormone (LH) receptors that are functional in increasing the synthesis of oviductal glycoprotein, which is known to increase the development of early embryos into blastocysts; they monitored the progress of cattle embryos after treating a bovine epithelial cell monolayer with an LH preparation. They recorded a significant increase in blastocyst yield after such treatment. The workers concluded that peri-ovulatory LH levels may promote development of early embryos in the oviduct by their actions on epithelial cells.

In York, Orsi *et al.* (2000) attempted to develop a more 'physiological' co-culture system by using a novel system that sought to provide for the needs of both the embryo and the supporting BOEC. In Germany, Rief *et al.* (2000) co-cultured cattle embryos on BOEC in a study of embryo–maternal interactions; they found the ATP levels of blastocysts from the co-culture group to be significantly lower than those obtained from the cell-free SOF + 5% OCS + 2% βME + 1% MEM. As noted in an earlier context (see Section 7.4.1 above), evidence supports the view that the process of ATP production is a key regulator of *in vitro* embryo development.

One study of a more practical nature was that of Tavares *et al.* (2000) in Brazil, who co-cultured zygotes in TCM-199 with bovine oviductal or granulosa cells, BRL or Vero cells; they found no significant difference in blastocyst yield among the culture systems (Table 7.7) but noted the need for efforts to improve such results.

7.5.4. Co-culture with non-oviductal cells

A wide range of different cell types and cell-conditioned media have been used with success

Table 7.7. Development of IVF bovine embryos co-cultured with different cell types (from Tavares *et al.*, 2000).

Cell type	Cultivated	Cleavage (%)	Blastocyst (%)
Oviductal	1068	829 (77.6)	174 (16.3)
Granulosa	1632	1071 (65.6)	213 (13.0)
VERO	392	277 (70.7)	28 (7.1)
BRL	406	288 (70.9)	29 (7.1)
Total	3498	2465 (70.5)	444 (12.7)

in the co-culture of cattle embryos, including trophoblastic vesicles (Stojkovic *et al*., 1995a,b), cumulus cells (Wang *et al*., 1995a), BRL cells (Carnegie *et al*., 1994, 1997; Funston *et al*., 1994, 1997; Hasler *et al*., 1994; Myers *et al*., 1994; Rehman *et al*., 1994b,c; Vansteenbrugge *et al*., 1994a,b; Farin, C.E. *et al*., 1995; Farin, P.W. *et al*., 1995; Van Inzen *et al*., 1995; Damiani *et al*., 1997; Krisher *et al*., 1998a,b), Vero cells (Grocholova *et al*., 1995; Pegoraro *et al*., 1996, 1998; Menck *et al*., 1997; Carnegie *et al*., 1999) and endometrial epithelial cells (Goff and Smith, 1998). A paper by Joo *et al*. (1998) dealt with several cell lines (BOEC, granulosa cells, BRL cells and Vero cells); under their culture conditions, BRL cells proved to be significantly more effective than the other cell lines.

It is believed that variable results achieved with BOEC may be due to heterogeneity in cell preparations from cow oviducts. In France, Menck *et al*. (1994) showed that Vero cells were as efficient as bovine oviductal cells in supporting embryos up to the blastocyst stage. Vero cells are a well-defined and established line of green monkey kidney epithelial cells used in the culture of human embryos; they also have the advantage of carrying less disease risk than bovine cells. In France, Fancsovits *et al*. (1998a,b) showed that production of cattle embryos in a Vero cell co-culture system that included B2 as the basic medium resulted in a greater proportion of oocytes that developed to the blastocyst stage after 7 days of culture than in a system utilizing TCM-199 as the basic medium. The higher cell numbers of the blastocysts produced in B2 (see Table 7.8) indicated that this medium was more efficient and resulted in a better-quality blastocyst. In the USA, Krisher *et al*. (1998a,b) reported on the effectiveness of B2 medium with BRL cells for developing cattle embryos; their modified B2 medium improved embryo development in comparison with modified TCM-199.

Cumulus and granulosa cells have been widely used in co-culture systems. A study by Goto *et al*. (1994) in Japan showed significantly better embryo development when one-cell cattle embryos were co-cultured with a granulosa cell monolayer than in cell-free culture. The authors were uncertain as to the mechanisms responsible for the improved blastocyst quality. Further studies in that country by Geshi *et al*. (1999) examined the effect of lowering carbon dioxide tension in their culture of cattle embryos with cumulus cells in TCM-199. It is known that co-culture with cumulus cells can result in increased levels of carbon dioxide in the medium, leading to a reduction in the pH of the medium which may have adverse effects on embryo development. The Japanese workers showed that reducing the carbon dioxide concentration from 5 to 2% and adding beta-mercaptoethanol (β-ME) to the IVC medium provided a more favourable environment and significantly increased blastocyst yield.

A study by Watanabe *et al*. (1999a,b) evaluated the ability of two culture media (B2 and CR2) and co-culture with two cell lines (oviduct and cumulus) to support cattle embryo development to the blastocyst stage; they recorded a significant and much higher blastocyst yield using cumulus cells in B2 medium (61% vs. 29%). For practical purposes, the authors noted the advantage of using cumulus cells, which neither needed the preparation nor carried the disease risks associated with oviductal cells.

In the USA, in a commercial setting, Long *et al*. (2000) compared three culture media (CR1aa; BARC-1; modified G1.2/G2.2) containing 10% FCS and BRL cells; no difference was evident among the culture systems in blastocyst yield and each resulted in acceptable pregnancy

Table 7.8. Effect of B2 and TCM-199 media on IVP bovine embryo development (from Fancsovits *et al*., 1998b).

Treatment	*n*	Cleavage (%)	8–16-cell (%)[a] – day 4	Blastocyst (%)[a] – day 7	Cell number ± SD – day 7 blastocysts
INRA-B2	176	91	84	35[b]	119[d] ± 39
TCM-199	274	90	78	27[c]	90[e] ± 32

[a]Percentage of cleaved embryos.
[bcde]Means with different superscripts differ significantly ($P < 0.05$).
SD, standard deviation.

rates at 60 days. In Norway, Bertheussen *et al.* (2000) compared serum-free M3 medium (used in human IVF clinics) with a standard bovine IVC medium (TCM-199 + 10% FCS); the two media were supplemented with granulosa cells. It was found that development beyond the morula stage was seriously impaired in the M3 medium. In a study reported by Hasler (2000a,b), embryos produced in B2–BRL co-culture with serum restriction (no serum for the first 72 h of IVC) resulted in pregnancy rates and calving sequelae similar to those found with embryos cultured without serum restriction. A study by Park, J.S. *et al.* (2000) in Korea showed improved cattle embryo development when mouse embryonic fibroblasts were added to their CR1aa medium during the last 5 days of a 7-day culture period. In Ireland, Enright *et al.* (2001b) demonstrated that the addition of granulosa cells to their TCM-199 culture medium improved embryo survival after cryopreservation; they also showed that the time period when embryos were exposed to granulosa cells was important in the acquisition of improved tolerance to cryopreservation.

A study by Duszewska *et al.* (2000a,b) in Poland tested a new 'mixed co-culture' system, using a monolayer composed of both Vero and BRL cells in B2 + 10%FCS medium; results showed that the mixed-cell monolayer yielded a significantly higher percentage of transferable quality embryos (67%) than on BRL cells (55%) or Vero cells (27%).

7.5.5. Serum-supplemented culture systems

FCS has been shown to inhibit the first cleavage divisions and to accelerate subsequent development of cultured cattle embryos. None the less, the serum continues to be widely used as a supplement in various culture media, despite its undefined and highly variable composition. In France, Tricoire *et al.* (1999) investigated the effect of FCS on embryo development, including survival after transfer, when added to SOFaa during the culture of cattle embryos; they showed that the presence of serum depressed cell proliferation in embryos and increased the expansion of the blastocoelic cavity, resulting in embryos at a more advanced stage of development with lower cell numbers. Such embryos were at least as viable as those produced in the absence of FCS; there was no evidence of problems during pregnancy or with the calves at birth.

Evidence that blastocyst formation in the bovine embryo is influenced by FCS was provided in the study of Northey *et al.* (1999) in Wisconsin; in the absence of FCS, embryos were slower to develop blastocoel cavities and there was a lower blastocyst yield. The same workers found that the very tight compaction observed with cattle embryos in the absence of FCS was obtained along with enhanced and earlier blastocyst formation if the addition of FCS to the culture medium (CR1aa) was delayed until day 6. In Ireland, work has demonstated a significant effect of the IVC system on the kinetics of embryo development and the sex ratio (Lonergan *et al.*, 2000a; Gutierrez-Adan *et al.*, 2001a,b); the highest blastocyst yield was achieved with SOF in the presence of FCS, with the male/female sex ratio significantly skewed in favour of males. Other work in Ireland examined the implications of culturing cattle zygotes *in vitro* in SOF or culturing them *in vivo* in the ligated sheep oviduct (Lonergan *et al.*, 2001a,b,c; Rizos *et al.*, 2001a,b,c,d); it was concluded that, while oocyte quality was a major factor influencing blastocyst yield, blastocyst quality was mainly influenced by the environment in which embryo culture took place.

In Brazil, Tavares *et al.* (2002) evaluated the ability of three different culture systems (CR2aa; KSOM with amino acids (KSOMaa); SOFaa), each supplemented with 5% FCS to support *in vitro* development of cattle blastocysts. All three media supported embryo development (see Table 7.9); CR2aa yielded lower numbers of blastocysts, whereas KSOMaa yielded the highest proportion of hatching blastocysts.

In Brazil, Assumpcao *et al.* (2002) examined the development of IVM and IVF cattle embryos cultured in SOFaa supplemented with either 5% or 7.5% FCS; while similar blastocyst yields were obtained with both serum concentrations, there was a significant increase in hatching rate with the 7.5% concentration (see Table 7.10).

In Connecticut, Nedambale *et al.* (2002) compared two culture systems (KSOM, SOFaaci) and found that changing from KSOM + 0.1% BSA to SOFaaci with 5% FCS after 4 days significantly improved early blastocyst formation and

hatching rate. Embryos cultured in KSOM with BSA appeared to develop slowly and most hatched late at day 9 (see Table 7.11).

In Wisconsin, Westberg *et al.* (2002) compared IVC medium containing 10% FCS, which had been found to give optimal blastocyst formation in their culture system, with BSA at different concentrations (3–10 mg/ml); they recorded a significant difference in the dynamics of blastocyst formation between the two protein sources. The dynamics in the BSA groups corresponded more closely to events in the live cow, with a lower proportion of blastocysts emerging with BSA on day 7 than with FCS. It was also found that there was a higher hatching rate (considered to be a measure of embryo quality) after BSA and that this hatching rate was influenced positively by an increasing concentration of albumin.

Table 7.9. Culture of IVP cattle embryos in different media +5% FCS (from Tavares *et al.*, 2002).

	Number of oocytes	Cleavage (%)	Blastocyst (%)	Hatched (%)
CR2aa	387	322 (83.2)[a]	128 (33.1)[a]	123 (31.8)[ab]
KSOMaa	285	195 (68.4)[b]	123 (43.2)[b]	106 (37.2)[b]
SOFaa	246	199 (80.9)[a]	113 (45.9)[b]	60 (24.4)[a]

[a,b]Values within a row differ from control ($P < 0.05$, chi-square test).
aa, with amino acids.

Table 7.10. Effect of serum concentration in SOFaa medium on IVP embryo development (from Assumpcao *et al.*, 2002).

	Number of oocytes	Cleavage *n* (%)	Blastocyst *n* (%)	Hatched *n* (%)
SOFaa 5% FCS	246	199 (80.9)	113 (45.9)	60 (24.4)[a]
SOFaa 7.5% FCS	223	179 (80.3)	111 (49.8)	101 (45.3)[b]

[a,b]Values within a row differ from control ($P < 0.05$, chi-square test).

Duration of serum treatment

Although the IVC of cattle and sheep embryos in the presence of serum may result in abnormal growth and development *in utero*, the effect of the time during which embryos are exposed to serum has not been clear. In Aberdeen, Sinclair *et al.* (2002) cultured sheep zygotes in SOF supplemented with 10% (v/v) steer serum for varying times; they found that continuous exposure to serum over a 5-day period or exposure during the early period of culture (days 0–3) increased the risk of abnormal fetal development.

Serum substitutes

Although there is evidence that the culture of bovine embryos *in vitro* may result in fetal overgrowth (large-offspring syndrome (LOS)), it is also recognized that serum supplementation is capable of generating a higher blastocyst yield than serum-free media. In Florida, Rodriguez-Sallaberry *et al.* (2002) tested a serum replacer (Knockout SR, Gibco) and on the basis of blastocyst yield showed that it could effectively replace fetal bovine serum in their KSOM culture medium. It remains to be seen what effect such replacement may have on the incidence of LOS.

Table 7.11. Effect of changing KSOM + BSA on day 4 to SOFaaci + 5% FCS (from Nedambale *et al.*, 2002).

Culture treatment	Number of oocytes (*n*)	Day 5 morulae (%)	Day 6 early blastocysts (%)	Day 8 blastocysts (%)	Day 8 hatched blastocysts (%)
1 (SOFaaci + FCS)	242	43 ± 4.05[a]	25 ± 2.02[a]	47 ± 3.44[a]	63 ± 5.38[ab]
2 (S + FCS to K + FCS)	242	43 ± 4.05[a]	21 ± 2.02[a]	42 ± 3.44[a]	54 ± 5.38[a]
3 (KSOM + BSA)	242	36 ± 4.05[a]	9 ± 2.02[b]	40 ± 3.44[a]	67 ± 5.38[ab]
4 (K + BSA to S + FCS)	242	46 ± 4.05[a]	24 ± 2.02[a]	44 ± 3.44[a]	83 ± 5.38[b]

[a,b]Values with different superscripts within columns are significantly different ($P < 0.01$).
5, SOFaaci; K, KSOM.

Supplementation with bovine follicular fluid

The literature contains information from workers who have employed follicular fluid rather than serum as the protein source in their IVC media. In Uruguay, Larocca *et al.* (1998) evaluated the effect of follicular fluid (10% added to CR1aa medium) taken either from large (> 15 mm diameter) or medium (5–7 mm) follicles; they found no difference between the two follicle sizes in regard to cleavage rate or early embryonic development.

7.5.6. Serum-free culture systems

The use of chemically defined, protein-free media is essential in providing a firm basis for achieving improvements in IVP culture methods (Maurer, 1992; Staines *et al.*, 1999). Although bovine serum may provide beneficial factors for the IVC medium, whether they be energy substrates, amino acids, vitamins, antioxidants or growth factors, it may also prove to be toxic. There are those who note that serum is not a naturally occurring biological product but a pathological fluid formed by blood clotting, a process that may lead to chemical changes that have detrimental effects on embryo culture. There is also the question of variations in the quality of serum preparations, which may result in successes achieved in one laboratory being unobtainable in another.

Bovine serum has a well-known biphasic effect on cattle embryo development (Bavister, 1995); while inhibiting the first cleavage division, even in embryos first exposed to serum near the end of their first cell cycle (Bredbacka and Bredbacka, 1995), it stimulates blastocyst development and/or hatching from the zona pellucida. There appears to be no beneficial effect of serum from the two-cell to the morula stage, but serum does enhance the development of morulae into blastocysts. Many undesirable low-molecular-weight components of serum may be eliminated by substituting BSA for bovine serum in the IVC medium and many reports on using this protein source have appeared in the recent decade. In Sweden, Shamsuddin *et al.* (1994) presented results suggesting that bovine zygotes could develop in a serum-free, somatic-cell-free culture system to the blastocyst stage; they replaced serum and somatic-cells by BSA and insulin, transferrin-sodium selenite (ITS). It should be noted, however, that embryo quality was much below that found with *in vivo*-produced embryos.

The ability of the bovine embryo cultured in the presence of serum to survive and hatch after standard cryopreservation treatment is held to be a reflection of its developmental quality; Semple *et al.* (1995) investigated whether cattle embryos cultured under serum-free conditions would give similar results. Serum was not found to be essential in their B2, BSA-containing medium; it was speculated that the elimination of serum lipids might influence the stage-dependent sensitivity to cryopreservation of IVP cattle embryos. In the USA, Damiani *et al.* (1997) sought to determine whether bovine embryos could be cultured to the blastocyst stage in a serum-free medium (B2 + BRL cells); they showed that the removal of serum during early development was not detrimental to embryonic development and had no subsequent effect on pregnancy rates after embryo transfer. In Scotland, Kuran *et al.* (1999a,b,c) found that blastocyst yield was poor in a protein-free culture system (SOF + PVA), although embryos appeared to be metabolically normal.

A study by Lim *et al.* (1994b) in Japan indicated that NaCl, Cl and osmolarity affected the development of cattle zygotes to the blastocyst stage in a simple, chemically defined medium containing pyruvate, lactate, amino acids and PVA. The authors concluded that NaCl concentration in a defined medium is one of the most important factors for the development of cattle embryos to the blastocyst stage, but the development of embryos to the morula stage is also regulated by osmolarity and/or Cl concentration. In Korea, Roh *et al.* (1999b) found that in a completely defined medium embryo development was greatly affected by high NaCl concentration whereas in medium containing BSA there was no such effect. The authors concluded that reduced NaCl concentration in completely defined medium is beneficial for the development of the bovine embryo; more needs to be known about the interaction between BSA and sodium or chloride ions.

In Japan, Abe *et al.* (1999), by histochemical and ultrastructural evaluations of lipid droplets, showed that cattle embryos cultured in serum-containing medium accumulated large amounts of lipid material in their cytoplasm; they

suggested that serum-free culture might produce more suitable embryos for cryopreservation and embryo transfer (see Fig. 7.10).

Further studies in the same laboratory showed that bovine embryos cultured in serum-containing medium abnormally accumulated cytoplasmic lipids into their cytoplasm (Abe *et al.*, 2002b); the authors suggested that the excessive accumulation of cytoplasmic lipid droplets may affect the cryotolerance of the embryos. The same authors noted that the lipid droplets in serum-supplemented media were much larger (2–6 μm vs. < 2 μm) and more numerous than in serum-free media; there was also evidence that the droplets contained a significant proportion of unsaturated lipids and that mitochondria in embryos were of an immature form (spherical or ovoid rather than elongated).

In Italy, Lazzari *et al.* (2000a,b) found evidence of a significant reduction in the compaction rate of cattle embryos developed in SOF–serum as compared with SOF–BSA; the yield of high-quality (freezable) blastocysts was also significantly lower in the SOF–serum medium. In New Zealand, Schurmann *et al.* (2002) reported that the use of fatty acid-free BSA (Immunochemical Products, New Zealand) resulted in more and better-quality embryos (31% vs. 6%) than with Sigma-BSA; it was speculated that this might be due to an altered ability to bind growth factors and ions or to remove toxic materials produced during embryo culture. Earlier reports had drawn attention to the fact that BSA variation was an important factor influencing the development of cattle embryos (Rorie *et al.*, 1994a,b).

Attempts to reduce the concentration of BSA in culture media by using other supplements have been made by various workers. In Brazil, Gilardi *et al.* (2002) used ITS and vitamins in CR2aa medium containing 1 mg/ml of BSA; although they found that the low concentration of BSA improved cleavage rate, further development was arrested or impaired, even with the ITS and vitamin supplements. They concluded that further studies are needed to adjust the nutrients of a semi-defined medium with reduced concentrations of BSA.

7.5.7. Defined culture systems

Although various workers have shown that bovine embryo development can be sustained in a chemically defined, protein-free medium, the outcome is generally unacceptable in terms of embryo yield and quality. In Japan, Lim *et al.* (1994a,b), for example, showed that, when PVA in their defined medium was replaced by 10% FCS, about 50% of inseminated oocytes developed to the blastocyst stage without co-culture with somatic cells. Defined culture systems for the IVP of cattle embryos have been designed to improve understanding of developmental biology and to decrease disease risks. A report by Rosenkrans and First (1994) reported on the effect of free amino acids and vitamins on cleavage and developmental rate of cattle zygotes *in vitro*; while vitamins had no effect, they showed a significant increase in blastocyst yield when EAA or NEAA were added to a simple medium

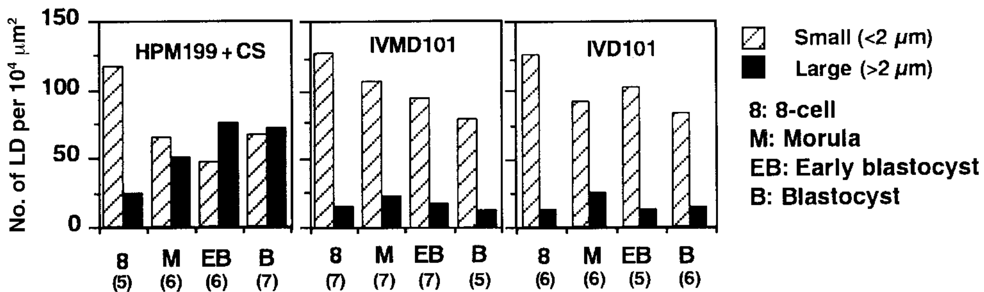


Fig. 7.10. Lipid droplets (LD) in bovine embryos cultured with and without serum. Fluctuation in the numbers of sudanophilic LD of different sizes of bovine embryos cultured in serum-supplemented (HPM199 + CS) and serum-free (IVMD101, IVD101) media. The numbers in parentheses represent the number of embryos examined. (From Abe *et al.*, 1999.)

(CR1). A study by Staines *et al.* (1997) evaluated embryo development in defined, protein-free culture conditions in SOF medium with various supplements. The highest blastocyst yield and blastocyst cell counts were with SOF plus BSA, with NEAA and EAA.

Growth factors

A report by Sirisathien *et al.* (2001) reported an improvement in bovine embryo development after adding EGF and IGF-1 to a chemically defined TCM-199 medium. The addition of 5 ng/ml of EGF resulted in a significant increase in blastocyst yield (50.6% vs. 36.5%); using 50 ng/ml IGF-1 led to a highly significant increase in blastocyst yield over controls (64.8% vs. 40.1%).

Hyaluronic acid supplementation

Hyaluronic acid (HA) is known to be present in low concentrations in bovine oviductal and uterine fluids. It is also known that the cattle embryo has a surface receptor for hyaluronan, CD44, throughout development from the oocyte to the blastocyst stage. Studies reported by Furnus *et al.* (1998a) used SOF supplemented with fatty acid-free BSA to culture cattle embryos in the absence or presence of HA; blastocyst yield was significantly increased by the additive (33% vs. 21–23%). Work reported by Stojkovic *et al.* (1999a) similarly investigated the effects of HA on the development of cattle embryos cultured in serum-free SOF, using a two-step culture procedure; they found that HA supported and increased blastocyst production when added during the second step of culture treatment (see Table 7.12). Although the mechanism by which HA affects the embryo remains unclear, the improvement in blastocyst yield when the glycosaminoglycan is present in the culture medium can be explained by the action of HA and its receptor, the glycoprotein CD44. In Canada, Palasz *et al.* (2000b) showed that the addition of hyaluronan to TCM-199 supplemented with BSA and ITS 5 days post-insemination had a positive effect on embryo development to the expanded- and hatched-blastocyst stages. In Germany, Stojkovic *et al.* (2002) demonstrated that a high concentration of HA (6 mg/ml) increased the viscosity of the culture medium and improved the *in vitro* development and the number of cells in bovine embryos; the survival rate of embryos after freezing was also improved by HA supplementation.

In pigs, supplementation of IVC medium with HA was found to improve the development of one- and two-cell porcine embryos to the blastocyst stage (Miyano *et al.*, 1994); a paper by Edwards *et al.* (1998) dealt with the effects of HA during IVC of IVP cattle and pig embryos. Studies reported by Gardner *et al.* (1997b, 1999) showed that fetal development after mouse embryo transfer was increased by replacing protein in their embryo culture medium with the glycosaminoglycan hyaluronate.

7.5.8. Sequential media

It is generally agreed that the changing needs of the developing bovine embryo are met to a large extent by programmed changes in the secretions of the cow's reproductive tract (see Lim *et al.*, 1996b, 1997; Hansel and Lim, 1998). In its simplest form, in the laboratory, this may take

Table 7.12. Effect of hyaluronic acid on development of IVP embryos (from Stojkovic *et al.*, 1999a).

	Treatment with HA			Blastocyst rate (% ± SD)			
Oocytes	Step one on day 1	Step two on day 5	Cleavage (% ± SD)	Blastocyst	Expanded blastocyst	Hatched blastocyst	Total
154	–	–	73.4 ± 2.2[a]	14.9 ± 1.7[a]	10.3 ± 7.2[a]	7.7 ± 3.6[a]	32.9 ± 9.5[a]
139	–	+	64.7 ± 1.6[b]	23.3 ± 2.1[b]	13.5 ± 2.3[a]	13.1 ± 1.1[b]	49.9 ± 1.6[b]
160	+	–	63.1 ± 1.7[b]	1.3 ± 1.2[c]	1.3 ± 1.2[b]	2.0 ± 2.0[c]	4.7 ± 4.1[c]
170	+	+	61.8 ± 5.1[b]	2.8 ± 2.5[c]	0[b]	0[c]	2.8 ± 2.5[c]

Means of percentage ± SD with different superscripts are significantly different ($P < 0.05$).
SD, standard deviation.

the form of replacing certain nutrients in the embryo culture medium at regular intervals (Fukui *et al.*, 1995, 1996; Poulin *et al.*, 1996). According to workers such as Gardner and Lane (1998) and Menezo *et al.* (1999), the use of sequential serum-free media now makes it possible to culture the human pronucleate embryo to the blastocyst stage at acceptable frequencies; resultant embryos have a high implantation rate, which gives a high pregnancy rate and reduces the need for the transfer of multiple embryos. During the first phase of culture, a simple culture medium, with or without amino acids but with low concentrations of glucose and phosphate, is recommended (see G1 in Table 7.13); EDTA is added to chelate unwanted cations, such as Fe and Cu, and to avoid free-radical formation. During the second phase, which takes the human embryo from the eight-cell to the blastocyst stage, the embryo starts its exponential demand for exogenous factors and the medium is adjusted accordingly.

In the sequential culture system developed by Gardner and Lane (1998), the concentrations of glucose, lactate and pyruvate in G1 and G2 were derived from measurements made of the concentrations of these metabolites in human reproductive tract fluid, both tubal and uterine, at various times during the menstrual cycle.

Table 7.13. Composition of human embryo culture media – G1 and G2. Non-essential and essential amino acids present in the concentrations specified by Eagle. Antibiotics and phenol red are optional constituents. (From Gardner and Lane, 1997.)

Component (mM)	G1	G2
NaCl	85.16	85.16
KCl	5.8	5.5
$NaH_2PO_4 2H_2O$	0.5	0.5
$CaCl_2$-$2H_2O$	1.8	1.8
$MoSO_4$ $7H_2O$	1.0	1.0
$NaHCO_3$	25.0	25.0
Na pyruvate	0.32	0.10
Na lactate (L isoform)	10.5	5.87
Glucose	0.50	3.15
Glutamine	1.0	1.0
Taurine	0.1	0.0
Non-essential amino acids	All	All
Essential amino acids	None	All
EDTA	0.01	0.0
Human serum albumin	2 g/l	2 g/l

The amino acid composition of each medium was based on extensive experimentation, using a mouse model system. The G1 medium was based on the concentrations of carbohydrates present in the human Fallopian tubes at the time when the cleavage stage embryo is present; this medium also contains those amino acids which have been shown to stimulate development of the cleavage stage embryo (i.e. the NEAA and glutamine). The chelator EDTA was also present, not only to sequester any toxic divalent cations present, but also to to help in minimizing glycolytic activity in the embryo, thereby lessening the risk of metabolic abnormalities; a paper by Gardner *et al.* (1997a) had shown that blastocyst cell number in cattle embryos was increased by EDTA for the first 72 h of development from the zygote stage. The G2 medium was based on the levels of carbohydrates present in the human uterus and contained both NEAA and EAA to facilitate both blastocyst development and differentiation. EDTA was not used, as it apparently selectively impaired ICM development and function, leading to a loss of embryo viability. Both G1 and G2 media were supplemented with human serum albumin.

Several commercial forms of sequential media have been employed in human assisted reproduction. Significantly greater blastocyst formation rates and pregnancy rates were recorded after using Cook's sequential media (Cook IVF) rather than G1.2/G2.2 media (Scandinavian IVF Science) by Janssens *et al.* (2000) in Belgium. In Japan, Kyono *et al.* (2000) found that Cook sequential culture media yielded the highest blastulation rate and highest embryo quality rating when compared with media from Irvine (USA) or Scandinavian IVF.

Culturing cattle embryos

The development of serum-free culture systems for the ruminant has been dealt with in several recent reviews (Gardner, 1999a,b). Workers with cattle embryos have examined the possible use of the G1/G2 media, which became available commercially (Scandinavian IVF Science) as a practical alternative to co-culture. In the USA, Hasler *et al.* (2000) found that bovine blastocysts cultured under this system possessed higher cell numbers than co-culture-derived blastocysts. Average cell number of expanded

blastocysts was 207 compared with 169 for co-culture; ICM number was 82 compared with 63 with co-culture.

In France, Heyman *et al.* (2002a) evaluated the potential of semi-defined sequential media developed for use with human embryos, ISM1/ISM2 (Medicult, Copenhagen), in studies with cattle embryos; they compared the system with their reference co-culture technique, which employed Vero cells and B2 medium supplemented with 2.5% FCS. Although embryo quality was similar, the yield of blastocysts was significantly lower using the sequential media; blastocyst formation occurred about 12 h later with the sequential media. The French group nevertheless concluded that the sequential culture system offered an interesting alternative to co-culture techniques and had the merit of minimizing disease risks arising from the use of serum or cells.

Pig embryos

A study reported by Durkin *et al.* (2002) in Indiana used metabolic parameters to formulate a sequential culture medium to meet the energy requirement of pig embryos more precisely and compared it with a standard culture medium (North Carolina State University 23 (NCSU-23)); blastocysts with greater viability were produced with the sequential medium but the culture system requires further refinement.

7.5.9. Embryo group size

There have been reports showing that mouse embryos produce factors that stimulate their development (Kato and Tsunoda, 1994); it has been shown that two-cell mouse embryos cultured singly in 25 μl microdroplets were of poorer quality than those cultured in groups. There has also been evidence that such inferior development could be improved by adding EGF or transforming growth factor alpha (TGF-α) or beta-1 (TGF-β_1) to the embryo culture medium. Reports in mice have also shown that the development of parthenogenetic embryos was affected by embryo density (Pinyopummin *et al.*, 1994); the authors suggested that embryo density might be considered for culturing manipulated embryos using parthenogenetic embryos to assist development. A further reference to the question of embryo density was in a paper by Gardner *et al.* (1997c), who noted that the development of the ICM in mouse blastocysts was stimulated by reducing the embryo : incubation volume. The role of TGF-β_1 in reproduction and development was the subject of a review by Shull and Doetschman (1994); it is possible that TGF-β_1 plays a role in regulating differentiation during early embryo development, including the morula-to-blastocyst transformation, blastocyst maturation and certain maternal–embryo interactions. In sheep, dealing with factors that enhanced cleavage and the development of embryos to the blastocyst stage, Gardner *et al.* (1994) observed that culturing embryos in groups stimulated development in that species.

In cattle, although EGF stimulated hatching of bovine embryos cultured singly from the eight-cell stage, the significant improvements in blastocyst yield and cell number seen in mouse embryos with the addition of EGF and TGF-β_1 were not evident in a study reported by Keefer *et al.* (1994a). Work reported by Poole *et al.* (1995) showed, however, that in singly cultured cattle embryos the supplementation of their TCM-199 medium with the combination of EGF/IGF-1/PDGF led to a significant increase in blastocyst yield in the absence of bovine serum. A study reported by Ferry *et al.* (1994) clearly demonstrated that single cattle zygotes were unable to develop to the blastocyst stage, whatever the volume or size of the culture drop, in a medium conditioned by oviduct cells without serum; when more than one zygote was present in the culture drop, blastocysts were obtained. In Canada, Blondin and Sirard (1994) developed *in vitro* cattle embryos either singly (one per 10 μl droplet) or in groups (ten per 50 μl droplet); they recorded significantly more morulae with group culture than with individual culture (45.9% vs. 28.0%). Other reports showed a favourable effect of increased embryo density, achieved either by decreasing the volume of culture medium or increasing the number of embryos incubated per unit volume. In Belgium, Donnay *et al.* (1997b) found that cell-free mSOF + 10% FCS did not permit single bovine embryos to develop to the blastocyst stage; development was also limited with three to six embryos per group (6% blastocysts) when compared with 20 embryos per group (23% blastocysts). There was no group

effect when embryos were co-cultured with BRL cells in TCM-199 or with cumulus cells in mSOF; the authors showed that embryo development or quality was enhanced by increasing the embryo : medium-volume ratio.

In cattle, the effects of adding growth factors to bovine embryo culture media have been limited, although PDGF and granulocyte–macrophage colony-stimulating factor (GM-CSF) have been shown to enhance development to the blastocyst stage in some reports (Thibodeaux *et al.*, 1995a,b; De Moraes and Hansen, 1997). A paper by Paula-Lopes *et al.* (1998) showed that the growth factor interleukin 1 beta (IL-1β) increased development to the blastocyst stage when bovine embryos were cultured at high density (~25–30 embryos per drop) but decreased or had no effect on development when cultured at low density (approximately ten embryos per drop); there was also evidence that IL-1β may increase blastocyst yield by its effect on embryo growth before day 5.

In cattle, the cooperative effects among embryos cultured in large groups have been described by a number of groups (Ahern and Gardner, 1998); it is not only during embryo culture that effects are evident. As previously noted, there are reports to show that group size in maturation can influence subsequent competence of oocytes. In Poland, Katska *et al.* (1998) co-cultured cattle oocytes either in large groups (>30 oocytes per well) or in small (one to five oocytes per drop of medium); they recorded higher blastocyst yields with the large group size. Arranging to use large embryo groups may present no problem to those engaged in large-scale embryo production, but in commercial practice, where many operators may be dealing with small numbers of oocytes and embryos, it may well be necessary to culture cattle embryos singly or in small groups. In New Zealand, Hagemann *et al.* (1998b) designed a system for the culture of individual cattle embryos; they achieved results similar to those obtained for group culture. All steps were carried out in 10–12 μl drops, using SOFaa/BSA medium under oil; the addition of bovine serum on day 5 of culture led to a significant increase in blastocyst yield. In Japan, Nagao *et al.* (1998) reported data indicating that the number of embryos in a droplet of IVC medium markedly influenced blastocyst yield; in low embryo density (one to five embryos per droplet), reducing the oxygen tension (1–2.5%) or excluding phosphate from the medium enhanced bovine blastocyst development.

A study by Hendriksen *et al.* (1999b) in Belgium involved the co-culture of either single or 35 oocytes with BRL cells in 500 μl volumes; cleavage, blastocyst and hatching rates with single embryos were significantly below those used in group culture (see Table 7.14). The effect of group size on the development and interferon tau (IFN-τ) secretion of cattle embryos was examined by Larson and Kubisch (1999) in the USA; they showed that culturing IVP cattle embryos in groups not only affected their developmental fate but also significantly altered the secretion of IFN-τ. In Japan, Fukui *et al.* (2000) presented evidence suggesting that individual bovine oocytes can be matured, fertilized and developed to the blastocyst stage in an IVP system using chemically semi-defined media as efficiently as oocytes cultured in groups; the problem with this, however, is that blastocyst yield using TCM + PVA is lower than in media supplemented with FCS or BSA.

In Belgium, Yuan *et al.* (2000) evaluated the ability of bovine embryos to develop in 50 μl droplets of SOF + 5% FCS medium, cultured either singly or in small (five) or larger (25)

Table 7.14. IVP embryo production with single or group embryo culture. IVP results using either one or 35 oocytes/embryos per well. The number of oocytes were 413 for single and 414 for group culture. (From Hendriksen *et al.*, 1999b.)

	% cleaved[a]	% blast. day 7[a]	% blast.day 9[a]	% hatching day 11[b]	% blast./cleaved
Single	67*	14*	23**	68**	34**
Group	74	20	33	82	45

[a]Of oocytes after IVF.
[b]Of blastocysts at day 9.
*$P < 0.05$; **$P < 0.01$ (chi-square test).
Blast., blastocyst.

groups. Results showed that blastocyst yield was significantly greater in droplets containing five and 25 embryos compared with single embryo culture (see Table 7.15); the hatching rate was also markedly reduced when embryos were cultured singly. The authors suggest that embryo density may be important after the critical maternal–embryonic transition because embryonic factors are involved in the activation or promotion of embryonic gene expression; they concluded that there is a need to elucidate the factors necessary for activating embryonic gene expression. In Ireland, on the basis of evidence obtained with cattle embryos cultured in SOF medium, Ward *et al.* (2000a) concluded that blastocyst yields were maximized when embryos were cultured in group sizes of not less than five; to optimize blastocyst quality, a group size of 25 was recommended. The Irish work made it clear that group size, rather than medium volume per oocyte, was the factor influencing blastocyst yield and quality.

Embryo density was one of the factors examined by Khurana and Niemann (2000a,b,c) in studies in which they used 12,533 bovine oocytes to evaluate various culture conditions that would increase IVP of cattle morulae/ blastocysts. Among their results they showed that the rate of development of one-cell embryos originating from inferior-quality oocytes was significantly improved when cultured in groups of 40 rather than 20 per 0.5 ml of medium.

The WOW culture system

A paper by Vajta *et al.* (2000b) noted that most commercial and human embryo culture systems require embryos to be cultured either singly or in small groups; the authors described a new approach to culturing under such conditions, which they described as the well of the well (WOW) culture system. Small conical and cylindrical wells, about 300 μm deep, were made in four-well Nunc dishes; one or five presumptive zygotes were placed in each well. The WOW system resulted in higher rates of embryo development (single embryo, 59%; group of five, 61%) than following culture in droplets or in the well. The authors concluded that the WOW system was simple, safe and easy to use. A study reported by Holm *et al.* (2000a,b) compared the development of cattle zygotes cultured singly in the WOW system with standard group culture; their results confirmed that the culture of single embryos in this system was highly successful in mSOF (SOFaaci) with 5% bovine serum. They also showed that in chemically defined SOF medium, blastocyst development was decreased and single culture further compromised blastocyst yield; there is an obvious need to clarify the role of serum in the WOW system. In Germany, Reichenbach and Weppert (2002), cultured cattle embryos in different systems, which included Nunc four-well dishes in which the bottom of each well contained five cylindrical subwells, each about 30 μl deep; in each well, groups of 5 × 5 zygotes were cultured in 400 μl SOF supplemented with 10% OCS. Results (see Table 7.16) led them to conclude that the 4 × 5 subwell system was suitable for the culture of small numbers of embryos.

7.5.10. Gas atmosphere

Oxygen

A number of reports have dealt with the effect of different gas phases used with IVC media. Various studies have shown oxygen tension in the mammalian oviduct to be lower than 10%;

Table 7.15. Embryo production in relation to group size. Total number of tested embryos and survival rates at first cleavage, morula, blastocyst, hatching blastocyst stages (data shown as mean % ± SEM). (From Yuan *et al.*, 2000.)

Group	Total embryos tested	Cleavage rate	Morula rate	Blastocyst rate	Hatched blastocyst rate
S1	457	$56.5^a \pm 7.2$	$19.4^a \pm 5.2$	$3.4^a \pm 2.5$	$0.3^a \pm 0.3$
S5	549	$67.5^a \pm 7.1$	$29.0^b \pm 3.9$	$14.5^b \pm 1.8$	$6.0^b \pm 1.9$
S25	535	$71.5^a \pm 4.3$	$36.5^c \pm 4.8$	$17.8^b \pm 2.6$	$15.9^c \pm 3.3$

[a,b,c]Difference superscripts within the same column differ significantly ($P < 0.05$; chi-squared).
SEM, standard error of the mean.

Table 7.16. Embryo development in different culture systems and group sizes (data shown as mean % ± SEM) (from Reichenbach and Weppert, 2002).

		No. zygotes		Cleavage rate		Blastocyst rate	
Group	Replicates	Total	Per well	Day 3	Day 7	Day 9	Hatched (day 11)
I	18	360	5	56 ± 2.6	23 ± 2.4^{a}	30 ± 2.4^{a}	25 ± 2.4^{a,c}
II	11	455	13	56 ± 3.7	25 ± 3.4^{a,c}	33 ± 3.4^{a,c}	27 ± 3.4^{a,b}
III	26	874	19	64 ± 3.2	32 ± 2.9^{c,d}	41 ± 3.0^{c,d}	33 ± 3.0^{b,d}
IV	10	480	(10 + 6)	62 ± 2.8	19 ± 2.6^{a,e}	23 ± 2.6^{a,e}	18 ± 2.6^{c,e}
V	7	225	(5 × 5)	60 ± 3.2	34 ± 2.9^{b,d}	41 ± 3.0^{b,d}	26 ± 3.0^{a,b}

Different superscripts within the same column differ significantly ($^{a,b,c}P < 0.05$; $^{d,e}P < 0.01$).
SEM, standard error of the mean.

such information implies that low oxygen may be preferable to atmospheric oxygen for IVC of cattle embryos. In Japan, Nagao *et al.* (1994) showed that a 5% oxygen level enhanced blastocyst formation in the absence of somatic cell support; their data also suggest that the beneficial role of somatic cells in co-culture may include the reduction of the oxygen concentration in the medium. In Argentina, Alberio *et al.* (1998b) examined the effectiveness of SOF and CR1aa media in cattle embryo development under 5% or atmospheric oxygen (20%) concentrations; significantly higher blastocyst yields were achieved using the 5% concentration. The mechanisms whereby oxygen concentration influences cattle embryo development remains unclear, but it is possible that reactive oxygen species produced in the embryos increase during culture at atmospheric oxygen concentration; reactive oxygen species, such as O_2 and H_2O_2, can cause lipid peroxidation and enzyme inactivation, resulting in cell damage, by promoting hydroxyl radical formation (Fujitani *et al.*, 1997).

Carbon dioxide

In the Netherlands, Merton *et al.* (1995) investigated the effect of two different culture media (TCM-199 and B2) with different CO_2 concentrations on embryo development. When B2 was used in 5% CO_2, the pH of the medium (6.5–6.8) affected embryo quality; by lowering the CO_2 level to 3% this effect was deleted. The authors concluded that embryo culture in B2, compared with TCM-199, required a lowering of the CO_2 concentration to achieve the highest blastocyst yield. In Japan, Geshi *et al.* (1999) recorded an improvement of IVC for cattle embryos using a low concentration of CO_2 (2%) and TCM-199 medium supplemented with 10 μM β-ME.

Ambient laboratory air

Few studies have examined the question of air quality in the embryo production laboratory. For such reasons, a study by Merton *et al.* (2002) investigated the effect of an intra-incubator carbon-activated air-filtration (CODA) system in their laboratory. They were able to show that air purification led to a significant increase in pregnancy rates established with IVP bovine embryos. The authors note that the improvement in embryo quality was achieved during the 6-day period of IVC and that the effect of air purification during the maturation and fertilization phases remains to be investigated.

7.5.11. Temperature and light

Experiments to determine the temperature and duration of heat exposure that would be detrimental for *in vitro* cattle embryo development were reported by Ju *et al.* (1999) in the USA. Embryos exposed to a mildly elevated temperature (40.5°C) for 30–60 min had no evident effect on cleavage, blastocyst formation or hatching. Increasing the temperature to 41.5°C for 30–60 min significantly reduced hatching rate. The development of IVC cattle embryos after exposure to high temperatures in the physiological range was studied by Rivera and Hansen (2001). When zygotes and two-cell embryos were exposed to temperatures ranging from 38.5 to 41.0°C for 3–12 h, heat shock did reduce blastocyst yield, but only after exposure

to 41°C for 9–12 h. Other studies in the same laboratory by Paula-Lopes and Hansen (2002) showed that exposure of eight- to 16-cell bovine embryos at day 4 to mild heat shock (40°C for 80 min) blocked the apoptotic response to subsequent, more severe heat shock (41°C for 9 h); it appeared that apoptosis could be prevented by induced thermotolerance.

In Utah, Bunch *et al.* (1999) tested the hypothesis that exposure of the bovine embryo to fluorescent light had an adverse effect and that yellow-filtering fluorescent light would protect embryos from damaging effects; the percentage of embryos that developed into morulae/blastocysts was 33.8% in direct light and 41% in filtered light. They concluded that exposure to fluorescent light had a significant retarding effect on embryo development.

7.5.12. Protection from oxidative stress

Cattle embryos cultured *in vitro* are susceptible to oxidative stress (Lequarre *et al.*, 1999) and various authors have reported attempts to alleviate this problem (Ealy *et al.*, 1994, 1995; Liu and Foote, 1994; Liu *et al.*, 1994; Luvoni *et al.*, 1994, 1996; Matsuyama and Fukui, 1994; Olson and Seidel, 1995; Lim *et al.*, 1996a; Takahashi, H. *et al.*, 1996; Takahashi, M. *et al.*, 1996; Takahashi, Y. *et al.*, 1996; Diez and Gomez, 1997; Takahashi, Y. and Kanagawa, 1998; Caamano *et al.*, 1998a,b,c; Carmargo *et al.*, 1998; Kato *et al.*, 1998b; Feugang *et al.*, 2001, 2002b,c). Similar studies have been reported with pig embryos (Grupen *et al.*, 1995a,b). One measure has been by way of reducing the oxygen concentration during culture from 20% to 5%, a level much more in keeping with the oviductal environment. There are many reports showing that culture of bovine embryos under reduced oxygen tension resulted in better blastocyst quality in comparison with 20% oxygen (O'Kearney-Flynn *et al.*, 1998a,b). A study was made by Iwata *et al.* (1998a,b) in Japan to determine the effects of glucose, antioxidants and different oxygen tensions on the development of bovine embryos cultured in mSOF medium; they showed that low concentrations of glucose during culture were beneficial and that generation of free oxygen radicals is partly caused by a high concentration of glucose in the medium. A report by Morales *et al.* (1999) examined the antioxidative capacity of chemicals in their mSOF medium; pyruvate improved bovine zygote and blastocyst development and displayed the highest hydrogen peroxide-degrading ability. A study by Takahashi *et al.* (1999) examined the effect of changing oxygen tension at several developmental stages on the yield of cattle blastocysts; their data suggested that changing the oxygen tension from 20 to 5% between day 4 and day 6 had the greatest effect.

In laboratories where it is not possible to reduce oxygen concentration, the addition of an antioxidant to the culture medium may offer a way of protecting embryos. In Texas, Lee *et al.* (1999a,b) documented the effect of supplementing SOF + 10% FCS with β-ME; the blastocyst yield was significantly increased by this addition (26.6% vs. 18.5%). Studies by Bing *et al.* (2000) in Japan evaluated the effect of different concentrations of recombinant human thioredoxin in their cattle IVC medium; they found that it was able to promote the bovine embryo at early stages but not when added 2 days after fertilization. Specific reducing agents may only be effective against oxidative stress at a certain stage of embryo development. Hamano *et al.* (1994) found that β-ME was only effective after the eight- to 16-cell stage.

In Belgium, Feugang *et al.* (2001) showed that Trolox (a water-soluble analogue of vitamin E) and β-ME in their SOF medium could prevent apoptosis induced by oxidative stress. Further work reported by Feugang *et al.* (2002c) presented evidence suggesting that the presence of a stimulator (β-ME) of glutathione synthesis at the time of embryo compaction/blastulation improved blastocyst quality in terms of hatching rate and cell number; the same authors noted that β-ME influenced cell allocation in hatched blastocysts by increasing the proportion of ICM cells. A study reported by Fischer-Brown *et al.* (2002) used β-ME in their semi-defined SOFaa + BSA culture system with or without 10% FCS; in FCS-supplemented medium, embryos formed blastocoels earlier and with 35% fewer cells than in supplemented medium. They found some evidence that the antioxidant increased hatching rate and day-6 development when FCS was present, although the nature of this interaction was unclear.

Takahashi *et al.* (2000a) used the COMET assay (single-cell microgel electrophoresis) to investigate the effect of oxidative stress on DNA in cattle embryos cultured in SOFaa + 5% FCS. Greater DNA damage was evident when a concentration of 20% oxygen rather than 5% was used. Retardation of embryo development was positively correlated with an increase in DNA damage. The influence of oxidative stress on bovine embryo development in culture media is dealt with in a report by Takahashi (2000b). A further report by Takahashi *et al.* (2002) demonstrated that β-ME has a protective effect on embryo development against oxidative stress and that the effect of the agent is associated with the promotion of cystine uptake in embryos.

The toxic effects of oxygen on the embryos of various animal species have been reviewed by Catt and Henman (2000). They suggested three possible methods of minimizing oxidative damage to embryos: (i) decreasing the oxygen in the gas phase used for culture; (ii) changing the formulation of culture media to include components designed to protect against oxidative damage; and (iii) reducing the co-incubation period for sperm and oocytes to minimize oxidative damage due to sperm metabolism.

7.5.13. Hormones, growth factors and cytokines

As observed by Erickson (1995), reviewing the role of growth factors and morphogens in developmental biology, there is now a realization that there are many more 'hormone-like' molecules involved in biology than was previously thought. Many of these molecules have relevance to the development of the bovine embryo *in vitro*. Hormones, growth factors and cytokines share many attributes; all are molecules released from one cell that can induce changes in other cells via binding to high-affinity molecule-specific receptors. Hormones are usually produced by specialized cells, are released into the circulation and act on a restricted set of target cells in another part of the body. Growth factors are usually considered to act primarily on non-haemopoietic cells whereas cytokines tend to act on cells of the immune system and to modulate inflammatory responses.

Growth hormone and insulin-like growth factors

Growth hormone (bovine somatotrophnin (BST)) and IGF-1 influence early embryonic development and the process of apoptosis in cattle; all components of the IGF system (IGF-I and IGF-II and their receptors and binding proteins) are known to be expressed in the early bovine embryo. Data reported by Prelle *et al.* (1999a,b) indicated that an interaction with IGF-binding proteins is necessary for the embryotrophic activity of IGF-I. Various reports have identified mRNA for growth hormone (Lechniak *et al.*, 1999) and IGF-1 receptors (Watson *et al.*, 1999a,b) on the developing embryo from the one- to two-cell stage. It is clear that the bovine embryo's early existence in the oviduct is spent in an IGF-rich environment. A study by Kolle *et al.* (2001a, 2002) in Germany analysed the effects of growth hormone on apoptosis and the ultrastructure of the bovine embryo; they showed that the percentage of apoptotic cells in 8-day-old blastocysts declined from 14% to 2.7% after growth-hormone supplementation (100 ng/ml recombinant BST (r-BST)) of TCM-199 medium. The same workers showed that growth-hormone treatment eliminated glycogen storage in the blastocyst and increased lipid exocytosis; they found a higher volume density of embryonic mitochondria in treated embryos, indicating that growth hormone influenced the metabolism of the early embryo. In pigs, it may also be mentioned, workers in the Netherlands showed that growth-hormone supplementation during embryo culture significantly reduced the percentage of blastocysts with apoptotic cells (Kidson *et al.*, 2002).

As noted by Kolle *et al.* (2001b), who showed that growth-hormone receptor mRNA and protein are synthesized from days 2 and 3 onwards in cattle embryos cultured *in vitro*, the effects of growth hormone during IVC of embryos may partly be a result of the higher availability of glucose, from both increased uptake and increased glycogen utilization. Studies on the expression of growth hormone in early cattle embryos reported by Joudrey *et al.* (2001) showed that transcription of the bovine growth-hormone gene begins at the eight- to 16-cell stage of development. Data

presented by Suzuki *et al.* (2000) showed that 200 ng/ml growth hormone in CR1aa medium significantly enhanced blastocyst yield but had no influence in CR2aa medium; they suggest that alanine and glycine, NEAA in CR2aa medium, may play a key role in embryo development.

In Germany, Prelle *et al.* (2000a,b) reported the expression of IGF-binding protein and IGF-1-receptor mRNA in cattle embryos; it is evident that various components of the IGF system are differently regulated by IGF peptides. Studies reported by Moreira *et al.* (2002) in Florida indicated that both BST and IGF-1 promoted embryonic development by increasing blastocyst yield and quality (see Fig. 7.11). The addition of an antibody to IGF-1 to the KSOM culture medium inhibited the effects of IGF-1 but did not alter the stimulatory effects of BST on embryonic development; the effect of growth hormone appeared to be independent of IGF-1 action. The authors note that such results may be relevant to the increased pregnancy rates observed in recipient cows receiving embryos from superovulated cattle that had received exogenous growth hormone; it is possible that such treatment increased embryo quality.

Interferon-tau/alpha

In early embryo development *in vitro*, it is known that expression of IFN-τ mRNA starts as early as the blastocyst stage and that a detectable amount of the growth factor can be found in culture medium; it appears that interferon may have a role in embryo development before it reaches the stage at which it becomes involved in maternal–embryo recognition. Support for the development of bovine embryos in culture by secretions from bovine trophoblastic vesicles was described by Stojkovic *et al.* (1997). A report by Takahashi *et al.* (2001), using TCM-199 + BSA medium, demonstrated a novel function of IFN-τ and IFN-α in promoting development from the morula to the blastocyst stage; the same workers used reverse-transcription polymerase chain reaction (RT-PCR) analysis to show that type 1 receptor mRNA was expressed in cattle embryos from the morula to the blastocyst stages.

Epidermal growth factor

An experiment reported by Poole *et al.* (1995) determined the effect of various growth factors on bovine embryo development beyond the

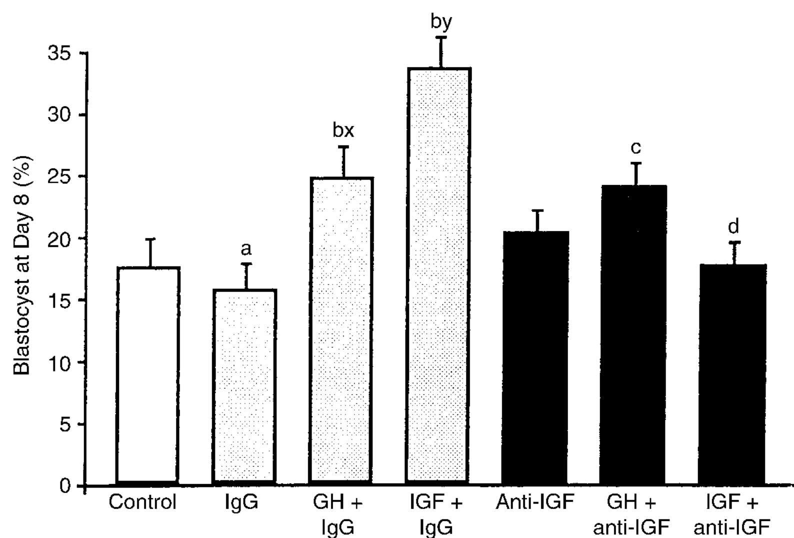


Fig. 7.11. Effect of BST and IGF-I on bovine embryo development. Percentage of inseminated oocytes that developed to blastocyst at day 8 after insemination (LSM ± SE). Controls are represented by an open box, IgG treatments by light grey boxes and anti-IGF-I groups by black boxes. Different letters indicate significant orthogonal contrasts: a vs. b, $P < 0.01$, x vs. y, $P < 0.02$: c vs. d, $P < 0.05$. LSM, least-square mean; SE, standard error; IgG, immunoglobulin G; GH, growth hormone. (From Moreira *et al.*, 2002.)

eight-cell stage in serum-free culture conditions; results indicated that the development of singly cultured embryos past the eight-cell stage was significantly increased by PDGF-α and PDGF-β (PDGF) and by a combination of EGF/IGF-1/ PDGF in the absence of bovine serum. A report by Viuff *et al.* (1995) showed that cells of the bovine oviduct (lamina epithelialis and lamina propria) transcribe PDGF-β mRNA, strengthening the likelihood that this growth factor is involved in normal embryo development. In a study of several growth factors, Lee and Fukui (1995) found evidence that fibroblast growth factor (FGF) and EGF may act synergistically on bovine embryo development when added to their SOF medium. The effect of several growth factors (IGF-1, TGF-α, EGF and PDGF) was studied by Palma and Brem (1995a), who recorded a significantly beneficial effect on cattle embryo development in the absence of serum; in medium supplemented with 10% OCS, there was no response. The significance of the EGF/TGF-α systems in early embryo development was discussed by Fischer (1996a,b). In a report by Park *et al.* (1997), it was shown that exposure of bovine oocytes to EGF during IVM enabled them to develop to the blastocyst stage in a chemically defined medium. A paper by Mohan *et al.* (1999) reported the detection of EGF-receptor mRNA at the two-cell to blastocyst stage in cattle; the presence of this receptor provides an explanation for the positive effects exerted by EGF on early bovine embryo development *in vitro*. A report by Makarevich and Markkula (2002) dealt with apoptosis and cell proliferation in IVP embryos stimulated with IGF-I during IVM and embryo culture; they found the requirement for IGF-I to be more critical during embryo culture than during oocyte maturation. They concluded that an assay for TUNEL in conjunction with cell-proliferation analysis could provide useful information about the quality of IVP embryos.

Effect of cytokines

There is evidence to show that early cattle embryos continuously express mRNAs encoding activin receptors from the one-cell to the hatched blastocyst stage; it is also evident that activin A and follistatin regulate the developmental competence of IVP cattle embryos (Yoshioka *et al.*, 1998a,b). Other studies by Yoshioka *et al.* (2000) in Japan used cinematographic analysis to show that the cytokines activin A and follistatin influenced the developmental kinetics of cultured cattle embryos produced *in vitro*; they showed that activin A shortened the duration of the third or fourth cell cycle by deferring the stage of developmental arrest (lag phase) and shortening the arrest period. The authors suggest that activin A enhanced the *in vitro* development of cattle embryos by improving the kinetics during early cleavage, especially at the stage of the onset of the lag phase, and by shortening the duration of the phase.

The development of IVP cattle embryos in SOF medium containing human leukaemia inhibitory factor (hLIF) was studied by Fukui and Matsuyama (1994a,b); their results indicate that hLIF improved embryo development when the medium was supplemented with BSA or PVA but not when serum was used. The implication is that serum contains growth factors that play an important role in embryo development. A study by Han *et al.* (1995a,b) found that the addition of hLIF did not influence blastocyst yield but did significantly improve the hatching rate of embryos when added to their SOF + BSA medium before freezing. A paper by Kauma and Matt (1995) dealt with work in mice showing that co-culture cells that expressed leukaemia inhibitory factor (LIF) enhanced mouse embryo development *in vitro*. The improved development of the early bovine embryo due to IL-1β was reported by Paula-Lopes *et al.* (1997, 1998) in Florida; they suggest that IL-1β probably increases blastocyst yield by its effect on embryo growth before day 5. The mRNA expression of LIF and its receptor subunits in IVP bovine embryos was dealt with in reports by Eckert and Niemann (1998) and Eckert *et al.* (1998). The effect of the glycoprotein bovine LIF (bLIF) on hatching and the numbers of ICM and TE cells in cattle blastocysts was examined by Yamanaka *et al.* (1999) in Japan; they recorded a significant increase in the number of TE cells and in the blastocyst hatching rate.

7.5.14. Culture media components

Antibiotics

Media used in the recovery, washing, maturation and fertilization of cattle oocytes and in the

IVC of the early embryo are generally supplemented with antibiotics to suppress the growth of contaminating microorganisms and to prevent the spread of pathogens. Various authors have reported on the effect of these antibiotics on the viability of the embryo. In the USA, using toxicity tests, Riddell *et al.* (1995) found no evidence of delay in development with one time or ten times the recommended concentration of penicillin G or streptomycin after 6 or 72 h exposure. The authors concluded that short-term exposure to even high concentrations of certain antibiotics is unlikely to result in toxicity and may help to ensure freedom from specific pathogens or contaminants.

Insulin

The stimulatory effects of insulin on the development of cattle embryos in a chemically defined, protein-free medium (mSOF supplemented with 1 mg/ml PVA with amino acids) was studied by Matsui *et al.* (1995a,b,c) in Japan; results suggested that insulin improved embryonic development by stimulating amino acid transport and/or glucose uptake.

Amino acids

It has been the practice for several years to add amino acids (EAA and NEAA; shown in Table 7.5) to embryo culture medium on the basis of protocols originally designed for somatic cell growth. A study by Elhassan *et al.* (1999a) measured amino acid levels in bovine oviductal and uterine fluids and compared them with concentrations found in a commonly used IVC medium (KSOM with EAA and NEAA). The authors presented results showing a wide difference in the concentrations of amino acids, which proved to be several times higher than those found in culture media. In a further report, Elhassan *et al.* (2001) showed that 23 of the 24 amino acids measured in uterine fluid were present in higher concentrations than in KSOM supplemented with either FCS or minimum essential medium with amino acids (MEM-aa); in oviductal fluid much the same result was found. The authors suggest that it would be worth testing a two-step embryo culture system in which a defined medium is supplemented initially with amino acids at oviductal-fluid concentrations, followed by a medium with amino acids at uterine-fluid levels.

Taurine and its precursor, hypotaurine, are sulphur-containing β-amino acids that are known to be present in high concentrations in the bovine reproductive tract; both are known to have antioxidant properties. As well as its role in oxidation and metabolism, taurine is thought to protect the embryo against high potassium concentrations. The amino acid has been shown to be beneficial in the culture of cattle embryos in some reports (Liu and Foote, 1994; Liu, Y. *et al.*, 1995; Liu, Z. *et al.*, 1995; Lu *et al.*, 1997; Lu, 1999) but not in others. In Ireland, Lonergan *et al.* (1999b) found that taurine supplementation of SOF had no effect on embryo development or quality; development of cattle zygotes to blastocyst in CR2 medium was significantly decreased by taurine plus glutamine in a study reported by Lu, S.S. *et al.* (1999).

Alanine and glycine are known to be two major amino acids in medium conditioned by bovine oviductal cells; high concentrations of these amino acids are present in the mammalian oviduct. At Beltsville, Bondioli *et al.* (1995) reported a beneficial effect of glycine and alanine in the co-culture of cattle embryos with BRL cells. In the USA, Suh *et al.* (1995) found that the addition of glycine to serum-free CR2 medium significantly increased blastocyst yield; alanine alone had no effect. A study by Shi *et al.* (1995) found that under certain culture conditions (10% OCS in TCM-199) the addition of alanine and glycine resulted in a significant increase in blastocyst yield. There has also been evidence from studies reported by Suzuki *et al.* (2000) that alanine and glycine may play a key role in embryo development *in vitro*.

In Aberdeen, a study by Kuran *et al.* (2002) investigated amino acid uptake or release, *de novo* protein synthesis and allocation of cells to the ICM and TE in blastocysts produced in a protein-free medium; the results demonstrated that depriving early bovine embryos of exogenous protein throughout the first week of life resulted in a halving of blastocyst yields in the absence of EAA.

Heparin

The glycosaminoglycan (GAG) heparin is widely used in cattle IVF to induce capacitation in

bovine spermatozoa; a lesser known effect of this GAG is in enhancing the developmental capacity of the bovine oocyte when added to the maturation medium (Lazzari and Galli, 1994; Lazzari *et al.*, 1994). In a later report, Lazzari *et al.* (1999a,b), using SOF medium supplemented with BSA and amino acids, demonstrated that the addition of heparin (10 μg/ml) resulted in a higher yield of day-7 blastocysts with lower cell numbers than those cultured without the GAG (see Table 7.17). It was also shown that, contrary to most reports, the cell number of IVC cattle embryos may be higher than that found in *in vivo*-derived embryos; in the present instance, heparin-cultured blastocysts were similar to those produced *in vivo*.

Hexoses

There is some evidence to suggest that replacing glucose with fructose during embryo culture may improve cattle embryo development. In Colorado, Chung, Y.G. *et al.* (2000) investigated this possibility with different doses of these hexoses; fructose proved to be slightly superior to glucose, the optimal concentration for the first 72 h of culture being 0.25 mM.

Vitamins

The effects of B-group vitamins on mouse embryo development were studied by Tsai and Gardner (1994) in Australia; their data indicated that nicotinamide inhibited development and reduced viability. McKiernan and Bavister (2000) drew attention to the fact that the role of vitamins in normal embryo development *in vitro* had not been thoroughly examined; they reported that a water-soluble vitamin, pantothenate, stimulated *in vitro* development of hamster embryos. The authors suggested that addition of this vitamin could have a significant effect on human embryo development *in vitro*, especially in the light of increasing emphasis on blastocyst transfers in human assisted reproduction. In Colorado, Olson and Seidel (2000a,b) found that the culture of IVP cattle embryos with vitamin E improved development; after spending a week in recipient cows, embryos cultured with vitamin E were approximately 63% larger in surface area than controls. In Aberdeen, Reis *et al.* (2002) showed that, when bovine zygotes were cultured in SOF in the presence of serum (10% FCS), blastocyst yields were significantly improved by vitamin E supplementation in comparison with controls (28% vs. 15%).

Surface-active components

The effect of macromolecular supplementation on the surface tension of TCM-199 and the utilization of growth factors by bovine embryos in culture was studied by Palasz *et al.* (2000a) in Canada; they concluded that surface-active components in media affected cattle embryos positively. Non-ionic surfactants, such as Twin-80 in TCM-199, may successfully replace the surface-active properties but not the growth-promoting properties of bovine serum in IVC media.

Mineral and silicone oils

Embryo toxicity in different batches of mineral oil was discussed in a paper by Pascual *et al.* (1997) in Spain. The influence of mineral and silicone oil on the development of cattle embryos in microdrops of CR1 medium was examined by Ferruzzi *et al.* (2000) in Brazil; the importance of using oil was confirmed but oil type had no effect

Table 7.17. Effect of heparin supplementation on blastocyst yield and cell number (from Lazzari *et al.*, 1999b).

	No. oocytes	No. cleaved (cleavage)	No. blast. day 7	% blast. day 7/ cleaved (mean ± SD)	No. blast. day 8	% blast. day 8/ cleaved (mean ± SD)	Total cell number (blast. day 7) (mean ± SD)
SOF	176	111 (64%)	26	23 ± 11[a]	44	40 ± 12[a]	183 ± 27[a]
SOF with heparin	177	112 (64%)	36	32 ± 8[b]	47	42 ± 10[a]	141 ± 22[b]

Values within columns with different letters differ significantly.
blast., blastocyst; SD, standard deviation.

on blastocyst yield. In Belgium, Van Soom *et al.* (2001) investigated the effect of silicone and paraffin oil used in microdroplet culture systems; embryonic development to the eight-cell and blastocyst stage was significantly impaired by one silicone oil batch in comparison with paraffin oil. It was also found that the oil had an adverse effect on the outcome of vitrification of blastocysts. It was thought that the oil overlay might have changed the relative abundance of fatty acids in the different lipid classes, which rendered cell membranes more susceptible to damage during cryopreservation.

7.5.15. Possible toxic agents

Ammonia

The number of cells in cattle embryos was reduced when Kuran *et al.* (1998) used a high ammonia-producing steer serum in their IVC culture system. The inclusion of the same steer serum in the first phase of a two-phase SOF culture system reduced the cleavage rate (Kuran *et al.*, 1999a); during the second phase of culture, it reduced the cell number and other measures of embryo quality. It may also be noted that, although the use of this same steer serum in the culture of sheep embryos caused fetal oversize (Carolan *et al.*, 1997; Sinclair *et al.*, 1998), there was no evidence in early cattle embryo development predictive of such adverse effects (Kuran *et al.*, 1999b). The effects of ammonia during different stages of culture on the development of IVP bovine embryos were recorded in a paper by Hammon *et al.* (2000a,b) in Utah; ammonia in the IVC medium significantly decreased blastocyst yield. The general conclusion, from their overall IVM, IVF and IVC studies, was that the effect of ammonia on development depended on the concentration and the stage of development when exposure occurred.

In New Zealand, Berg *et al.* (2002d), working with IVP cattle embryos cultured in modified SOF with amino acids (mSOFaa) supplemented with either BSA or PVA, reported data supporting the view that increased ammonium production is the product of protein-free culture conditions; they note that ammonium is a known teratogen and has been implicated as a mediator of fetal abnormalities following embryo culture. In Colorado, Lane *et al.* (2002) noted that care should be taken to reduce the exposure of early bovine embryos to ammonium; they showed that ammonium preferentially inhibited ICM development of cultured bovine blastocysts and reduced embryo metabolism by disturbing intracellular pH regulation.

Nitric oxide

Various attempts have been made to remove embryo-toxic nitric oxide from IVC co-culture systems (Lim and Hansel, 1998; Lim *et al.*, 1999a,b,c). Bovine embryo culture medium (mBECM) was supplemented with a nitric oxide scavenger (haemoglobin, 1 μg/ml); it was concluded that the addition of haemoglobin was a useful method of eliminating this embryotoxic factor from the culture system. In York, Orsi and Leese (2002) reported data indicating that excess nitric oxide in their IVC medium (SOFaa/BSA) depressed the activity of key metabolic enzymes and induced oxidative damage, leading to reduced ATP content, which impaired bovine embryo development.

7.5.16. Simplifying culture systems

What is possible in the research laboratory, in terms of equipment, personnel and facilities, may not be feasible under field conditions where commercial cattle embryo production is being attempted. For such reasons, there may a need to consult reports on possible ways of simplifying the production process. In Japan, Suzuki *et al.* (1995) developed what they describe as a simple, economical and portable CO_2 incubator for the production of cattle embryos in the field; water added to effervescent granules was used to generate CO_2. The incubator was employed in all stages of embryo production, including maturation, fertilization and embryo culture; embryo yield and quality were comparable to that in a conventional CO_2 incubator. In sheep, a report by Byrd *et al.* (1995) dealt with successful embryo development using a portable incubator in the absence of CO_2. Alberio *et al.* (1997) were also able to show that cattle embryo production was not affected by replacement of a CO_2 incubator by a simple sealed system. A paper by Palma

et al. (1998a) reported that IVP embryo production in a bag system without a gassed incubator did not adversely affect developmental capacity and viability of embryos; the authors concluded that environmental conditions in the bag (pH, temperature and humidity) during the various stages of embryo production were such as to give acceptable results.

In Denmark, Avery *et al.* (2000a,b,c) reported on their use of a 2.5 l Oxoid AnaeroJar (AG25) as an alternative and inexpensive culture system for *in vitro* cattle embryo production. The AG25 system is a simple, commercially available system for the growth of microorganisms; the authors concluded that the system could be substituted for expensive incubators and gas mixtures necessary for IVM, IVF and IVC. In Japan, Dong *et al.* (2001) showed that bovine embryo production results achieved in a simple portable CO_2 incubator were within the range of those obtained in an ordinary incubator; they concluded that the portable incubator could be successfully employed for embryo production under field conditions. In looking at methods of simplifying embryo production, it might be as well to remember that the top priority should be to devise effective and repeatable systems, whether complex or simple, that provide high-quality cattle embryos.

7.6. Evaluating Embryo Quality

A serious difficulty in the laboratory production of cattle embryos is that adverse effects may not be evident in the mature oocyte, in the early cleaving embryo or even at the time of embryo transfer. Those who view the effectiveness of their IVM/IVF systems in terms of fertilization and early cleavage rates ignore at their peril the possibility that their methods may not necessarily be capable of establishing viable pregnancies. It is important that careful attention be given to methods of accurately evaluating the normality and viability of embryos at the time of transfer or before cryopreservation. The methods of embryo evaluation used in conventional cattle embryo transfer are many and varied, including dye exclusion tests, measures of enzyme activity, glucose uptake and live/dead stains (see Overstrom, 1996; Van Soom and Boerjan, 2002). Most embryo assessment schemes depend on a subjective appraisal; many practitioners of cattle embryo transfer will be familiar with the guidelines laid down in the International Embryo Transfer Society (IETS) manual (see Table 7.18). Apart from the need for appropriate grading procedures, it is also important to monitor carefully the laboratory procedures used in embryo production; a paper by Riddell *et al.* (1997) noted that quality-control trials provide security for the laboratory and the client and serve as a useful tool in decision-making.

7.6.1. Morphological and morphometric parameters

The important feature of any embryo classification scheme is that it should be based on easily

Table 7.18. Embryo grading scheme (International Embryo Transfer Society Manual) (from Stringfellow and Seidel, 1998).

Code	Description
Code 1	Excellent or good. Symmetrical and spherical embryo mass with individual blastomeres (cells) that are uniform in size, colour, and density. This embryo is consistent with its expected stage of development. Irregularities should be relatively minor, and at least 85% of the cellular material should be an intact, viable embryonic mass. This judgement should be based on the percentage of embryonic cells represented by the extruded material in the perivitelline space. The zona pellucida should be smooth and have no concave or flat surfaces that might cause the embryo to adhere to a Petri dish or a straw
Code 2	Fair. Moderate irregularities in overall shape of the embryonic mass or in size, colour and density of individual cells. At least 50% of the cellular material should be an intact, viable embryonic mass
Code 3	Poor. Major irregularities in shape of the embryonic mass or in size, colour and density of individual cells. At least 25% of the cellular material should be an intact, viable embryonic mass
Code 4	Dead or degenerating. Degenerating embryos, oocytes or one-cell embryos: non-viable

recognizable morphological features and should be backed by reassuring evidence on pregnancy rates to be expected with each of the grades. In the years since embryo transfer has been used in commercial practice, many reports have appeared on methods of evaluating embryos recovered from superovulated donors; the most widely used method has been based on morphological and morphometric evaluation.

In the USA, Lindner and Wright (1983) described a classification scheme that has been widely used in commercial practice; they identified four categories based on morphological characteristics, namely, excellent, good, fair and poor; embryos with excellent and good qualities yielded the highest pregnancy rates, whereas poor embryos gave the lowest rates. The method involved the identification of various morphological features, including shape, colour and size of the perivitelline space, the number of extruded and degenerate cells and the number and nature of vesicles. Many different classification systems along similar lines have been reported during the past two decades. In Slovenia, for example, Mrkun and Kosec (2000) classified *in vivo*-produced cattle embryos according to morphological characteristics and developmental stages; the pregnancy rate after transfer of 'excellent' embryos was 68.6%, with 'fair' embryos it was 54.7% and with 'poor' embryos it was 14.3%.

Variability in embryo grading

Video recordings were used by De Leeuw (1996) in attempts to evaluate uniformity among laboratory personnel engaged in the grading of cattle embryos. A study by Farin *et al.* (1999) examined the degree of variation among evaluators when selecting cattle embryos for transfer based on morphology. Six evaluators graded day-7 embryos produced *in vivo* or *in vitro* (15 of each) by viewing video-recorded images of the embryos and classifying each for stage of development and quality. Pregnancy outcome was based on results at the facility of one evaluator, where the percentages of cattle pregnant after transfer of grade 1, 2 and 3 *in vivo* embryos were 76, 65 and 54, respectively, and 59, 45 and 30 for IVP embryos. Within most grades, the proportion of embryos selected for transfer differed significantly among the six evaluators. There was marked variation among evaluators in the proportion of cattle becoming pregnant, especially for IVP embryos. The pregnancy rates for grade 1, 2 or 3 *in vivo*-produced embryos were 66–76, 62–69 and 54–60%, respectively; the corresponding values for IVP embryos were 39–59, 15–45 and 24–32%, respectively. Almost twice as many transfers were required per pregnancy for IVP embryos as for those produced *in vivo*, regardless of the grade.

Ultrastructural features

The ultrastructure of cattle compact morulae produced *in vivo* or *in vitro* was quantified by Crosier *et al.* (1999, 2000b, 2001a) in North Carolina, using morphometric analysis. They showed that the volume density of lipid was significantly greater in compact morulae cultured in IVC media than in those from live animals. There was a significantly reduced proportional volume of total mitochondria in compact morulae cultured in TCM-199 + 10% OCS compared with those produced *in vivo*. The general conclusion was that IVP morulae differed ultrastructurally from those produced *in vivo*; the greatest deviations in cellular ultrastructure were evident in TCM-199 + 10% OCS. Information on the ultrastructural morphometry of cattle blastocysts produced *in vivo* and *in vitro* is given in Table 7.19.

A study by Abe *et al.* (2002a) in Japan examined the ultrastructural features of cattle morulae recovered from superovulated animals and classified by the scheme described by Lindner and Wright (1983). Junctional complexes between cells in the low-quality embryos were less developed than those in high-quality morulae. The high-quality morulae showed numerous microvilli projecting from the cells into the perivitelline space; in low-quality embryos, microvilli were poorly developed. It is known that microvilli are essential for fluid transport activity in the embryos; it is possible that morulae with poorly developed microvilli have a poor absorptive capacity. There were also other conspicuous ultrastructural differences between morulae of high and low quality, including evidence that fair- and poor-quality embryos contained nucleoli with low transcriptional activity, a large number of lipid droplets and immature mitochondria.

In Ireland, Rizos *et al.* (2002b) described ultrastructural differences between sheep and

Table 7.19. Volume density (%)* of cellular components in cattle blastocysts produced under different culture conditions (from Crosier *et al.*, 2001a).

	Treatment†			
Cellular component	MO	IVPS	IVPSR	mSOF
Cytoplasm	55 ± 4[a]	68 ± 4[ab]	72 ± 4[b]	61 ± 4[a]
Total mitochondria	8.7 ± 1.2[a]	4.3 ± 1.2[b]	4.6 ± 1.3[b]	3.8 ± 1.2[b]
Mature	2.7 ± 0.4	1.6 ± 0.4	2.0 ± 0.4	1.3 ± 0.4
Immature	3.8 ± 0.7[c]	1.9 ± 0.7[d]	1.8 ± 0.8[d]	1.7 ± 0.7[d]
Vacuolated	2.3 ± 0.5[c]	0.8 ± 0.5[d]	0.8 ± 0.6[d]	0.8 ± 0.5[d]
Nuclei	20 ± 2[a]	13 ± 2[b]	11 ± 2[b]	15 ± 2[ab]
Apoptotic bodies	0.5 ± 2.4[c]	7.4 ± 2.4[d]	5.6 ± 2.6[d]	7.4 ± 2.4[d]
Inclusion bodies	1.6 ± 0.5	0.4 ± 0.5	0.9 ± 0.5	0.5 ± 0.5
Debris within blastocoel	2.9 ± 1.0	0.2 ± 1.0	0.8 ± 1.1	0.4 ± 1.0
Blastocoel	23 ± 4	16 ± 4	16 ± 4	15 ± 4
Intercellular space	7 ± 3	7 ± 3	9 ± 3	10 ± 3
Cytoplasmic-to-nuclear ratio	5 ± 2[a]	11 ± 2[ab]	14 ± 3[b]	8 ± 2[ab]

*Values are least-squares means ± SEM, statistical model 1.
†n = 7, 7, 6 and 7 for MO, IVPS, IVPSR and mSOF, respectively. Within rows, values with different superscripts differ, [ab]$P < 0.05$; [cd]$P < 0.10$.
SEM, standard error of the mean; MO, embryos produced by multiple ovulations; IVPS, *in vitro* produced with serum; IVPSR *in vitro* produced with serum restriction.

cattle embryos produced *in vivo* and *in vitro*. They showed that, in comparison with their *in vivo* counterparts, IVP embryos were characterized by a lack of desmosomal junctions, a reduction in the microvilli population, an increase in the average number of lipid droplets and increased debris in the perivitelline space and intercellular cavities. Such differences were more marked in cattle IVP blastocysts, which also displayed electron-lucent mitochondria and large intercellular cavities. It is suggested that such differences may explain species differences in their tolerance of cryopreservation.

Human embryo quality considerations

In Germany, Ozornek *et al.* (1997) attempted to define an embryo scoring system to use with their human patients. They derived a 'total transferred embryo score' (TTES) from blastomere number, fragmentation and symmetry of the blastomere; they found the TTES to be a simple and useful method of estimating embryo quality. One of the morphological criteria used in selecting embryos for transfer is the absence of multinucleated blastomeres; such blastomeres are usually arrested cells, and embryos with one or more are expected to be developmentally incompetent. Multinuclearity occurs at the start of human embryo development and relatively frequently at the two-cell stage (Joris *et al.*, 1997). Other studies in Belgium evaluated the impact of transfer of multinucleated embryos on pregnancy rates (Pelinck *et al.*, 1998); these were found to be lower than those with mononucleated embryos. Wherever possible, embryos with multinucleated blastomeres should be avoided for transfers.

7.6.2. Age and developmental stage attained

Two factors are usually considered when visually evaluating the IVP cattle embryo under the light microscope: (i) its general appearance, in terms of features such as the presence of irregular or degenerate cells; and (ii) the stage of development of the embryo in relation to the time since fertilization (hpi). Time-lapse cinematography has been shown to be a useful tool in analysing the progress of embryos cultured *in vitro* by several groups (Grisart *et al.*, 1994).

Time of first cleavage

The time of the first cleavage after IVF is correlated directly with developmental potential, as

assessed by the developmental rates of cattle embryos to the compacted-morula, blastocyst and hatched blastocyst stages (Van Soom *et al.*, 1997a,b,c,d; Holm *et al.*, 1998a,b; Lonergan, 1999; Lonergan *et al.*, 1999a,b,c; Yoshioka *et al.*, 2000). In Ireland, as shown in Table 7.20, a close correlation was observed between the time interval from insemination to first cleavage and subsequent embryo developmental competence (Lonergan *et al.*, 1999a,b,c; Rieger *et al.*, 1999); later cleavage resulted in significantly reduced development to the blastocyst stage. A study by Dinnyes *et al.* (1999) compared the influence of the timing of first cleavage post-insemination on the *in vitro* survival of IVP cattle blastocysts following vitrification; they confirmed that early-cleaving embryos are more likely to develop to the blastocyst stage and showed that early cleavage had a positive effect on the cryopreservation of the embryos. The authors suggest that incorporating the timing of first cleavage in a non-invasive embryo quality evaluation system might improve the cryo-survival potential of the selected embryos. A study by Brevini-Gandolfi *et al.* (2000) suggested a correlation between the timing of first cleavage and polyadenylation levels of maternal mRNA.

Early cleavage and pregnancy rates in human assisted reproduction

Various workers have shown that early cleavage to the two-cell stage can indicate embryo viability and be a strong prognostic factor of the likelihood of pregnancy after IVF in humans (Sakkas *et al.*, 1998; Shoukir *et al.*, 1998); the simple act of selecting early-cleaving two-cell embryos may alleviate the problem of estimating which are the most advanced at the time of transfer. It appears that early cleavage is influenced by intrinsic factors within the human oocyte or embryo that promote cleavage after fertilization. In Columbia, Jimenez *et al.* (1999) evaluated the association between early cleavage and pregnancy rate; two-cell embryos at 28 hpi were associated with a 25% implantation rate, in contrast to a 10% value for zygotes uncleaved at that time. In Turkey, Demircan *et al.* (1999) similarly found that cleavage (at 27 h) was highly predictive of pregnancy and decisive in selecting embryos for transfer. The same group also showed that cell number at 48 hpi and the number of nucleolar precursor bodies were significantly higher in early-cleaving embryos (Demircan *et al.*, 2000).

A report by Fenwick *et al.* (2002) presented data showing that human patients who produced embryos that cleaved within 25 h of insemination yielded higher pregnancy and implantation rates than those that did not; as in cattle, early onset of first cleavage was associated with increased blastocyst formation. In view of its ease of application and its objectivity, early cleavage could possibly be of value in selecting embryos for transfer.

Assessing embryo quality at morula stage

Compaction is the first differentiation event in the bovine embryo and is crucial for subsequent development. In IVP embryos, the process of compaction is known to occur to a lesser degree than in morulae produced *in vivo* (Van Soom, 1996); some morulae do not even compact but proceed directly from the non-compacted stage to the blastocyst stage (see Fig. 7.12). A study by

Table 7.20. Time of first cleavage and bovine embryo development (from Rieger *et al.*, 1999).

Time of cleavage (hpi)	No. of fertilized oocytes	% of total	Blastocysts	
			No.	% of cleaved
27	350	41.7	204	58.3
30	200	23.8	89	44.5
33	76	9.1	23	30.3
36	26	3.1	5	19.2
42	51	6.1	6	11.8
NC	136	16.2	1	0.7
Total	839	100.0	328	39.1

hpi, hours post-insemination; NC, not cleaved.

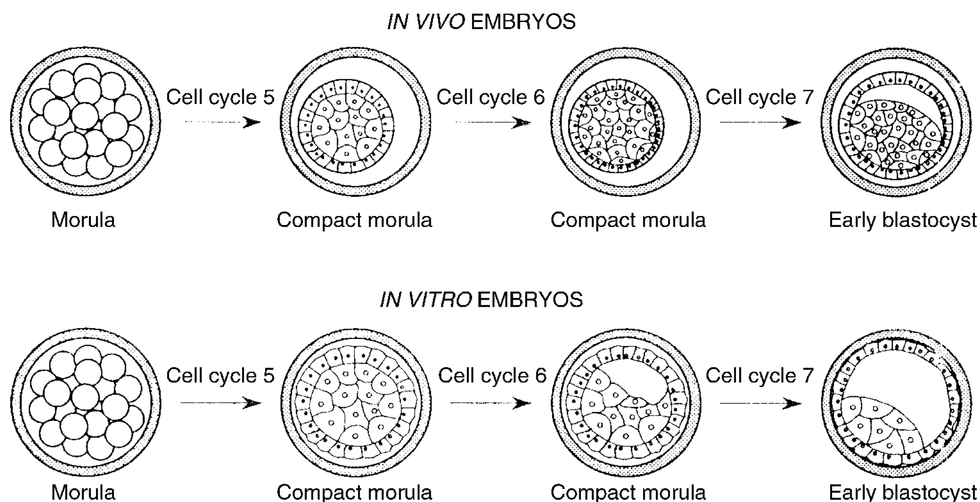


Fig. 7.12. Development of *in vivo* vs. *in vitro* bovine embryos. More pronounced compaction and later blastulation in the *in vivo*-produced embryos was associated with more reliable ICM cell numbers in comparison with *in vitro*-produced embryos. (From Van Soom, 1996.)

Lazzari *et al.* (2000a,b) indicated that most blastocysts that developed on day 7 were derived from cattle embryos at the compacted morula stage on day 5 or on day 6. In contrast to such results, about half of the blastocysts forming on day 8 were derived from embryos that did not compact before blastulation. The authors suggest that this may partly explain why blastocysts forming on day 8 are of lower quality than those forming on day 7 (Hasler *et al.*, 1995a,b; Van Soom *et al.*, 1997c). Evidence provided by Hue *et al.* (2000) may also be noted; this showed that IVP embryos developing to blastocysts on day 6 are more similar to *in vivo*-produced blastocysts than those that appear later in culture (days 7 and 8).

Timing of blastocyst formation

It is now clear that the expression of developmentally important gene transcripts can be used as a quality parameter of early-stage cattle embryos. It is also clear that faster-developing blastocysts are of higher quality than slower-developing blastocysts, based on cell number, end-stage development and pregnancy rate after transfer. A study by Knijn *et al.* (2001) presented convincing evidence that embryos reaching the blastocyst stage at day 7 differed in the expression of six developmentally important gene transcripts from blastocysts formed at day 8; the authors were tempted to suggest that such differences could be related to embryo quality. The authors further noted that the mRNA levels of day-7 blastocysts were not unlike those of *in vivo*-produced counterparts. Further work by Knijn *et al.* (2002a) produced evidence suggesting that, between 45 and 100 h after ovulation, the number of cells that will appear in the *in vivo*-derived blastocyst is defined; this coincides with the time when embryos migrate from the oviduct to the uterus.

7.6.3. Metabolic tests

Papers by Donnay *et al.* (1999a,b,c) discussed the development of a metabolic marker for embryo viability. The authors concluded that the best strategy is to develop several non-invasive assays, each measuring a specific cellular activity/process, with an individual embryo and, if possible, simultaneously. Similar views were expressed by Gardner (1999a,b) in a paper dealing with ways and means of assessing blastocyst viability (see Fig. 7.13). Further work on bovine embryo metabolism as a predictive marker of developmental potential was reported by Gopichandran and Leese (2002) in York;

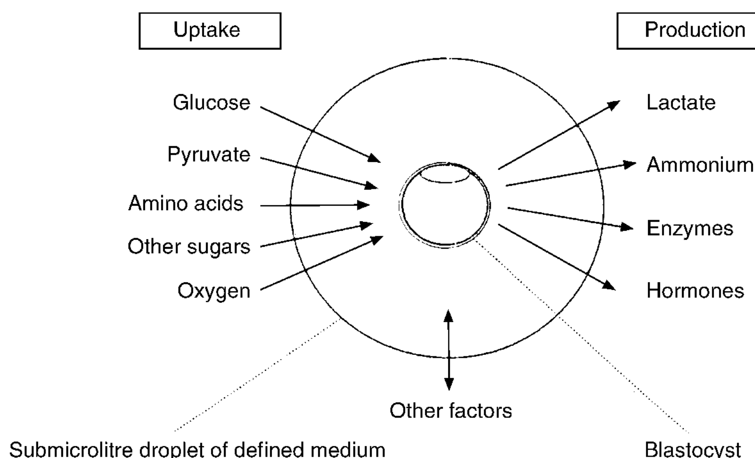


Fig. 7.13. Non-invasive assessment of blastocyst viability. Blastocysts are incubated individually in a known volume, for example 0.5 μl, of defined medium. Serial nanolitre samples can then be taken and analysed for carbohydrates, amino acids, ammonium, oxygen and enzymes. The concomitant measurement of glucose consumption and lactate production can give an indirect measure of glycolytic activity. The concomitant measurement of amino acid consumption and ammonium production can give an indirect measure of amino acid utilization. The release of enzymes, such as lactate dehydrogenase, into the surrounding culture medium reflects impairment in membrane integrity and, as such, may be useful in assessing freezing damage. (From Gardner, 1999b.)

cattle embryos cultured individually in 1 μl droplets of SOFaa/BSA medium were analysed ultramicrofluorometrically for pyruvate, glucose and lactate concentration. The workers suggest that their data may make it possible to select single, viable cattle embryos on the basis of their pyruvate consumption at the two-cell stage and grow them in groups to enable them to develop to the blastocyst stage.

7.6.4. Indications of embryo normality

Numerous reports that have appeared over the past decade have drawn attention to differences between IVP embryos and their *in vivo*-produced counterparts (see review by Holm and Callesen, 1998a,b; and research reports by Holm *et al.*, 1998a,b; Leibfried-Rutledge *et al.*, 1999; Hue *et al.*, 2000; Khurana and Niemann, 2000a,c).

Staining tests

An assessment of viability of cattle embryos produced *in vitro* by using trypan blue dye was made by Coelho *et al.* (1998a,b) in Brazil; they reported that blastocysts classified morphologically as quality 3 presented inferior survival rates (3-day culture period) to those classsified as quality 1 and 2.

Chromosomal abnormalities and cell numbers

A paper by Kawarsky *et al.* (1996) dealt with chromosomal abnormalities in bovine embryos and their influence on development. Elsewhere, workers reported on the incidence of bovine diploid oocytes matured *in vitro* and the incidence of polyploidy and mixoploidy in early bovine embryos (Lechniak, 1996; Lechniak *et al.*, 1996). Methods of chromosome preparation and the examination of such preparations for evidence of normality have been reported by Yoshizawa *et al.* (1998, 1999); the same group in Japan concluded that bovine chromosomal mosaicism (mixoploidy) in IVP embryos arose from polyspermy (Yoshizawa *et al.*, 1999). A study by Slimane *et al.* (2000) in which they assessed chromosomal abnormalities by fluorescence *in situ* hybridization (FISH) analysis in two-cell cattle embryos (*in vitro*- and *in vivo*-derived) led them to conclude that abnormalities occurred as early as first cleavage and could be mainly associated with the fertilization process

rather than with culture conditions. A study by Viuff *et al.* (2001) in France presented evidence on the frequency of chromosome abnormalities in 256 *in vivo*-developed cattle embryos, ranging from 2 to 5 days of age; results of interphase FISH-based chromosome analysis revealed that mixoploidy frequency increased from 5% on day 2 up to 31% on day 5 and that polyploidy was a rare phenomenon. In comparison with data reported in previous reports with IVP embryos (Viuff *et al.*, 1999a,b,c, 2000), the *in vivo*-developed embryos showed a significantly lower incidence of mixoploidy (16% vs. 25%) and polyploidy (1% vs. 6%).

Viuff *et al.* (2001) assessed the incidence of chromosomal abnormalities in IVP cattle embryos and those produced *in vivo*; a similar incidence was apparent in both categories. They concluded that chromosomal abnormalities may occur as early as the first cleavage and suggested that they might be associated with the fertilization process rather than with culture conditions. The study of Viuff (2001) also involved a precise count of cell numbers in the *in vivo* embryos; *in vivo* embryos contained significantly more cells at day 3, day 4 and day 5 than the IVP embryos studied in their earlier report. A study by Hyttel *et al.* (2001) reported that blastocysts produced *in vitro* display a significantly higher rate of mixoploidy, with the embryo consisting of both normal diploid and abnormal polyploid cells, than that found in embryos developed *in vivo*; they also showed that the rate of mixoploidy among embryos produced *in vitro* increases with increasing developmental stage. The same authors note that, after fertilization *in vitro*, initially there may be a high rate of 'true' polyploidy (all cells polyploid) but such embryos are eliminated before they cleave beyond the eight-cell stage, the stage at which major activation of the embryonic genome occurs in cattle.

Intercellular communication

A study by Boni *et al.* (1999a,b) in Italy aimed to identify differences between *in vitro*- and *in vivo*-derived cattle embryos in cell-to-cell communication; they recorded late and reduced expression of intercellular communication in the embryos produced *in vitro* and suggested that this may be partly responsible for their low developmental capacity. Compaction in the bovine embryo is believed to depend on the coordinated expression of a series of cytoskeletal and intercellular adhesion molecules; the localization of such molecules was studied by Baraldi-Scesi *et al.* (1999). They found that the distribution of these molecules in the bovine embryo showed a similar pattern to that already reported in mice and pig embryos.

Lipid droplets

A study by Abe *et al.* (1999) in Japan evaluated the quantitative fluctuation and the ultrastructure of cytoplasmic lipid droplets in IVP embryos cultured in serum-free or serum-supplemented media; embryos cultured in a serum-containing medium accumulated large amounts of cytoplasmic lipid, in contrast to those cultured without serum. On that basis, serum-free cultures could be a means of producing good-quality embryos for cryopreservation.

Interferon-tau secretion

Various workers have reported on the production of factors by bovine embryos in culture that may be indicative of their normality. A paper by Kubisch *et al.* (1998) dealt with the relationship between the age of IVP bovine blastocyst formation and the secretion of IFN-τ. In Germany, Stojkovic *et al.* (1999c) studied the secretion of IFN-τ in bovine embryos in long-term culture (up to day 23); they found that the trophoblastic area of *in vivo*-derived embryos was more than twice as large as that of IVP embryos. The authors suggested that such differences might be partly responsible for the differences in pregnancy rates after transfer to recipients. In the USA, Larson *et al.* (1999) sought to determine whether culture conditions affected IFN-τ production; they found that while hatching and cell number were affected by culture conditions, IFN-τ secretion by IVP-produced blastocysts in TCM-199 medium was similar to that in embryos in the live animal. Other studies in the same laboratory reported by Kubisch *et al.* (2001a,b) assessed the effects of genotype and various culture media on IFN-τ secretion by cattle blastocysts; they found that paternal genotype was a significant determinant of the embryo's ability to develop to the blastocyst stage and of IFN-τ secretion. In the UK, Mann

et al. (2002) presented results demonstrating a dramatic increase in IFN-τ during blastocyst elongation; this was due to increased embryo size rather than any change in the intensity of interferon production. It is also now apparent that female cattle blastocysts produce more than twice as much IFN-τ as males at the same stage of development, whether produced *in vitro* or *in vivo* (Kimura *et al.*, 2002); the significance of such a gender difference is not apparent.

Golgi apparatus

It is known that microtubule-associated membranes and proteins have an important role during the cell cycle during early embryo development and that organelles such as the Golgi apparatus exhibit dynamics that reflect the environment within cells. In Oregon, Payne and Schatten (2002) attempted to characterize the structure and dynamics of the Golgi apparatus during early embryonic development and the role they play in the development of the embryo to the blastocyst stage; they suggest that an intact Golgi apparatus is not essential for early development and may not be required by the embryo until it hatches from the zona pellucida and attaches itself to the endometrium.

Proliferating cell nuclear antigen

Studies reported by Markkula *et al.* (1999b, 2000, 2001) and Raty *et al.* (2000) demonstrated that the rate of cattle embryo development at the blastocyst stage was associated with the presence of proliferating cell nuclear antigen (PCNA), a component of the leading strand of DNA-polymerase delta; they suggest that the PCNA marker can be used for estimation of the developmental potential of IVP embryos.

Embryo cryosurvival

There have been many studies that have drawn attention to the poorer survival rate of IVP cattle embryos in comparison with those recovered from superovulated cattle or after *in vivo* culture. In Ireland, Enright *et al.* (2000a,b) showed that, after cryopreservation by freezing in 10% glycerol, VS3a vitrification or solid-surface vitrification, the survival of IVC cattle embryos was significantly lower than the survival of embryos cultured in the sheep oviduct or produced by superovulation of donors.

Proteins involved in embryo developmental competence

It is known that bovine oocytes store proteins and mRNA to support early embryo development; it is also clear that a large part of the stored mRNA disappears through degradation or translation. In Canada, Massicotte *et al.* (2002) compared protein synthesis in competent and incompetent bovine two-cell embryos to identify proteins involved in developmental competence; they found four proteins that were present in all highly competent embryos but absent in the incompetent embryos.

7.6.5. mRNA expression patterns

The investigation of gene expression patterns in various stages of development of bovine embryos is an important approach in gaining an understanding of the molecular mechanism of embryogenesis. Numerous reports have appeared during the past decade that may be consulted on mRNA expression in cattle embryos (Collins *et al.*, 1995; Renard *et al.*, 1995; Barcroft *et al.*, 1998; McDougall *et al.*, 1998; Natale *et al.*, 1999; Crosier *et al.*, 2000b; Prelle *et al.*, 2000a,b; Knijn *et al.*, 2002b; Mohan *et al.*, 2002; Robert *et al.*, 2002; Tesfaye *et al.*, 2002a,b). RT-PCR techniques, in which there have been recent advances, are among the tools used to detect and characterize mRNA transcripts that play an important role during early embryo development. According to Niemann and Wrenzycki (2000), the application of advanced molecular tools to bovine embryos opens up exciting new horizons for research; they also note that, because the rate of bovine embryo development is similar to that of humans, it may be that the bovine embryo will supplant the mouse as the new model for mammalian embryo development. The expression of sex-chromosome-linked genes during early embryo development in cattle was studied by Peippo *et al.* (2000a,b); they showed that mRNAs for three X-linked genes are present throughout development up to and including

the hatched blastocyst stage, either as maternal- or as embryo-derived transcripts. It is well documented that the sex chromosome composition of the cattle embryo has an influence on its early development.

In terms of *in vitro* embryo production, a review by Natale *et al.* (2001) noted that the embryo may attempt to respond to a suboptimal culture environment by adjusting its gene expression pattern; by investigating such responses, it may be possible to design improved culture media. A study by Wrenzycki *et al.* (1998a,b,c) with IVP cattle embryos showed that alterations in mRNA expression are associated with the culture environment. In a further report, Wrenzycki *et al.* (2000a,b) investigated the effects of two culture media (TCM-199 vs. SOF) supplemented with different protein sources (bovine serum vs. BSA) on the relative abundance of ten specific gene transcripts. The authors detected significant differences between *in vivo*- and *in vitro*-derived embryos, especially at the morula stage. Culture medium was found to have a profound effect on the transcriptional activity of cattle embryos, whereas the effect of protein source was less pronounced. Although SOF provided an environment more similar to that found *in vivo*, differences between SOF- and *in vivo*-derived embryos were evident and further work is needed to optimize the culture system.

In other studies in Germany, Schmoll *et al.* (2000) investigated the level of transcription of genes important for early embryonic development; they showed that the amount of Na^+–K^+-ATPase (α_1) and IFN-τ transcipts showed a positive correlation to zona pellucida diameter in embryos produced *in vitro* but not in those produced *in vivo*. The expression of specific imprinted genes is required for normal early embryonic development, with abnormal expression leading to aberrant growth of the fetus and placental membranes. As noted by Ruddock *et al.* (2002), it is necessary to determine the expression patterns of these genes during the normal development of the bovine embryo so that they can be used for comparison purposes with IVP embryos produced by different protocols or after various manipulations, such as nuclear transfer.

In Ireland, studies have analysed differential mRNA expression in cattle blastocysts produced under different conditions of culture known to result in blastocysts of different quality, as shown by their survival after cryopreservation (Gutierrez-Adan *et al.*, 2002; Rizos *et al.*, 2002b). Results demonstrated clear differences between the relative abundance of some developmentally important gene transcripts at the blastocyst stage which may explain the differential quality of such embryos. It remains to be seen how such information may be used to improve the yield and quality of IVP cattle embryos; the challenge is to modify the culture conditions *in vitro* to induce the expression of genes as shown in embryos produced in the live cow.

Other work in Ireland has attempted to identify differentially expressed genes, using suppressive subtractive hybridization, in early- and late-cleaving cattle embryos (Fair *et al.*, 2002); maternal RNA from competent and incompetent two-cell embryos was characterized and genes that are differentially expressed were identified. As noted earlier, early-cleaving two-cell embryos are more likely to develop to the blastocyst stage than their later-cleaving counterparts.

In Germany, Rief *et al.* (2002a.b) investigated the effect of co-culture with oviductal cells on embryonic expression of three developmentally important genes; the differences they recorded in the levels of expression of these genes between embryos co-cultured in this way and standard IVP embryos (cultured in SOF + 5% OCS + 1%NEAA + 2%EAA) emphasized the profound effect of culture system on the transcriptional activity of cattle embryos. Elsewhere in that country, using IVP cattle embryos, Ponsuksili *et al.* (2002a) sought to identify genes that are differentially expressed during the development of morulae to the blastocyst stage; they did this by application of suppression subtractive hybridization to detect stage-specific expression-sequence tags. They concluded that such methods can be employed with cattle embryos of different ages to elucidate stage-specific gene activity. A further study by Ponsuksili *et al.* (2002b) demonstrated the feasibility of constructing representative complementary DNA (cDNA) expression libraries from single embryos, thereby providing the means of elucidating the expression profiles of genes during early embryonic development in cattle.

Until the eight- to 16-cell stage, the bovine embryo is maintained by the mRNA and proteins that had been stored in the oocyte; new transcription is believed to start at the eight- to 16-cell

stage, although some minor transcription has been reported from as early as the one- to two-cell stage (see Section 7.4.3). It is known that the maternal-to-zygotic transition requires several factors to remodel the chromatin and start the transcription machinery; the regulating transcription factors are believed to be present in the oocyte as mRNA or are expressed early in development to enhance the maternal-to-zygotic transition. In Canada, Vigneault *et al.* (2002) attempted to quantify five transcription factors and one translation factor expressed in early embryo development (cultured in SOF medium); they concluded that important transcription factors display different expression patterns, suggesting their possible role in the zygotic activation. In Germany, Tesfaye *et al.* (2002a,b) applied the mRNA differential-display RT-PCR method to characterize the mRNA expression patterns in IVP cattle embryos (eight-cell to blastocyst stage) and to generate embryonic expressed-sequence tags, which can be used in the identification of active genes responsible for early development. They presented findings that indicated potential target genes for future research.

At compaction, the bovine embryo's cells start to differentiate into two cell types, the ICM cells and the TE; the ICM cells give rise to the embryo and the TE forms the majority of the extraembryonic membranes. During compaction and blastocyst formation, changes in the utilization of energy substrates occur, with glucose becoming the predominant nutritive source. The expression pattern of genes associated with the differentiation process of the two cell types was examined in blastocysts produced *in vitro* (SOF + OCS medium) by Wrenzycki *et al.* (2002); they investigated the relative abundance of gene transcripts involved in compaction and cavitation and in glucose transport. Their results indicated that gene expression becomes restricted to one of the two cell layers of the IVP bovine blastocyst; it remains to be seen whether IVC may alter the spatial expression pattern of genes.

Embryos under stress

The culture of the bovine embryo *in vitro* exposes it to various stresses that are not normally encountered within the cow's reproductive tract. Even with the most efficient culture systems, it is believed that a proportion of embryos show changes in the expression of genes that reflect a stress response to suboptimal conditions. In Aberdeen, Fontanier-Razzaq *et al.* (2001) presented results showing that there are changes in gene expression in cattle IVP blastocysts in response to DNA-damaging agents; it appears that an increase in gadd153 mRNA is a useful marker of DNA damage and metabolic stress in bovine embryos.

7.6.6. Post-hatching evaluation

Hatching of the bovine blastocyst can be expected to occur 8–10 days after ovulation and fertilization in the cow (see Fig. 7.14). Various workers have examined cattle embryos several days or even weeks after hatching. A paper by Galli *et al.* (1997b) demonstrated that elongated bovine blastocysts can be obtained from embryos cultured *in vivo* from day 2 to day 7 in the sheep oviduct and from day 7 to day 14–16 in the cow uterus. Farin *et al.* (1999) sought to determine the effect of production method (*in vivo* or *in vitro*) on the development of conceptuses at day 17 (10 days after transfer to recipient cattle); they recorded that conceptuses derived from embryos produced in a serum or a serum-restricted environment were longer at 17 days than those produced *in vivo*. Studies reported by Hue *et al.* (2000, 2002) involved the transfer of IVP blastocysts from days 6, 7 or 8 of culture to recipient cows and recovering them at day 18; evidence was found that blastocysts developing on day 6 of IVC developed in the cow in a way more similar to *in vivo*-derived embryos than did blastocysts that appeared later in culture (days 7 and 8). The authors suggest that their approach provided a new basis for analysing the developmental potential of IVP embryos and could also be used in evaluating embryos derived from somatic nuclear transfer.

Work elsewhere has examined the possibility of long-term embryo culture in the laboratory. Vajta *et al.* (2000c) reported attempts to establish an IVC system capable of supporting cattle embryos to a more advanced stage of development; they saw such a system as being of value in evaluating the developmental potential of cattle embryos without this involving transfer to recipient animals. Although growth, elongation

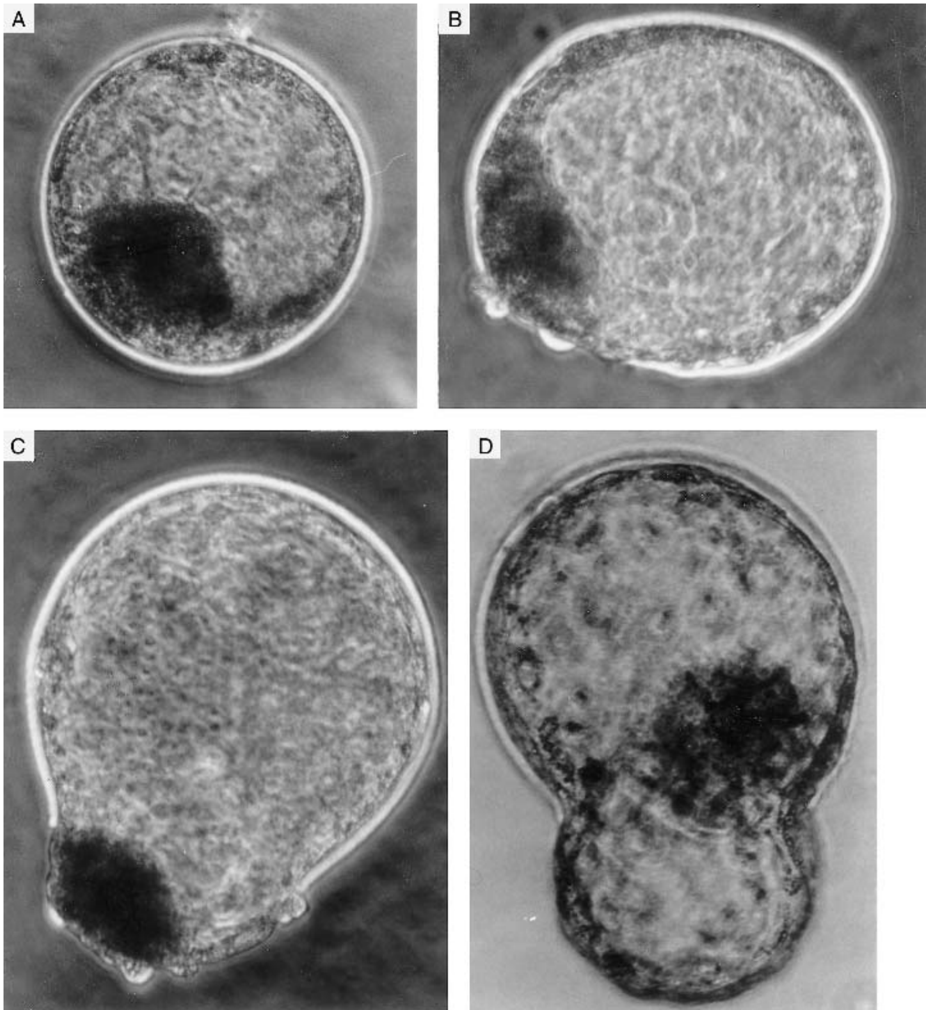


Fig. 7.14. Stages in the hatching of the bovine blastocyst. Hatching usually occurs when the bovine blastocyst has reached the 8- to 10-day stage of development. In this instance, the blastocyst has reached the expanded stage within the zona pellucida in (A); the subsequent photographs (B–D) show stages in the hatching process. All photographs to the same scale.

and ICM differentiation could be induced in embryos during more than 3 weeks of culture on agar and collagen gels, they concluded that further studies to optimize the system were necessary.

Other workers have attempted to define milestones of bovine post-hatching embryonic development in embryos produced *in vivo* for comparison with IVP counterparts. A paper by Hyttel *et al.* (2002) dealt with embryos collected from superovulated cattle on day 14 or day 21 and with IVP hatched blastocysts cultured on a collagen matrix in SOFaaci. At day 14, developing embryos were ovoid to filamentous and displayed a complete hypoblast lining of the TE; at 21 days, embryos ranged from those presenting a primitive streak to those showing a neural tube, up to 14 somites and allantois development. The IVP embryos remained spherical and, up to day 11, half of the embryos displayed hypoblast formation; from day 12, the ICM had degenerated in all embryos. Under the conditions of long-term culture employed, only hypoblast formation could be achieved.

7.6.7. Post-transfer evaluation

A matter of considerable concern to those engaged in the laboratory production of cattle embryos is the high incidence of early fetal loss recorded in some reports dealing with the transfer of IVP embryos. In New Zealand, workers have identified, in recipients of IVP embryos produced in a SOF-based system, that a major cause of fetal loss in the first trimester of pregnancy is malformation of allantoic development (see Thompson and Peterson, 2000); growth and differentiation of the other extraembryonic membranes (amnion, yolk-sac and trophoblast) appeared to be normal. In the USA, Farin *et al.* (2000) examined the gross and histological morphology of placentas at day 222 of gestation, using embryos from superovulated animals or produced *in vitro* in serum or serum-restricted culture systems; they found that placentas from *in vitro* embryos exhibited distinct alterations in both gross and histological characteristics compared with those from *in vivo* embryos. The factors responsible for allantoic pathology remain unknown and an association between this defect and the other malformation of major concern, fetal oversize, has not been established.

Insulin-like growth factors

A study by Blondin *et al.* (2000) reported that the IVP of cattle embryos altered the levels of IGF-II mRNA in bovine fetuses examined 63 days after transfer; they compared fetuses derived from cultured embryos (using TCM-199 medium supplemented either with 10% OCS or with 1% BSA) with fetuses produced from *in vivo* embryos.

Heavy calves

There are a number of reviews and reports that report average calf birth weight and duration of pregnancy to be greater when using IVP cattle embryos than when using *in vivo* embryos (Kruip and Den Daas, 1997; Stacchezzini *et al.*, 1997; Numabe *et al.*, 1999, 2000a,b). In the Netherlands, Merton *et al.* (1998) dealt with 2228 IVP embryos transferred to recipients on 120 farms; average birth weight was 47 kg (range 20–78 kg); it appeared that the higher the embryo viability (a higher grade and unfrozen), the lower the birth weight of the IVP calves. In Ireland, among various factors influencing the commercial acceptability of IVM/IVF-derived embryos in the late 1980s was a low incidence (< 2%; 13/644 single/twin births) of what appeared to be fetal oversize; some singleton calves weighed 70 kg and more. Apart from large birth weights, subsequent growth and health appeared to be normal. In looking at the possible origin of the problem that occurred in the 2% overweight calves, it should be noted that these animals were derived from cattle embryos that had been cultured *in vivo* (ligated sheep oviduct) from the zygote to the blastocyst stage. It has also to be noted that in subsequent studies in Ireland involving IVP cattle embryos from a granulosa cell co-culture system, Bourke *et al.* (1995) reported on the outcome of 469 calvings in which young were born, apparently without the occurrence of fetal oversize.

In the context of the Irish 1980s results, the possibility was not dismissed that the origin of fetal overweight may have been due to the genetics of the embryos (Continental sires and Continental-cross-bred oocyte donors) rather than to some unusual epigenetic effects. It was remembered that the introduction of the Charollais to dairy cattle breeding in the UK in the 1960s had been followed by alarmist reports about large calves and difficult calvings. By the 1990s, the bulls used in embryo production possessed a proven easy-calving record. There is the possibility that antigenic dissimilarity of the embryo and foster-mother may be a factor in explaining some instances of heavy calves. There is evidence that calf birth-weight depression may follow inbreeding and that this is associated with markedly lighter placental weights; it has also been noted that larger placenta and placental areas in cattle have been observed with increasing genetic dissimilarity. There is also the possibility that part of the birth-weight problem stems from the preponderance of bull calves that have been recorded in some reports after transfer of IVP embryos (Reinders *et al.*, 1995).

IVP and nuclear-transfer cattle embryos

There is probably a case for making a clear distinction between calves produced by standard IVM/IVF/IVC techniques and those derived from nuclear transfer and nuclear reprogramming. It

has been evident for more than a decade that cloning technology is associated with a proportion of calves that are heavier than normal (Wilson *et al.*, 1995) and to animals showing evidence of defects in metabolic regulation (Adams *et al.*, 1994; Garry *et al.*, 1996). Nuclear transfer has been clearly associated with abnormally low pregnancy rates, which stem from substantial embryo and fetal losses occurring in the first trimester of pregnancy. It has to be kept in mind that nuclear transfer for the production of cattle clones relies on the reprogramming of a donor nucleus so that it behaves in the same way as a zygotic nucleus; such reprogramming constitutes an essential element in the initiation of normal embryonic development. However, there is evidence to suggest that nucleus reprogramming does not direct embryogenesis in a manner identical to that of normal bovine embryos; studies by Smith *et al.* (1996) have shown that the gene expression pattern for a number of growth factors differed for blastocysts developed from cloned embryos in comparison with IVP and *in vivo*-produced embryos. It would not be unexpected to find a higher incidence of anomalies in gene expression occurring with nuclear transfer than with normal culture procedures.

8

Preservation of Embryos and Oocytes

8.1. Introduction

The aim of oocyte and embryo storage *in vitro* is to preserve the organism in a state of suspended animation, from which it can be revived after a short or long period to continue its normal development. It is believed that spermatozoa and embryonic cells can probably remain viable at a temperature of −196°C (liquid nitrogen) for perhaps 1000 years or more, the only source of damage at such a temperature being direct ionization from background radiation. In normal commercial practice, however, there is little need to think beyond storage for more than a limited number of months or years. There is no reason to believe that the usual length of cryopreservation of cattle embryos in liquid nitrogen should be a factor affecting their viability after thawing. There may, on the other hand, be good practical reasons for attempting the relatively short-term storage (days) of cattle embryos, at refrigerator temperatures; the duration of such storage may well be an important consideration.

Quite apart from the practical implications of storing cattle embryos for future use, it is likely that, in research, cryopreservation techniques have an important part to play in assessing the quality of *in vitro*-produced (IVP) cattle embryos; differences that may not be evident between fresh embryos produced *in vivo* and *in vitro* may become all too apparent when the same embryos are exposed to cooling and freezing. In talking about improvements in the cryopreservation of cattle embryos, regardless of their origin, it is worth noting the considerable expense of carrying out field trials to confirm the effectiveness of a new cryopreservation protocol. A review by Hasler (2002) has noted that it would be necessary to transfer more than 1700 embryos to have a 90% certainty of detecting a 5% true difference in the pregnancy rate of two freezing methods.

8.1.1. Embryo cryopreservation: past and present

The freezing of mammalian embryos was first shown to be possible in 1971, when David Whittingham and colleagues in London obtained live mice pups after the transfer of frozen–thawed embryos that had been frozen using either glycerol or dimethyl sulphoxide (DMSO). Some of the milestones in the freeze–thawing of mammalian embryos are detailed in Table 8.1. The frozen storage of cattle embryos has been part of the commercial farming scene since the mid-1970s and, in humans, the first births from frozen embryos were reported in the early 1980s. It should be mentioned, in passing, that, 20 years earlier, the application of artificial insemination (AI) in dairy cattle had been greatly facilitated by the development of effective sperm-freezing procedures by Chris Polge and co-workers at Cambridge; this was a milestone development in farming practice around the world.

In the early days of cattle embryo transfer (ET), it was evident that effective exploitation of

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Table 8.1. Milestones in the birth of young after transfer of frozen–thawed embryos.

Year	Species	Researcher(s)
1971	Mouse	Whittingham *et al.*
1973	Cow	Wilmut and Rowson
1974	Rabbit	Bank and Maurer
1974	Sheep	Willadsen
1975	Rat	Whittingham
1976	Goat	Bilton and Moore
1982	Horse	Yamamoto *et al.*
1984	Human	Zeilmaker *et al.*
1985	Hamster	Ridha and Dukelow
1988	Cat	Dresser *et al.*
1989	Pig	Hayashi *et al.*
1989	Rhesus monkey	Wolf *et al.*

genetically valuable embryos would depend on the development of effective techniques for their storage at low temperatures. The first calf born after transfer of a frozen embryo in the early 1970s at Cambridge (Frosty II) did much to stimulate research into finding simple and reliable methods to facilitate commercial applications. In the course of the past three decades, countless thousands of cattle embryos have been frozen and thawed for transfer in countries around the world and a considerable literature on cryopreservation techniques has been built up (see reviews by Dobrinsky, 1996, 2002; Palasz and Mapletoft, 1996; Kaidi *et al.*, 1997, 1999a,b; Massip, 1999, 2001; Rall, 2001; Leibo and Songsasen, 2002). The pregnancy results that can be anticipated with frozen–thawed cattle embryos obtained by way of conventional superovulation techniques are well established; the general view is that pregnancy rates using high-quality embryos should be no more than about 10% below those found with fresh embryos.

Cambridge workers were at the forefront in developing effective techniques for the freezing of cattle embryos, the work of Chris Polge, Ian Wilmut and Steen Willadsen being particularly important. It was found that slow freezing (0.3°C/min) of cattle embryos to low subzero temperatures (–80°C) required slow thawing; slow freezing of cattle embryos to relatively high subzero temperatures (–25 to –35°C), on the other hand, required rapid thawing (360°C/min). Such findings, initially demonstrated by Willadsen at Cambridge in the freezing of sheep embryos, subsequently formed the basis of the cryopreservation technique that was to be widely used in commercial practice; since 1977, the method has been the standard technique in freezing the embryos of many species other than cattle, including human embryos. Embryos are exposed to cryopreservative at +20°C, cooled to –7°C and seeded to induce ice formation; they are then cooled at about 0.3 to 0.5°C/min to –30 to –35°C, before being plunged into liquid nitrogen.

In vitro*-produced embryos*

Although frozen–thawed IVP blastocysts have resulted in the birth of many thousands of normal healthy calves throughout the world by commercial transfer, it is well established that such embryos are much more sensitive to freezing than *in vivo*-derived embryos, especially at stages earlier than the blastocyst. Pregnancy rates using IVP embryos have been inconsistent and generally lower than those reported for *in vivo*-derived embryos, which can be related to a number of well-documented differences at the morphological, ultrastructural, metabolic, biochemical and genomic level between the two categories of embryos (Pollard and Leibo, 1994; Massip *et al.*, 1995a,b; Holm and Callesen, 1998a,b). There are clear indications that sensitivity to chilling of cattle embryos depends on their developmental stage and on culture conditions, as well as on the presence of numerous lipid droplets in the cytoplasm in the IVP embryos. Although pregnancy rates with fresh IVP blastocysts can be of the order of 50–60% (Reinders *et al.*, 1995), such percentages may be halved with frozen–thawed embryos. Similar evidence was reported in sheep; Tervit *et al.* (1994) showed that the freezability of IVP sheep embryos was markedly below that of their *in vivo*-produced counterparts.

It has to be said that the freezability problems currently experienced with IVP embryos were not entirely anticipated in some laboratories. In large-scale field trials in Ireland in the late 1980s, embryos cultured in the sheep oviduct gave rise to pregnancy rates comparable to those achieved with embryos from superovulated donors; this is also the well-recorded experiences of workers in Italy (Galli *et al.*, 1995; Galli and Lazzari, 1995, 1996).

Vitrification

The most significant steps in the cryopreservation of cattle embryos in more recent times include the ability to freeze and transfer embryos in straws without dilution and the development of the open-pulled-straw (OPS) method for efficient vitrification of embryos and oocytes (Vajta *et al.*, 1997a,b,c,d,e,f,g,h, 1998a,b,c,d). Although vitrification appeared on the scene of embryo cryopreservation in the mid-1980s as an alternative to traditional slow-rate freezing of cattle embryos (see Table 8.2), its possible advantages (simplicity, speed, low cost) had little impact on commercial cattle ET operations and its application was generally confined to research studies. In vitrification, ice-crystal formation is prevented by using high concentrations of cryoprotectants and high cooling and warming rates.

Table 8.2. Milestones in vitrification of mammalian embryos

Year	Species	Researcher(s)
1985	Mouse	Rall and Fahy
1986	Cow	Massip *et al.*
1986	Hamster	Critser *et al.*
1988	Rat	Kono *et al.*
1989	Rabbit	Smorag *et al.*
1990	Sheep/goat	Scieve *et al.*
1994	Horse	Hochi *et al.*
1998	Pig	Kobayashi *et al.*

Vitrification techniques, using the standard French mini-straw (0.25 ml capacity) to contain the embryos, limit the maximum cooling rate to about −2000°C/min. A recent innovative vitrification procedure, on the other hand, the OPS method, enables a much faster cooling and warming rate (> 20,000°C/min); this involves the bovine embryo being loaded into a mini-straw previously heat-pulled to half the diameter and thickness of the wall. The OPS vitrification method is regarded as a convenient, simple and effective way of cryopreserving 7–9-day-old cattle blastocysts (Vajta *et al.*, 1997a, 1998a). The same method appears to be particularly effective in the cryopreservation of bovine oocytes, which may have particular relevance in certain fields of embryo-based biotechnology (cloning, transgenesis).

Quite apart from using vitrification as a method of embryo preservation, the technique may be valuable in investigating quality factors involved in IVP cattle embryo production. In Ireland, Lonergan *et al.* (2002) used vitrification to study the effect of different postfertilization (day 1 to day 7) culture methods on the ability of blastocysts to survive freezing. As shown in Table 8.3, *in vivo*-produced blastocysts or those cultured in the sheep oviduct had the highest survival rates; the embryos that had spent the longest period in synthetic oviductal fluid (SOF) culture had the lowest survival rate. The data suggested that certain windows of embryo development result in greater ability to survive cryopreservation than others.

Table 8.3. Development and survival of vitrified bovine embryos produced under different culture conditions (from Lonergan *et al.*, 2002).

Treatment	*N*	% cleaved	Blastocyst yield[a] Day 7	Blastocyst yield[a] Day 8	% survival 24 h after vitrification/ warming[a]
SOF 6 days	354	82.5	26.5[ab]	30.2[a]	32.3[b]
SOF 2 days/ewe 4 days	400	82.2	3.8[c]	8.2[b]	100[a]
SOF 4 days/ewe 2 days	334	79.9	0[d]	0[c]	–
Ewe 2 days/SOF 4 days	400	78.6	20.2[a]	22.6[d]	6.7[c]
Ewe 4 days/SOF 2 days	400	–	31.0[b]	32.5[a]	40.6[b]
Ewe 6 days	400	–	24.2[ab]	28.9[ad]	100[a]
In vivo		–	–	–	100[a]
Significance		NS	$P < 0.05$	$P < 0.05$	$P < 0.05$

NS, not significant.
[a]Values with different letters within a column are significantly different.

Advances in other farm mammals

The greatest progress in long-term storage in recent years has been in the development of pig embryo cryopreservation (Nagashima *et al.*, 1994a,b, 1995a,b). It had long been speculated that intracellular lipid was responsible for the extreme sensitivity of the pig embryo to chilling and low temperatures; the development of delipation technology was to provide conclusive evidence that intracellular lipid was linked to hypothermic sensitivity. The possible relevance of this to the problems experienced in the freezing of IVP cattle embryos is not difficult to see. It has been a characteristic of *in vitro*-cultured (IVC) cattle embryos that there is an excess of lipid material and they have shown a similar response to the pig embryo when delipation techniques have been employed. In the bovine embryo, in contrast to the pig, and indeed the horse, the lipid material constitutes an abnormality, mainly derived from the culture method employed and its possible effect on mitochondrial activity.

8.1.2. Advantages of embryo storage

The development of embryo freezing methods had a major impact on cattle breeding worldwide. It became possible to ship suitably screened embryos to far-distant continents and to follow breeding programmes that hitherto had not been possible. On the farm itself, valuable genetic resources were saved by virtue of the ET practitioner no longer having to depend on gathering sufficient recipient cattle to utilize every embryo recovered on a particular day.

8.2. Storing the Fresh Embryo

During the period in which cattle ET has been in operation commercially, relatively few studies have been devoted to the short-term storage of embryos at temperatures of zero to +10°C. However, for several good practical reasons, the ability to store embryos at temperatures above freezing for relatively short periods (1–2 days) may occasionally be preferable to freezing or vitrification. There are those who observe that the disease risk with frozen embryos may be somewhat greater than with fresh embryos; an intact zona pellucida affords protection against pathogens and this membrane may occasionally be cracked during the freeze–thaw process. It must also be noted that the markedly reduced viability of IVP cattle embryos after cryopreservation has been a serious factor limiting the commercial applicability of this new form of cattle embryo production.

8.2.1. Embryo storage at ambient temperature

As part of normal cattle ET operations, there is an obvious need for short-term storage of embryos recovered from donor cattle until such time as they are either transferred to a recipient or stored by cryopreservation. The general view is that the viability of the bovine embryo, usually at the blastocyst stage, begins to decline after 12 h storage at normal room temperature (20–30°C) in an appropriate medium. It is believed that temperature may be of greater concern if embryos are to be cryopreserved after being held for an extended period; there are also those who hold that cattle embryos should be maintained at refrigerator temperature prior to freezing, although there appears to be no clear evidence in support of this view (Mapletoft and Stookey, 1998). The choice of media for embryo collection and temporary storage has ranged from complex media such as tissue-culture medium 199 (TCM-199) and Ham's F-10 to much simpler formulations such as Dulbecco's phosphate-buffered medium + bovine serum (D-PBS). A study reported by Schneider *et al.* (1998) in Germany showed that IVP cattle embryos could be effectively stored for at least 10 h in phosphate-buffered medium + bovine serum (PBS) or bicarbonate-free TCM-199, both supplemented with 0.4% bovine serum albumin (BSA); the authors noted that storage in culture medium was less successful, probably because the medium was unable to maintain pH.

For those operating under farm conditions, the requirement may simply be for PBS containing antibiotics and 2% fetal calf serum (FCS) for

embryo collection and PBS with 10–20% FCS for temporary storage prior to transfer. As to storage conditions, room temperature (20–30°C) is usually found to be satisfactory; where embryos are held for any length of time, the culture medium should be changed occasionally. In the USA, Nelson and Nelson (2001) surveyed culture media used by 26 ET companies for the recovery, holding and freezing of embryos flushed from superovulated donors (see Table 8.4); they noted that the most widely utilized alternatives to D-PBS were two commercially available media (Emcare, zwitterion-buffered, IPC, Auckland, New Zealand and ViGro Plus, HEPES-buffered, AbTechnology, Pullman, Washington State).

8.2.2. Embryo sensitivity to cooling

As with sperm, mammalian embryos experience a cessation of metabolism when maintained *in vitro* below body temperatures; studies by Sreenan in Ireland more than 30 years ago were among the first to show that it was possible to store cattle embryos on a short-term basis at 10°C for 24 h or longer and that such embryos were able to resume normal development on transfer to the rabbit oviduct. Work at Cambridge in the mid-1970s revealed a clear relationship between embryo developmental stage and its ability to survive chilling and freezing. It was shown by Chris Polge and associates that eight-cell cattle embryos failed to survive cooling to 0°C, whereas sheep embryos of the same stage all survived. In other comparisons, it was found that eight- to 16-cell cattle embryos failed to survive cooling to 0°C whereas all late morulae survived; it was shown that bovine morulae survived cooling to 7.5°C, 5°C and as low as 0°C for 24 h and it became clear that blastocysts were even more resistant to cooling. In the USA, in the early 1980s, it was commercially possible to chill bovine blastocysts to 0°C, ship them many miles on an airliner and transfer them 12–24 h later with an acceptable pregnancy rate.

Table 8.4. Media employed by 26 commercial cattle ET companies in the USA (from Nelson and Nelson, 2001).

Number of companies	Procedure		
	Flush	Holding	Freezing
2	A	A	A
2	A	B	B
1	A	A	B
2	A	C	C
1	A	B + C	C
1	B	A	A
4	B	B	B
5	C	C	C
1	B + C	B + C	B + C
1	C	C	D
3	B	C	C
1	B + C	A + C	C
1	C	C	B
1	B	B + C	B + C

A, D-PBS, B, EMCARE™, C, ViGro™, D, other.

IVP cattle embryos

Many reports have dealt with the chilling sensivity of IVP cattle embryos (see Pollard and Leibo, 1994; Balasubramanian *et al*., 1998). Comparative studies of *in vitro*-derived vs. *in vivo*-derived cattle embryos have shown the relevance of work on chilling sensitivity to embryo cryopreservation; it appears that bovine *in vivo* morulae are resistant to chilling but IVP morulae are not (see Table 8.5). It is clear that the sensitivity to chilling is not inherent in that embryonic stage, but is due to the manner in which the embryo was produced. Cattle embryos from superovulated donors can be frozen by slow cooling as effectively in propylene glycol or in ethylene glycol (EG) as in glycerol.

Chilling sensitivity of cattle embryos in their early development stage was studied by Arav *et al*. (1999) in Israel, who evaluated differences in lipid-phase transition. They found that IVP embryos at the first three cleavage stages (two-, four- and eight-cell stage) presented similar thermal responses, whereas the blastocyst stage did not; the authors suggest that such differences between early-stage embryos and blastocyst might be due to embryonic gene expression. It was noted that lipid phase transition in early embryos was similar to that previously described for bovine oocytes at the germinal vesicle (GV) stage; the transition was between 20 and 10°C and centred around 15°C.

Table 8.5. Development of cattle embryos chilled at different cleavage stages (from Balasubramanian *et al.*, 1998).

Stage	Group	Embryos	% Hatched	Norm % Surv	Cells/blast. at 192 h
Two-cell	Control	77	28.6[a]	100	–
	Chilled	77	0[b]	0	–
Four-cell	Control	59	32.2[a]	100	–
	Chilled	59	0[b]	0	–
Eight-cell	Control	54	33.3[a]	100	160.8 ± 7.0[a]
	Chilled	54	9.3[b]	27.9	119.4 ± 3.3[b]
Morula	Control	73	34.2[a]	100	156.9 ± 7.8[a]
	Chilled	73	16.4[c]	48.0	125.3 ± 4.6[b]
Blastocyst	Control	71	83.1[d]	100	191.8 ± 2.2[c]
	Chilled	71	56.3[e]	67.7	188.1 ± 3.3[c]

[a–e]Values with different letters within a column are significantly different ($P < 0.05$).
Blast., blastocysts.

8.2.3. Embryo storage at refrigerator temperature

A practical alternative to facilitate transport may be to arrest development by chilling. It is known that cysteamine treatment of bovine oocytes during *in vitro* maturation (IVM), which increases intracellular glutathione (GSH) levels, can enhance the ability of embryos to survive chilling (De Matos *et al.*, 1995, 1996a,b). A report by Balasubramanian *et al.* (2000) dealt with the effect of cysteamine supplementation of IVM cattle oocytes on chilling sensitivity and embryo development. In the study, IVP embryos were chilled to 0°C for 30 min at various stages of development; there was a high survival rate of blastocysts after such chilling and the authors suggested that cysteamine supplementation offered promise as a viable alternative to freezing for commercial applications requiring short-term storage to facilitate transport of IVP cattle embryos.

Such a routine is not uncommon in horse ET applications. According to Squires *et al.* (1999), one of the major improvements in horse ET in recent times has been the ability to store embryos at 5°C and ship them to a centralized station for transfer into recipient mares; embryos can be collected by practitioners on the farm, cooled to 5°C in a cooling unit and stored for a short time without a major decrease in fertility (see Table 8.6). The majority of embryos transferred at Colorado State University are shipped to that station from breeders and veterinarians all over the country. In France, Moussa *et al.* (2002) used commercially available media (Emcare Holding solution; ViGro Holding Plus) as alternatives to Ham's F-10 medium to store horse embryos at 5°C in an Equitainer unit for 24 h. Elsewhere, short-term storage of horse embryos has been attempted, but at ambient rather than refrigerator temperature. Fleury *et al.* (2002) in Brazil reported on the storage of equine embryos in HEPES-buffered Ham's F-10 medium at 15–18°C for periods of up to 18 h without evidence of an adverse effect on pregnancy rate; it is suggested that such methods may enable specialized recipient farms to become an effective option for owners or veterinarians that do not have their own recipients.

Table 8.6. Pregnancy rates (%) after transfer of cooled and transported horse embryos (from Squires *et al.*, 1999).

		Cooled/shipped	
Year	Fresh	Cooled, shipped counter to counter	Cooled, shipped overnight
1995	89/127 (70.0)	38/64 (59.4)	27/52 (51.9)
1996	58/86 (67.4)	45/70 (64.3)	18/27 (66.7)
1997	36/59 (61.0)	29/43 (67.4)	45/72 (62.5)
1998	49/70 (70.0)	24/38 (63.2)	45/79 (57.0)
Overall	232/342 (67.8)	136/215 (63.2)	135/230 (58.7)

Short-term storage (24 h) of pig embryos may occasionally be required for transportation in connection with ET and breeding programmes; pig embryos, however, are sensitive to

cooling and it is necessary to store them at much higher temperatures. In the Netherlands, Rubio *et al.* (2002) tested different storage temperatures and media for holding embryos; they recorded TCM-199 and 38°C as being the best combination of medium and temperature and that day-5 embryos were preferable to day-4 embryos (see Table 8.7).

8.3. Conventional Freeze–Thaw Protocols

Improvements in various areas of cattle ET technology, including cryopreservation, are continually being made in research laboratories and by commercial concerns that are carrying out transfers on a regular basis (see Hasler, 2002). The principles of cryopreservation are believed to be the same for all living cells, the most important consideration being the removal of most of the water from cells before they freeze intracellularly. Research in freeze–thaw procedures has included numerous studies dealing with the type and concentration of cryoprotectant, cooling and freezing rates, seeding and plunging temperatures as well as techniques to ensure cryoprotectant removal.

8.3.1. Cryoprotectants

The literature reveals many different methods for the cryopreservation of embryos that have been developed by researchers around the world. Cryoprotectants generally fall into two categories, intracellular and extracellular. Of the intracellular cryoprotectants, which are of low molecular weight, glycerol and DMSO were initially those most commonly used in freezing cattle embryos; these agents permeate all cells of the embryo. There are species differences in the permeability characteristics of embryos and it is known that cattle embryos are more permeable to glycerol than to DMSO, which was the cryoprotectant used in the early 1970s to produce Frosty II; towards the end of the 1970s, glycerol emerged as a superior cryoprotectant for the freezing of compact morulae and blastocysts. In the most recent decade, EG has emerged as a particularly useful cryoprotectant for dealing with cattle embryos, used at concentrations of 1.5–1.8 M.

The extracellular cryoprotectants are larger molecules, such as sugars and proteins (e.g. sucrose, hyaluronic acid, BSA); the way in which such molecules provide cryoprotection is not well understood. It is thought that a molecule such as BSA protects the embryo in the immediate post-thaw phase by helping to stabilize the cell membranes. Among the more novel forms of extracellular cryoprotectants to appear in the recent decade are the antifreeze glycoproteins extracted from Antarctic fish.

According to Isachenko *et al.* (1994a,b), a cryoprotectant with less permeability (e.g. glycerol) has a primary protective action on cytoplasmic membranes whereas a more permeable agent (for example, EG) protects all the membranes of intracellular structures, including lysosomes as the most hydrated structures of the cell. Where lysosome destruction occurs in the freezing process, lytic enzymes are released, with adverse effects on cell structures and the breakdown of blastomeres.

Table 8.7. Evaluation of pig embryos stored at different temperatures and in different media. TCM-199 and 38 °C were the best combination of medium and temperature. (From Rubio *et al.*, 2002.)

Groups	*n*	Embryos with ≥ 1 damaged cells or nuclei (%)	No. of cells per embryo (mean ± SD)	Dead cells (%) (mean ± SD)
0 h – Control day 4	37	3	42 ± 18	3 ± 0.0
0 h – Control day 5	23	0	60 ± 23	0 ± 0.0
24 h – all day 4	196	35	47 ± 24	9 ± 0.2
24 h – all day 5	156	22	70 ± 27	3 ± 0.2
day 4-E-25 °C	23	65	34 ± 18	31 ± 0.4
day 5-T-38 °C	15	0	50 ± 14	0 ± 0.0

SD, standard deviation.

8.3.2. Two-step to one-step temperature decrease

A couple of decades ago would have found many cattle ET operators adding the cryoprotectant DMSO to the medium containing embryos in six steps of increasing concentration and using a two-step control of temperature to provide a fall, first at 0.3°C and then 0.1°C/min; such freezing protocols occupied 2 h or more from start to finish. An improvement came with the use of glycerol (1.4 M) as the cryoprotectant and the finding that there was no evident damage to cattle blastocysts when they were equilibrated directly in the glycerol at the final concentration. Satisfactory results have been achieved when the embryos are seeded at −6°C, then cooled in a one-step freezing curve at a rate between 0.3 and 0.6°C/min (further details in Table 8.8).

8.3.3. Straws for storage

The introduction of the 0.25 ml plastic insemination straw as a freezing container allowed for easy storage of the bovine embryo; although the 0.5 ml straw is generally used in semen storage in the USA, ET practitioners use the smaller size in embryo cryopreservation. The advantages of the straw over the previous containers (usually 1 ml glass ampoules) included better exposure of the embryo and medium to the cooling effect of liquid nitrogen and the ease of storing large numbers of straws in nitrogen storage units. The embryo is usually loaded into the straw using a 1 ml syringe with an adaptor that ensures a tight seal with the end of the straw; the various columns of medium drawn into the straw are separated by air bubbles during the loading process (see Fig. 8.1). The loaded straw is sealed by heat, by polyvinyl powder or by means of a plastic sealing device. As with any device that comes in contact with the embryo, the straw has been subjected to stringent quality tests by its manufacturer to ensure its non-toxicity. In more recent years, a new 0.25 ml straw (CBS) produced by IMV Technologies in Aigle and marketed around the world has been investigated and reported on by Lavergne *et al.* (2000) in France; they incubated bovine zygotes in straws for periods at least three times greater than that required in the conventional freeze–thawing process. The authors confirmed the absence of toxicity of the straws and concluded that they should have no deleterious effects on the survival of embryos.

Table 8.8. Freezing protocol for bovine embryos using glycerol as cryoprotectant (from Hasler, 2002).

1. Equilibrate embryos in 10% glycerol for 10–20 min
2. Load embryos in 0.25 ml straws: seal and label straws (embryos can be loaded while they are equilibrating)
3. Place straws in freezer that is maintaining a holding temperature of −6°C
4. Seed straws and maintain at −6°C for 15 min
5. Decrease temperature at 0.5°C/min to −32°C.
6. Plunge straws into liquid nitrogen

8.3.4. One-step thawing procedures

A major problem in the early years of cattle embryo freezing was the requirement for the stepwise removal of cryoprotectant from the thawed embryo; the multistep dilution procedure was designed to expose the embryo to decreasing concentrations of the protective agent, which usually called for a microscope and a minimum of 1–2 h work under laboratory-like conditions. In the early 1980s, however, alternatives to the multistep thaw procedure were described and tested, notably by Stan Leibo and colleagues in North America, Renard and co-workers in France and Massip and associates in Belgium. In one alternative, the straw was filled with hypertonic sucrose as a diluent as well as with cryoprotectant containing the embryo, the two solutions separated from each other by an air bubble (see Fig. 8.2); after thawing, the two fractions were mixed by shaking the straw before transfer. The embryo was then incubated within the straw for an appropriate period (2–20 min) at a controlled temperature (20–37°C) before being transferred to the waiting recipient animal. An even simpler alternative was to incorporate sucrose in the freezing medium (1.36 M glycerol in PBS) at a

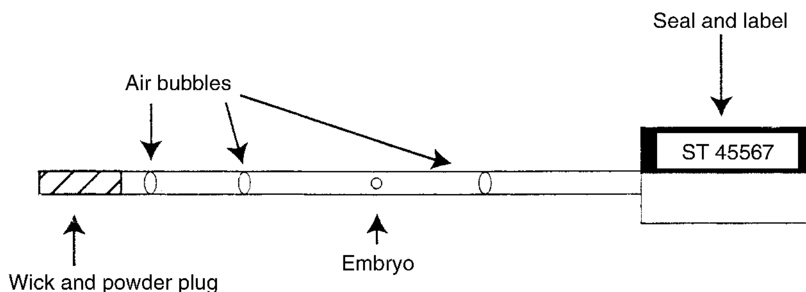

Fig. 8.1. The plastic straw as an embryo-freezing container. Diagram of a plastic straw loaded with an embryo and columns of either glycerol or ethylene glycol cryoprotectant solution separated by air bubbles. (From Hasler, 2002.)

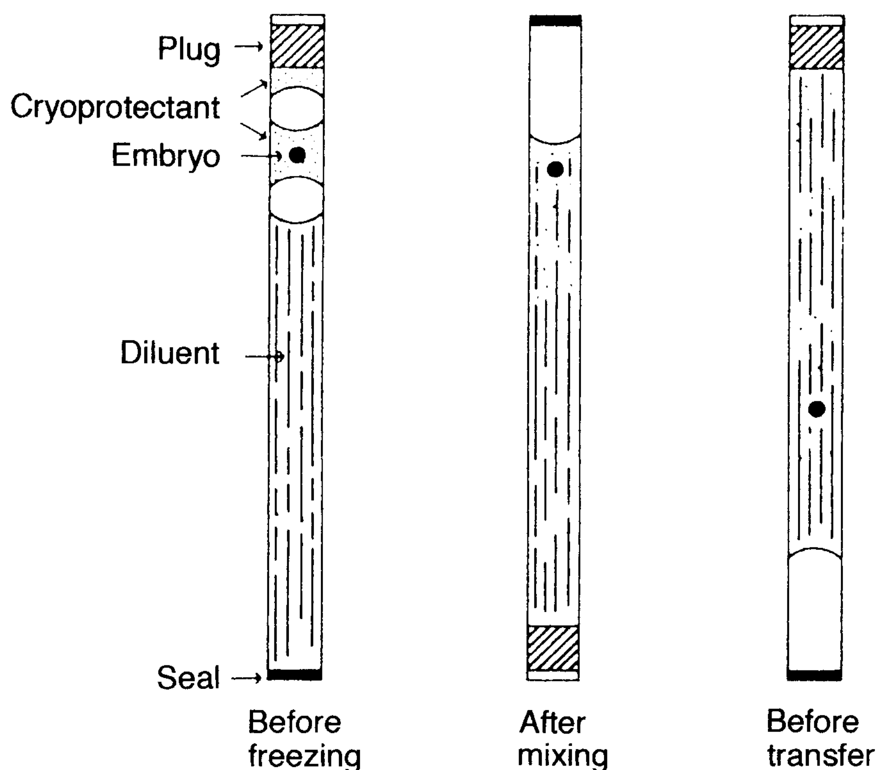

Fig. 8.2. One-step thawing of an embryo within a straw. Illustrated is the one-step *in situ* thawing method. Before freezing and after thawing, the embryo in its cryoprotectant solution is isolated from the diluent by an air bubble. The bovine embryo is mixed with the diluent by shaking the straw upside down towards the plug, producing a single column of fluid. The embryo is then allowed to respond osmotically to the diluent prior to transfer.

concentration of 0.25 M; the fact that embryos were prehydrated by the mixture prior to cooling meant that slow cooling could be terminated at −25°C rather than at lower subzero temperatures.

For those working in the field, the one-step thawing procedure clearly offered important advantages, particularly those in countries where no laboratory facilities were available for embryo handling. Direct transfer of the embryo

from the storage straw eliminated the need for trained embryologists to be present at the time of ET; trained inseminators could readily perform the transfers on the farm. This was to bring ET on the farm almost to the same level of technical complexity as that involved in the insemination of frozen–thawed semen. It was now possible to think in terms of do-it-yourself (DIY) ET in cattle, after appropriate training and certification, in the way that DIY AI has been applied on the farm for many years. According to a review by Jainudeen *et al.* (2000), however, Leibo's glycerol + sucrose method was not widely adopted by the ET industry because pregnancy rates were lower than with conventional thawing procedures.

8.3.5. Ethylene glycol as the cryoprotectant

During the past decade, EG has been effectively employed as a cryoprotectant for cattle embryo preservation. The molecular weight of this agent (62.1) is lower than that of glycerol (92.1), propylene glycol (76.1) and DMSO (78.1) and it is probable that its beneficial effect is partly due to its high permeability; the fact that it permeates the embryo rapidly also eliminates the need for the stepwise dilution of the cryoprotectant at the time of thawing. Its first use was by Japanese workers in the late 1970s in protecting rat and mice embryos against freezing damage. A decade ago, Voelkel and Hu (1992) reported acceptable pregnancy rates after the transfer of cattle embryos frozen in EG and transferred after thawing directly from the straws. In Canada, McIntosh and Hazeleger (1994) adopted EG as the cryoprotectant for routine operations in their commercial cattle ET operations on the basis of results achieved. In Macedonia, Kocoski *et al.* (1994a,b) reported results of a trial on the direct transfer method using 10% EG as the cryoprotectant; they concluded that the method could be widely used in the field as a reliable and promising method for expansion of the ET industry.

In the Netherlands, Aarts *et al.* (1994) recorded similar pregnancy rates for embryos frozen in 1.5 M EG and in glycerol; embryos were directly rehydrated after EG in contrast to the time-consuming three-step removal procedure used with glycerol. In Japan, Dochi *et al.* (1995) reported satisfactory pregnancy rates and the birth of calves after the direct transfer of thawed cattle embryos preserved with EG. In Germany, Bracke and Niemann (1995) employed 1.5 M EG and achieved satisfactory pregnancy rates after transferring embryos directly to recipients; the same authors suggested that higher concentrations of EG (3.6 M) might be appropriate for the freezing of IVP embryos. A report by Galli *et al.* (1995) in Italy presented data on cattle embryos transferred directly after freezing in 1.5 M EG; pregnancy rates were comparable to those found after stepwise rehydration of embryos frozen in 10% glycerol. In the UK, May (1996) described the procedures for the thawing and transfer of embryos frozen in straws in EG; the author suggested that the direct transfer of an embryo may be a useful technique when dealing with repeat-breeder cattle. A paper by Otter *et al.* (1998) dealt with pregnancy rates of bovine embryos frozen in 1.5 M EG, making a comparison between direct transfer and transfer after one-step dilution. In a survey of the cattle ET industry in North America, Leibo and Mapletoft (1998) reported the growing popularity of EG and direct transfers, recording that, in 1997, 55.4% of frozen–thawed embryos transferred in the USA and 87.6% of those in Canada were frozen using EG. A review of literature by Hasler (2002) presented a comparison between EG and glycerol in several reports (Table 8.9).

In Europe, a review by Bracke and Niemann (1995) reported pregnancy rates between 50 and 70% in cattle receiving embryos that had been frozen with EG as the cryoprotectant. In France, a study by Nibart and Humblot (1997) presented the results of field trials in which 6085 day-7 embryos were employed; the pregnancy rate with EG as the protectant (50.5%) was somewhat greater than when glycerol + sucrose was employed (48.5%). In other work in France, Ponsart *et al.* (2000) dealt with 2134 transfers

Table 8.9. Pregnancy rates after transfer of embryos frozen in glycerol or ethylene glycol (from Hasler, 2002).

Glycerol		Ethylene glycol	
No. Cattle	% Pregnancy	No. Cattle	% Pregnancy
3428	55.4	12,944	58.8

$P > 0.05$.

in which EG was compared with glycerol + sucrose; they recorded a pregnancy rate of 55.4% with EG and 47.2% with the glycerol + sucrose combination. The authors concluded that the EG method had improved the success of transfer. There have been ET operators who have combined sucrose (0.1–0.25 M) with EG. Those who have carried out comparisons (Hasler *et al*., 1997) and conducted surveys of practitioners (Leibo and Mapletoft, 1998) have not found any benefit from the combination treatment. In a paper from China, Bai *et al*. (2000) reported a high survival rate and hatching rate after freezing embryos in 1.8 M EG + 0.05 M sucrose.

The preparation of straws using EG as the cryoprotectant is described by Hasler (2002). For equilibration, the embryos can be placed directly in the final EG concentration of 1.5 M at room temperature and straws loaded during the equilibration period; holding the prefreezing EG exposure time to less than 10 min has been widely practised in commercial ET. The author noted that some of the reported problems with EG may have resulted from equilibration under high-temperature summer conditions on the farm; the cryoprotectant may be more toxic to embryos during extended exposure at higher than normal ambient temperatures.

Prefreezing additives

A paper by Otoi *et al*. (2000) reported on the developmental competence of frozen–thawed blastocysts that had been cultured for 24 h before freezing with media containing beta-mercaptoethanol (β-ME) (100 μM concentration); the inclusion of β-ME improved the development of blastocysts but not the pregnancy rate after transfer to recipients. The use of agarose in the freezing of biopsied and sexed cattle embryos was the subject of a paper by Dupras and Laurence (2000); they concluded that exposure to a 1% solution of agarose before freezing in medium containing 1.5 M EG + 0.1 M sucrose resulted in pregnancy rates no different from those with intact embryos.

Ultrastructural studies

In Brazil, Visintin *et al*. (2002a,b) described studies of the ultrastructure of *in vivo*-produced cattle embryos (compact morulae/early blastocysts) from Friesian and Nellore (zebu) cattle in which they examined fresh morphology as well as that after exposure to EG and after cryopreservation; they concluded that the use of EG provided a non-toxic method for rapid and controlled freezing of such embryos. In sheep, Cocero *et al*. (2002) found that EG protected membrane and cytoplasmic structures of embryonic cells from cryo-injury much better than glycerol; they concluded that lack of success in the freeze–thawing of sheep embryos could be attributed to the lack of protection of inner cells. The Spanish authors provided morphological and ultrastructural evidence of dramatic alterations in organelles and cell components in the less accessible areas of the sheep embryo.

Demi-embryos

A paper by Lange (1995) dealt with the cryopreservation of cattle embryos and demi-embryos using EG for direct transfer after thawing; the author concluded that the direct transfer of demi-embryos is feasible in a commercial ET programme. In Finland, a study by Bredbacka *et al*. (1996) reported the birth of calves after non-surgical transfer of fresh and refrigerated demi-embryos.

Trophoblastic vesicles

There are practical reasons for storing bovine trophoblastic vesicles (bTVs); some suggest that they may be the means of enhancing pregnancy rate in cattle after AI or ET. A study reported by Lester *et al*. (1994) indicated that EG was the least toxic of the cryoprotectants evaluated and that cryopreservation in 1.5 M EG resulted in bTVs of higher quality than when other agents (glycerol, propylene glycol, DMSO) were employed.

8.3.6. Thawing and cryoprotectant removal

Rapid thawing of the straw is necessary for optimal survival of the bovine embryo, regardless of whether it has been frozen with glycerol or EG as the cryoprotectant. As described by Hasler (2002), a typical protocol involves thawing straws for 5–10 s in air, followed by submersion in water at 25–30°C until the ice

is completely melted. The same author notes evidence that completely thawing in air results in reduced embryo viability, whereas thawing directly in water results in a higher incidence of cracked zonae pellucidae.

For embryos frozen in glycerol, removal of the cryoprotectant is necessary before transfer; for those frozen in EG, the agent is diluted out of the embryo within the uterus of the recipient animal. In the early days of freezing, glycerol was diluted out in several steps, each step involving a small decrease in glycerol concentration. Using glycerol in combination with sucrose eliminated the need for multiple concentrations of the cryoprotectant; sucrose prevented osmotic swelling as glycerol was being replaced by water.

8.4. Freezing the IVP Bovine Embryo

The viability of the IVP embryo after freeze–thawing or other means of cryopreservation is of crucial importance to the practical exploitation of this technology. Commercial requirements are for a cryopreserved IVP embryo that can be thawed by a one-step procedure on the farm with a guaranteed survival rate approaching as closely as possible to 100%. It is also highly desirable for direct transfer to the recipient to be carried out immediately after thawing rather than after a delay.

8.4.1. Morphological and functional differences

There are now numerous reports that provide direct comparisons of the morphological and functional characteristics that distinguish IVP from *in vivo*-produced embryos (Holm and Callesen, 1998a,b). In many instances, such comparisons have revealed clear and measurable differences in the morphology and dimensions of the inner cell mass (ICM) (Rexroad *et al.*, 1995), in the nature of the compaction process (Carolan *et al.*, 2000), in the lower metabolic activity of embryonic cells (Partridge *et al.*, 1994), in the sensitivity of morulae to reduced temperature (Pollard and Leibo, 1994) and in embryo survival after conventional freeze–thawing procedures. Measurable differences have been demonstrated in the permeability of blastomeres and in the susceptibility of the zona pellucida to digestion by pronase. Many investigators have commented on the darkness of blastomeres, giving the IVP embryo a characteristic ‘sunburnt’ appearance.

In Ireland, Rizos *et al.* (2002a) examined the effect of bovine oocyte maturation, fertilization or culture *in vivo* or *in vitro* on the proportion of oocytes reaching the blastocyst stage and on blastocyst quality as measured by survival after vitrification; they recorded a dramatic effect on blastocyst quality, with those blastocysts produced by *in vivo* culture surviving vitrification at significantly higher rates than their IVC counterparts. The authors note that their results indicate that the intrinsic quality of the oocyte is the main factor affecting blastocyst yields, whereas the conditions of embryo culture have an important role in determining blastocyst quality. As evident in results reported by Lonergan *et al.* (2002), embryo culture as currently practised is an important element influencing the survival of IVP cattle embryos after vitrification.

Embryo ultrastructure after cryopreservation

A report by Fair *et al.* (2001a) described the ultrastructure of blastocysts derived by *in vivo* and *in vitro* methods and to investigate how the morphology was affected by exposure to cryoprotectant (10% glycerol) or cryopreservation by conventional slow freezing; it was evident that *in vivo*-derived blastocysts possessed structural characteristics that conferred a greater tolerance to the cooling and freezing process. The authors noted that the characteristics of IVM/*in vitro*-fertilized (IVF) embryos cultured in the sheep oviduct were similar to those of embryos from superovulated donors, although they displayed a wider perivitelline space and appeared to possess less stacked microvilli. The totally IVP blastocysts displayed a wider perivitelline space, no stacking of microvilli, increased numbers of lipid droplets and a reduction in the junctional contacts between trophoblastic cells.

8.4.2. Embryo survival and pregnancy rates

The first pregnancy in cattle from a frozen–thawed embryo was reported some three

decades ago. Several hundred papers dealing with various aspects of embryo cryobiology have appeared since that time and cattle ET practitioners are well accustomed to the results they may expect with frozen *in vivo*-produced embryos. The general view would be that pregnancy rates using high-quality freeze–thawed embryos should not be more than about 10% below that found with fresh embryos.

In many studies reported with IVP cattle embryos, however, there is often evidence of a marked deterioration in morphological quality and a much reduced viability after cryopreservation when compared with *in vivo* embryos (Den Daas and Merton, 1994; Mahmoudzadeh *et al.*, 1994a,b; Reinders *et al.*, 1995; Ohboshi *et al.*, 1997; Lee, E.S. *et al.*, 1998); similar evidence has come from those working with sheep (Tervit *et al.*, 1994). Differential fluorochrome staining was used by Iwasaki *et al.* (1994) to evaluate the effect of freezing treatments (glycerol + sucrose) on the ICM of IVP embryos at the morula/blastocyst stage; their one-step freezing technique resulted in a higher proportion of dead ICM cells compared with the three-step method. They pointed to the need for work to clarify the cause of the damage to the ICM cells.

The freezability problems experienced by many with cattle IVP embryos were not entirely anticipated on the basis of early studies in Ireland carried out by Lu and associates. During 1988, the Irish commercial company, Ovamass, carried out large-scale field trials in the Republic and in Northern Ireland in which experienced AI technicians transferred IVM/IVF embryos non-surgically to more than 1000 recipient cattle in 58 herds. These embryos were produced by IVM (TCM-199 + oestrous cow serum (OCS)) and fertilization (Tyrode/albumin/sodium lactate/sodium pyruvate (TALP) medium) with subsequent culture in the sheep oviduct; frozen embryos yielded results little different from those commonly experienced with *in vivo*-produced embryos (pregnancy rates of 50.3% for 803 fresh ETs vs. 43.1% for 308 frozen ETs). Such embryos had been exposed to an appropriate oviductal environment from 20 h after IVF until the compacted morula/blastocyst stage. With the elimination, under commercial pressure, of the sheep oviduct as the early embryo culture system and the substitution of somatic-cell co-culture systems came the realization that the embryo's ability to survive the freezing processing had deteriorated markedly; this difference in viability between IVC and *in vivo* culture has been borne out in many experiments in Ireland.

Elsewhere, there were those who demonstrated that pregnancy rates after transfer of fresh IVP cattle embryos were of the order of 50–60% (Den Daas and Merton, 1994) but that these percentages were halved with frozen–thawed embryos. In studies reported by Reinders *et al.* (1995), the pregnancy rate with frozen *in vivo* embryos was 87% of that achieved with fresh; with IVP embryos, the corresponding figure was 33%. Such evidence made it all too clear that there were specific defects in the IVP embryo that seriously compromised its ability to tolerate low-temperature conditions. Many reports have been published on freezing with either glycerol or EG as the cryoprotectant.

Glycerol

The effect of the stage of IVP embryo development and embryo quality on survival after freezing, using glycerol as the cryoprotectant, has been reported in several papers (Han, H.Y. *et al.*, 1994; Han, Y.M. *et al.*, 1994; Carvalho *et al.*, 1995). In Denmark, Holm *et al.* (1994a,b) noted that cryopreservation with 10% glycerol revealed differences in IVP cattle embryo quality that were not evident in simple morphological evaluation at day 7 after IVF. In Germany, with glycerol as the cryoprotectant, Boxhammer *et al.* (1998) evaluated the survival rate of IVP embryos that had been cultured with insulin-like growth factor I (IGF-I) or its analogues in chemically defined medium; expanded blastocysts cultured with IGF-I–peptides tended to have a better survival rate than controls. After freezing in 10% glycerol, a study in Ireland showed the survival of IVC embryos to be significantly lower than the survival of embryos cultured in the sheep oviduct or those produced *in vivo* (Enright *et al.*, 1999, 2000b); the authors noted that survival after cryopreservation could be a useful indicator of embryo quality.

Ethylene glycol

Data presented by Cseh *et al.* (1995) indicated that age and time of blastocyst formation were

important factors in the freezing of IVP embryos, using 1.8 M EG as the cryoprotectant; they concluded that the most appropriate age and developmental stage for freezing were the day-7/8 blastocysts. A study by Rodrigues *et al.* (1995a,b) demonstrated a greater effectiveness when a concentration of 3.6 M EG was used to protect IVP embryos rather than the conventional 1.5 M concentration. In Sweden, factors affecting the survival of IVP embryos after freezing with either glycerol or EG were studied by Gustafsson *et al.* (2001); they demonstrated that day-7 blastocysts were three times more likely to survive freeze–thawing than day-8/9 blastocysts. In Brazil, a study by Pereira *et al.* (2002) compared glycerol and EG in conventional freezing (two-step for glycerol; one-step for EG); they concluded, on the basis of significantly different pregnancy rates, that the EG one-step procedure was the more useful for freezing IVP embryos at the expanded blastocyst stage.

Prefreezing additives

Various attempts have been made to improve the survival of IVP embryos after cryopreservation by modifications of their culture conditions. A paper by Imai *et al.* (1997) reported that the addition of an unsaturated fatty acid, linoleic acid, as bound to BSA (linoleic acid-albumin LAA), to their medium for culturing IVM/IVF cattle zygotes improved the survival of blastocysts after conventional freezing; they found that LAA improved post-thaw survival of blastocysts from 44% to 72%. Further studies in the same area were reported by Hochi *et al.* (1999), who demonstrated that up to 76% of frozen–thawed bovine morulae survived cryopreservation when presumptive zygotes were cultured in the presence of 0.1% LAA; the authors suggested that LAA may improve membrane stability. Elsewhere, it was shown by Okazaki *et al.* (1997) that LAA added to the freezing medium had no effect on embryo survival; it seemed probable that LAA altered the quality of cell membranes during IVC but not during the freezing process. In Japan, Tominaga *et al.* (2000a) studied the effect of adding LAA to Charles Rosenkrans 1 with amino acids (CR1aa) IVC medium for 4 days after insemination; the developmental rate of 16-cell embryos to the blastocyst stage was significantly enhanced by the addition. In New Zealand, Pugh *et al.* (1998) showed that the protein composition of the freezing medium could significantly affect embryo survival after freezing and that the survival of late morulae could be improved by additional BSA (10 mg/ml).

The objective of a study by Guyader-Joly *et al.* (1999) was to test the view that phospholipids with the potential for lowering membrane-phase transition temperatures could minimize membrane damage and thereby enhance cattle embryo development after thawing; they found that addition of lecithin did not improve embryo survival and speculated that its efficacy may have been reduced by the negative effects of spontaneous lipid peroxidation.

Freezing zygotes and early-cleavage embryos

A report by Pugh and Tervit (2000) in New Zealand presented results confirming the low tolerance of bovine zygotes to chilling and showing that their resistance to chilling may be improved by exposing them to certain polyamines (putrescine and spermine) prior to and during cooling. It is known that some polyamines are ubiquitous in cells and are involved in many cellular functions, such as cleavage and differentiation; in the present instance, it was thought that the polyamines provided some protection for the pronuclei.

8.4.3. Delipidizing the embryo

The opaque appearance of blastomeres commonly observed in the IVP embryo is known to be due the presence of lipid material. Ultrastructual studies have shown the presence of many lipid droplets in the cells of the trophectoderm and ICM of the IVP embryo. The presence of lipid is responsible for the clear differences in buoyant density that have been demonstrated between IVP and *in vivo*-produced embryos. Authors started to comment on the resemblance of the IVP cattle embryo to the pig embryo. In pigs, it had long been recognized that the early embryo showed extreme sensitivity to cooling and low temperatures; it was also known that the lipid droplets abundant in one- to eight-cell-stage pig embryos declined markedly at the

blastocyst stage. Such findings were relevant when it was found that pig embryos at the expanded and hatched-blastocyst stage had a higher tolerance to cooling in comparison with morulae and early blastocysts.

Delipidizing the pig embryo

It is now clear that early-cleavage stage pig embryos are quite capable of surviving the freeze–thawing procedure after the mechanical removal of cytoplasmic lipid (Nagashima *et al.*, 1994a,b, 1995a,b). In this procedure, two- to four-cell pig embryos were centrifuged at 12,500 × *g* for 8 min in the presence of a cytoskeletal inhibitor (cytochalasin B) to polarize the lipid droplets within the blastomeres; the resultant lipid layer is then removed with a pipette. The same workers showed that the piglets born from the frozen–thawed delipidated embryos were normal in appearance and had birth weights and postnatal growth rates normal for the breed. The possible implication of such results was that cytoplasmic lipid droplets were probably responsible for the problems experienced in the cryopreservation of pig embryos, rather than more obscure problems of cell-membrane permeability or structure.

Delipidizing the IVP bovine embryo

A report by Leibo *et al.* (1995) showed that intracellular lipid droplets, which can be displaced and largely removed, were at least partly responsible for the decreased buoyancy and increased chilling/sensitivity associated with the IVP cattle embryo. Elsewhere, workers were able to demonstrate that the tolerance of IVP embryos to freeze–thawing could be increased by removing cytoplasmic lipid droplets from the one-cell zygote (Diez *et al.*, 1996; Ushijima *et al.*, 1996). In Japan, Tominaga *et al.* (1998, 2000b) demonstrated that lipid removal by centrifugation (15,500 × *g* for 7 min) at the two-cell stage without micromanipulation resulted in the production of high-quality cattle embryos with a high survival rate after freezing and thawing. In France, Diez *et al.* (2001) demonstrated that delipidation of one-cell bovine embryos (by micromanipulation) was compatible with their normal development to term and had a beneficial effect on their tolerance to freezing and thawing at the blastocyst stage; electron microscopy showed much fewer lipid droplets (and smaller) in delipidated blastocysts than in controls (see Table 8.10).

Lipids and mitochondria

It is clear that farm-animal species vary in the lipid content of their embryos; pigs and horses are in a high-lipid category, while sheep and cattle are in a low-lipid category (Khandoker *et al.*, 1997). In Aberdeen, McEvoy *et al.* (2000c) showed that the mean fatty acid content in pig oocytes was significantly greater than that in cattle and sheep oocytes; of 24 fatty acids detected, palmitic, stearic and oleic acids were the most prominent in the three species. It was concluded that the greater fatty acid content of pig oocytes is primarily due to more abundant triglyceride reserves. The authors suggested that this species-specific difference and that in respect of polyunsaturated fatty acid reserves may underlie the chilling, culture and cryopreservation sensitivies of embryos derived from pig and cattle/sheep oocytes. In France, a study by Abd El Razek *et al.* (2000) compared lipid composition of *in vivo*-produced and IVP cattle embryos (SOF + 10% FCS); they showed that

Table 8.10. Survival of bovine blastocysts developed from delipidated zygotes (from Diez *et al.*, 2001).

Group	Recipients	Number of pregnancies (%)			
		P4 test	Ultrasonic echography		
		day 21	day 35	day 90	Calves born
Delipidated blastocysts	17	11 (64.7)	9 (52.9)	7 (41.2)	8 (47.0)[a]
Control IVF blastocysts	22	13 (59.0)	11 (50.0)	10 (45.4)	10 (45.4)

[a]One recipient transferred with a single blastocyst gave birth to a pair of monozygotic twins. Chi-square test: no significant difference was found.

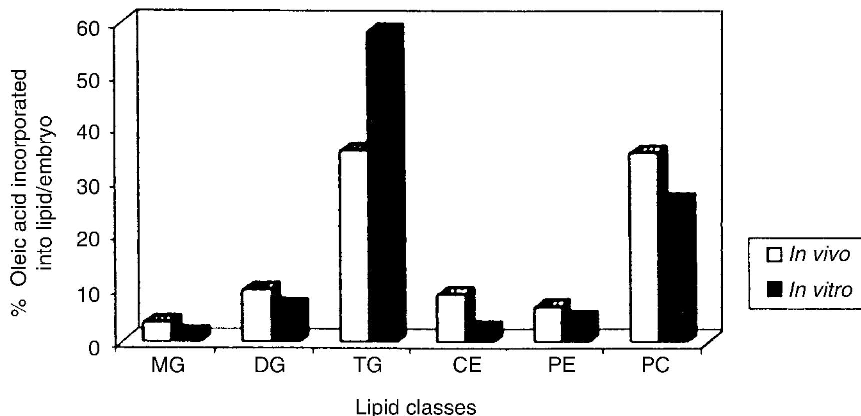

Fig. 8.3. Lipid classes in IVP embryos and *in vivo* embryos (from Abd El Razek *et al.*, 2000).

the IVP embryos contained more triglycerides and fewer lipids from other classes than *in vivo* embryos (see Fig. 8.3).

In sheep, where IVC conditions have led to embryos with abundant lipid inclusions, this has been associated with problems in fetal development (Thompson *et al.*, 1994). What factors involved in the *in vitro* system are likely to be responsible for the accumulation of 'abnormal' amounts of cytoplasmic lipid in the ruminant embryo? In the studies of Thompson *et al.* (1994), culture of early sheep embryos in SOF supplemented with serum was apparently associated with the problem; an alternative culture system, in which serum was replaced with amino acids and albumin, resulted in embryos lacking abundant lipid inclusions and in normal lambs. In the same laboratory, there was evidence that IVC of *in vivo*-produced sheep embryos in SOF medium supplemented with serum may, under certain conditions, have adverse effects on the structure of mitochondria (Dorland *et al.*, 1994). In cattle, it was evident from one study that the mitochondria of IVP cattle embryos were structurally different from mitochondria of *in vivo*-produced embryos (Plante and King, 1994); it was observed that half the number of mitochondria in IVP blastocysts were swollen and lacking matrix and intact cristae at the time of hatching. It is known that mitochondrial activity is an integral part of early embryo metabolism, and serum in the culture medium may have an adverse effect on such metabolism, resulting in some of the problems found in the IVP embryo.

8.4.4. Effect of culture medium

A paper by Holm *et al.* (1994a) reported that post-thaw survival was higher for IVP blastocysts that developed in CR1 medium compared with those cultured in SOF medium; in this, the authors found that cryopreservation revealed differences in embryo quality that were not apparent in simple morphological examination before freezing. Studies reported by Imai *et al.* (1997) demonstrated that the addition of unsaturated fatty acid, LAA to CR1aa medium supplemented with 5% calf serum enhanced the survival rate of IVP cattle blastocysts and expanded blastocysts after cryopreservation.

In Spain, Diaz *et al.* (1999) studied the effect of different cryoprotectants for freezing IVP embryos produced in different culture systems; their evidence showed that blastocysts obtained in modified SOF (mSOF) had lower survival rates after thawing than blastocysts co-cultured with Vero cells. They also showed that EG led to survival rates higher than glycerol when freezing blastocysts obtained in co-culture with Vero.

8.4.5. Rapid freezing of IVP embryos

Several workers have attempted to develop simple, rapid-freezing procedures which may be applied to the IVP embryo as a means of achieving higher survival and development. Rapid freezing involves the immersion of the embryo container (straw) in liquid nitrogen

within a minute or so of completion of equilibration. In Japan, Matsuoka *et al.* (1995) froze IVP cattle embryos ultrarapidly; after equilibation, blastocysts were loaded into 0.25 ml straws, placed in liquid nitrogen vapour for 20 s and then plunged into liquid nitrogen. The same workers showed that embryos could be successfully frozen using EG and trehalose or sucrose and could be transferred directly without cryoprotectant dilution. Two rapid-freezing procedures were described by Nowshari and Brem (1998); they used EG at three concentrations (4.4, 6.0 and 7.0 M), each in combination with 0.5 M sucrose. A significant improvement in embryo survival occurred with the 7.0 M concentration of EG in comparison with controls (1.5 M EG + 0.25 M sucrose). The authors suggested that the rapid-freezing protocol offered a simple, less time-consuming and effective method than conventional slow freezing in dealing with day-8 IVP blastocysts. In a subsequent paper, Nowshari and Brem (1999) reported that IVP embryos could be successfully refrozen by the same rapid-freezing procedure.

8.5. Vitrification of *in Vivo*-produced Embryos

Vitrification is a process of solidification in which crystalline ice does not separate and there is no concentration of solutes, as in conventional freezing; there is an abrupt increase in the viscosity of the holding medium, producing a solid, glasslike state. High cooling rates can be employed but initial exposure to the vitrifying solution has to be at refrigerator temperature and very brief to avoid adverse effects from cryoprotectant toxicity. Warming rate has to be rapid to avoid crystal formation as the temperature returns to normal. The merit of vitrification is seen in terms of simplifying and speeding up the cryopreservation process, avoiding the need for a lengthy cooling period and expensive freezing equipment.

8.5.1. Early studies

In the mid-1980s, vitrification as an alternative to the conventional freezing technique was described by Rall and Fahy; the new technique involved a mixture of solutes (DMSO, acetamide, propylene glycol as permeating agents; polyethylene glycol as the macromolecule) that permitted vitrification of the embryo holding solution when cooled to very low temperatures. The whole procedure required about 35 min to allow stepwise equilibration before embryos were plunged into liquid nitrogen (see Fig. 8.4).

The first success in preserving mouse embryos by vitrification was followed by many other studies with that species. For use in cattle, a series of papers on embryo vitrification were published by Saha and other workers in Japan (Saha *et al.*, 1994a,b, 1995, 1996a,b, 1997; Saito *et al.*, 1994; Saha and Suzuki, 1997). Elsewhere in the same country, Kasai (1996) described a typical low-toxicity vitrification solution (EFS 40) containing three cryoprotective agents: (i) a rapidly permeating, low-toxicity agent (e.g. 40% EG); (ii) a macromolecule (e.g. 18% Ficoll 70); and (iii) a non-permeating hexose sugar (e.g. sucrose). Cattle embryos were briefly (1 min) exposed to the solution before plunging into liquid nitrogen. A review of the cryopreservation of mammalian embryos by vitrification was given by Ohboshi (1998); in an earlier paper, the same author and colleagues dealt with the use of polyethylene glycol for IVP embryo preservation by vitrification (Ohboshi *et al.*, 1997).

As noted by Massip (1999), potential osmotic and toxic effects can be limited by selecting appropriate cryoprotectants; EG, as noted above, is suitable, as are glycerol and DMSO. Damaging effects of solutes can be lessened by using a mixture of two or more cryoprotectants or by stepwise equilibration (two or three steps) in solutions of intermediate concentrations at room temperature or after cooling to refrigerator temperature. Among the macromolecules employed in vitrification, Ficoll 70, polyvinyl pyroolidone (PVP) and BSA have been used; non-permeating agents, such as sucrose, reduce the toxicity of the vitrification solution.

Vitrification involves a high cooling rate; this was achieved in early studies by plunging a 0.25 ml straw directly into liquid nitrogen. The maximum cooling rate using this approach has been estimated as 2500°C/min, although this will vary with the volume of the solute mixture and other factors.

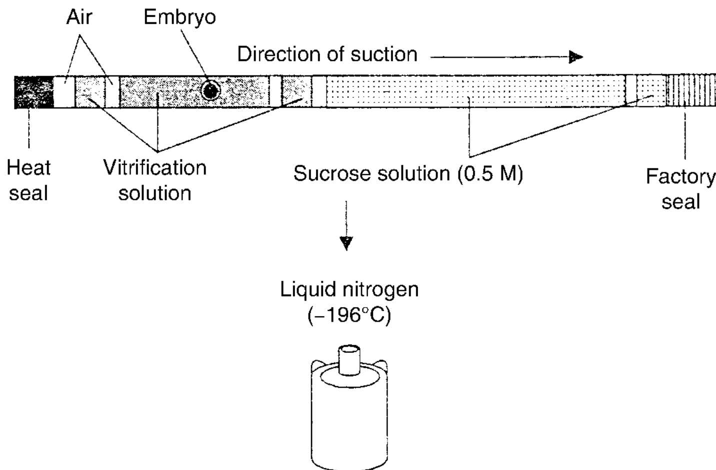

Fig. 8.4. Steps in the vitrification of a bovine embryo. The mini-straw (0.25 ml) is loaded by applying suction → sucrose solution → air → vitrification solution + embryo → air → heat seal. The straw is then plunged into liquid nitrogen for vitrification.

A large field trial, involving the transfer of 728 embryos, reported by Van Wagtendonk-De Leeuw *et al.* (1997a,b), compared pregnancy rates in recipient cattle receiving *in vivo*-produced embryos frozen by conventional slow cooling or vitrified by the Rall method; the two methods yielded similar results (slow freezing, 45%; vitrification, 44.5%). However, although the vitrification method is simple, fast and requires no freezing machine, embryos are very sensitive to the vitrification solution, so the time window for processing them is narrow and a successful outcome is dependent on a stringent technique. According to Massip (1999), despite its apparent attractions (time-saving and low cost), vitrification has failed to make any great headway in commercial cattle ET applications. Compared with the conventional slow-freezing method, it appears to be more difficult to apply on the farm because of the care required; the highly concentrated vitrification solutions require careful control of time and temperature of equilibration and rehydration media (Vajta *et al.*, 1999a,b,c). The vitrification of *in vivo* embryos collected in the field from Jersey cows by the OPS method was reported by Saalfeld *et al.* (2002); evaluating embryos after thawing, they reported re-expansion and hatching rates of 84.6% and 66.6%, respectively.

Cleavage-stage embryos

It is well known that precompaction-stage cattle embryos are particularly difficult to cryopreserve. A report by Shimizu *et al.* (1998) dealt with survival and development of *in vitro*-produced bovine 16-cell embryos after vitrification; they showed that this could be very successful, using a three-step technique (Step 1, 10% glycerol; Step 2, 10% glycerol + 10% EG; Step 3, 20% EG + 20% glycerol + 20% PVP). A study by Yang and Leibo (1999) investigated the viability of bovine zygotes suspended in various concentrations of EG and cryopreserved in 10 μl microdrops to achieve very high cooling rates; their results indicated that this was possible, although cleavage rates were markedly below those of controls.

8.5.2. Vitrification and slow freezing as alternatives

Although slow-freezing cryopreservation protocols are used widely in the commercial cattle ET industry, there are those who view vitrification as a viable alternative capable of reducing the costs involved in cryopreservation. In California, using both *in vivo*-produced and IVP blastocysts,

Campos-Chillon *et al.* (2002) looked at the possibility of vitrification being used successfully on the farm; they recorded similar pregnancy rates for vitrification (56%) and slow freezing (44%) after transfer of *in vivo*-produced embryos. Their vitrification method permitted greater time flexibility during the transfer period, which may be an advantage in dealing with multiple recipients. The vitrified embryos could be stored in the straw for 15 min after thawing, whereas the usual recommendation for slow-frozen embryos is for immediate transfer. The authors suggest that vitrification offers an alternative to slow freezing but note that large-scale field trials would be necessary to extend their findings. In the Czech Republic, Lopatarova *et al.* (2002) reported similar survival rates for *in vivo*-derived bovine embryos vitrified by the OPS method and those preserved by conventional freezing methods; they concluded that OPS vitrification was an effective and rapid method of cryopreserving cattle embryos.

A study by Kong *et al.* (2000a,b) in Korea investigated the use of a glass micropipette (GMP) rather than the OPS method; they considered that this might allow faster cooling and warming rates to be employed. Using mouse embryos, they showed that both methods of vitrification yielded high rates of re-expanded embryos (93.5% for OPS; 95% for GMP). However, using the OPS method, straws float during cooling and storage in liquid nitrogen, which complicates long-term storage using traditional cane/goblet systems. With the higher density of glass, the GMP containers do not float.

8.6. Vitrification of IVP Embryos

Much information is available for consultation on the vitrification of IVP embryos as a result of efforts during the recent decade (Brandenhoff *et al.*, 1994; Dinnyes *et al.*, 1994, 1995, 1996; Agca *et al.*, 1995, 1998a,b,c,d; Delval *et al.*, 1995, 1998; Dobrinsky *et al.*, 1995; Kuwayama, 1995; Vajta *et al.*, 1995a,b, 1996a,b, 1997b,c,d,e,f,g,h; Van Wagtendonk-de Leeuw *et al.*, 1997a,b; Donnay *et al.*, 1998b; Lee and Fukui, 1998; Martinez *et al.*, 1998; Vajta, 1999).

The efficiency of IVP embryo cryopreservation has been greatly improved by increasing the speed of cooling and warming. Techniques that have been developed to permit this include direct immersion of droplets in liquid nitrogen, use of an electron-microscope grid to provide support (Martino *et al.*, 1996b), the OPS (Vajta *et al.*, 1997a) and the cryo-loop method (Lane *et al.*, 1999a). According to the latter authors, the OPS method, although giving promising results, remained technically difficult and used straws that had to be pulled to a specific diameter. They developed a method, adapted from a proved method for flash-cooling protein crystals, for the cryopreservation of embryonic cells (Lane *et al.*, 1999b) in which hamster embryos in cryoprotectant were placed in a small loop (0.3–1.00 mm diameter) and rapidly submerged in liquid nitrogen to achieve near-instantaneous vitrification; the loop was then sealed in a cryo-vial of liquid nitrogen and stored.

8.6.1. Developing an effective vitrification procedure

Experiments designed to optimize conditions for the successful preservation of cattle IVP embryos by vitrification have been reported by several laboratories. In Belgium, Mahmoudzadeh *et al.* (1995) used a vitrification solution consisting of 40% (v/v) EG, 18% (w/v) Ficoll and 10.26% (w/v) sucrose; embryos exhibited significantly higher survival and hatching rates after two-step vitrification, especially at the blastocyst and expanded blastocyst stage. In Austria, Willemsen *et al.* (1995), Willemsen (1996) evaluated the effect of IGF-I in culture medium on embryo survival after vitrification; neither a beneficial nor an adverse effect was recorded.

Until recently, early-stage cattle embryos produced *in vitro* have been difficult to cryopreserve because of their extreme sensitivity to cooling below physiological temperatures (Martino *et al.*, 1996a,b). One way of minimizing this problem is to traverse the damaging temperature zone as fast as possible (Vajta *et al.*, 1998a). As noted earlier, the fastest cooling rate achievable with the 0.25 ml straw is 2500°C/min; by modifying the straw and the volume of cryoprotectant, using the OPS method, it is possible to achieve an eightfold increase in cooling and warming rates (20,000°C/min). For IVP

embryos, vitrification appears to be the method of choice. A report by Wurth *et al.* (1994) reported a pregnancy rate of 23% with vitrified embryos but only 14% for those frozen by conventional means. A report by Avery *et al.* (1995a,b) in Denmark reported on conventional glycerol freezing and vitrification of IVP cattle blastocysts; their results (see Table 8.11) showed the vitrification method to be superior, on the basis of post-thaw survival. Two papers published by Agca *et al.* (1998a,b) also demonstrated the effectiveness of the vitrification procedure.

A comparison between the OPS vitrification method and conventional slow freezing of IVP cattle embryos was reported by Lane *et al.* (1998); they found the vitrification method to be suitable for direct transfer of embryos; further reports from the same laboratory indicated that the OPS method might also be suitable for nuclear-transfer embryos (Lewis *et al.*, 1998a; Peura *et al.*, 1998). In Belgium, Kaidi *et al.* (2001) compared conventional freezing with vitrification in regard to blastocyst quality, evaluating membrane permeabilization, number of cells and their allocation to ICM and trophectoderm; although the overall rate of blastocyst hatching was similar for frozen and vitrified embryos, the cell damage induced by the two methods appeared to differ (e.g. the decrease in trophectoderm cells was more prominent in frozen than in vitrified blastocysts). In Denmark, Holm *et al.* (1999b) reported that more expanded blastocysts re-expanded after OPS vitrification than after EG conventional slow freezing. In Slovakia, Lazar *et al.* (2000) used the OPS method in vitrifying IVP embryos; after thawing, embryos were cultured for 24 h and showed a re-expansion rate of 69%. After transfer to six recipient cows, three became pregnant and produced calves; the authors concluded that the OPS method was convenient, simple and effective for dealing with 7–9-day-old blastocysts.

Table 8.11. Comparison of conventional freezing and vitrification. Survival of frozen/thawed *in vitro*-derived day-7 bovine blastocysts. The embryos ($n = 242$) were randomly allocated to either conventional freezing or vitrification. Mean ± SD; three replicates. (From Avery *et al.*, 1995b.)

Freezing method	24 h (%)	48 h (%)	72 h (%)
Vitrification ($n = 114$)	47 ± 9^a	33 ± 9^c	28 ± 10^e
Conventional ($n = 128$)	8 ± 6^b	9 ± 7^d	2 ± 2^f

ANOVA $P < 0.0001$. Tukey–Kramer multiple comparisons test: ^{a,c,e}:NS; ^{b,d,f}:NS; ^{a,b}:$P < 0.001$; ^{c,d} and ^{e,f}:$P < 0.05$.
SD, standard deviation; ANOVA, analysis of variance; NS, not significant.

The introduction of the OPS method for vitrifying cattle embryos was followed by work on the in-straw dilution of the vitrification solution. Studies reported by Vajta *et al.* (1998b, 1999a) demonstrated that in-straw dilution did not decrease embryo survival rates and could be carried out at ambient temperature; sucrose supplementation of the vitrification solution proved unnecessary. The authors suggested that OPS vitrification with in-straw dilution might prove to be an appealing alternative to the conventional slow-rate freezing protocol. Results presented by Lewis *et al.* (1999) indicated that vitrification could be combined with in-straw dilution to achieve acceptable pregnancy rates after transfer of cryopreserved IVP blastocysts; they saw such results to be the means of facilitating commercial adoption of accelerated breeding technologies in cattle. In Japan, Hamawaki *et al.* (1999) investigated whether embryo survival could be improved by minimizing the volume of vitrification solution or by using a direct cooling method, which involved immersing embryos in liquid nitrogen directly; they found that significantly more embryos survived vitrification using the minimum volume method than by general cooling. The authors suggest that this modification may improve the success of the vitrification method. In other studies reported by Hamawaki *et al.* (2000), they examined pregnancy rates after in-straw dilution and transfer of vitrified bovine IVP embryos; recipients with vitrified or slow-cooled embryos showed satisfactory pregnancy rates. In this work, the vitrification solution (30% EG + 0.5 M sucrose) was mixed with the diluent column (0.5 M sucrose) by gentle shaking; transfer was carried out within 5 min of thawing.

Assisted hatching

Some authors have attempted to improve post-thaw hatching rates of IVP cattle embryos that

have been vitrified. In Korea, Kong *et al.* (2000a,b) presented results indicating that assisted hatching (0.05% pronase for 1 min) could increase the hatching rate.

Avoiding contamination of embryos

One disadvantage of most vitrification methods is the direct contact of the embryo suspension with liquid nitrogen contaminated with virus and other microorganisms. Although there are those who suggest that contamination can be prevented by cooling OPS straws in filtered liquid nitrogen (Vajta *et al.*, 1998a; Lane *et al.*, 1999c), such filtering may not be capable of dealing with viruses. In human assisted reproduction, although many articles have been published about human gamete and embryo cryopreservation, an issue of concern is minimizing the possibility of microbiological cross-contamination in nitrogen storage units (Clarke, 1999; Rall, 2001).

A review by Vajta (2000) refers to a possible solution in the minimum-volume cooling method of Hamawaki *et al.* (1999), where embryos in extremely low volume are loaded on to the wall of a 0.25 ml insemination straw, which is sealed before cooling. Another possible solution mentioned by the author is to use even narrower and thinner-walled straws than the OPS and heat-sealing them as with normal straws.

Factors relevant to the success of vitrification

Many reports have appeared during the 1990s dealing with factors that may influence the success of the vitrification process. In Belgium, DeRoover *et al.* (1999) found that COC quality had no effect on subsequent IVP embryonic development *in vitro* after cryopreservation by vitrification. A paper by Baguisi *et al.* (1999b) examined the incidence of necrosis and apoptosis in vitrified IVP blastocysts; they concluded that post-thaw necrotic effects needed to be further reduced for vitrification to become an acceptable cryopreservation protocol. A double-staining technique that may be used in determining optimum equilibration and dilution conditions during vitrification was reported by Kaidi *et al.* (1998b). The same author used cinematography to define criteria that could be used during post-thaw dilution to select IVP embryos that could survive after vitrification (Kaidi *et al.*, 1999b); they were able to show that blastocysts with an initial diameter < 200 μm have less chance of survival than those of greater size.

In Spain, Gutierrez-Adan *et al.* (1999d) reported studies indicating that, for vitrified IVP embryos, direct in-straw rehydration at 4°C for 10 min could be a practical procedure for use under field conditions where a suitable interval between thawing and transfer can be arranged. The effect of the time interval between biopsy and vitrification was the subject of a study by Ito *et al.* (1999b) in Japan; they reported that the survival rates of *in vitro*-derived cattle embryos vitrified 2.5 and 5 h after biopsy were comparable to those for non-vitrified controls (73 and 78% vs. 96%, respectively). In Poland, Papis *et al.* (1999a,b) vitrified day-3 embryos in droplets or in straws; the highest blastocyst yield after thawing was with embryos vitrified in droplets (71.2 vs. 43.9%). In Ireland, Dinnyes *et al.* (1999) studied the influence of the time of the first cleavage after insemination on the *in vitro* survival of cattle blastocysts after vitrification; their vitrification solution consisted of 6.5 M glycerol + 6% BSA in PBS. They showed survival and hatching of embryos to be significantly higher for blastocysts formed by day 7 than for those formed by day 8; in terms of cleavage post-insemination, day-7 blastocysts from 27 and 30 h cleavage groups survived significantly better than those from the 36 h group (63 and 66 vs. 25%, respectively).

8.7. Cryopreservation of the Bovine Oocyte

During the past half-century, there has been considerable progress in the freezing of bull spermatozoa and in the low temperature of cattle embryos; oocyte cryopreservation, on the other hand, is not yet an established technology. None the less, many reports are available for consultation on the storage of the bovine oocyte (Arav *et al.*, 1994; Goncalves *et al.*, 1994; De Lille *et al.*, 1995; Mahr-Behm, 1995; Ohboshi *et al.*, 1997; Dochi *et al.*, 1998a; Hochi *et al.*, 1998; Metzdorf *et al.*, 1998; Ullah *et al.*, 1999; Shaw *et al.*, 2000); success in this area could have important practical implications both in cattle and in

human assisted reproduction. However, it must be borne in mind that mammalian oocytes are among the most difficult cell types to cryopreserve. It should be noted that ovarian tissue cryobanking represents an alternative method for preserving female genetic resources which may be of considerable importance in preserving the fertility of human patients and the gametes of endangered species (see Ledda *et al.*, 2001).

Vitrification of cattle oocytes has been performed using several different cryoprotectants, including combinations of DMSO/actamide/propylene glycol, EG/DMSO, EG/Ficoll/sucrose and glycerol/1, 2-propanediol; such agents have been used in varying concentrations and with different equilibration times.

8.7.1. Factors relevant to oocyte cryopreservation

The effect of cooling and rewarming can have disastrous effects on the meiotic spindle and chromosomes of the bovine oocyte; in the USA, Aman and Parks (1994) showed that exposure to 4°C for 10–20 min caused complete disappearance of the spindle, with chromosome dispersion after a longer interval. Papers have been published on factors relevant to the cryopreservation of mammalian oocyte, such as membrane-transport properties (Gao *et al.*, 1994). Elsewhere, studies have demonstrated that exposure of bovine oocytes to 0°C for as little as 30 s may significantly affect their ability to continue development (Martino *et al.*, 1996a,b; Azambuja *et al.*, 1997, 1998). A study by Isachenko and Michelmann (1999) compared the ultrastructure of lipid droplets and the effect of cooling on intracellular lipid vesicles of cattle and pig GV oocytes. In the bovine oocyte, they noted that lipid droplets had a homogeneous structure and that utilization of lipids took place directly from these vesicles without formation of interim lipid compounds. In contrast, in pig oocytes they found two kinds of lipid droplets, dark and grey vesicles that were connected to each other. Apparently, dark vesicles changed into grey, followed by utilization of these 'grey' lipids. Changes in the morphology of lipid droplets were evident during cooling in pig oocytes but not in those of cattle.

8.7.2. Freeze–thawing

Although mouse oocytes have proved to be relatively tolerant to chilling, bovine oocytes are extremely chilling-sensitive and have shown very low survival rates when cryopreserved by standard slow-cooling protocols (Otoi *et al.*, 1995a,b, 1996; Arav *et al.*, 1996; Im *et al.*, 1997); human oocytes are also known to be chilling-sensitive. This latter observation could make studies with cattle oocytes particularly relevant to work with human oocytes. A study by Martino *et al.* (1996a) characterized the high chilling sensitivity of bovine oocytes at both the GV and the metaphase II (MII) stages. The authors noted that most attempts to cryopreserve bovine oocytes had used conventional slow-freezing protocols that exposed them to temperatures of zero or below zero for long periods of time. It was evident that the damage sustained at such temperatures, even before freezing began, probably explained the low success rates achieved.

It became clear that special techniques were required to cryopreserve bovine and human oocytes in view of their chilling sensitivity. Studies reported by Martino *et al.* (1996b), using extremely rapid cooling, improved the survival rate of cattle oocytes to the point where 30% cleaved after IVF and one-half of these developed to the blastocyst stage, a survival rate comparable to that in oocytes exposed to cryoprotectants but without cooling. Elsewhere, workers looked at ways in which the maturation of the bovine oocyte may be influenced to lessen their chilling sensitivity. A report by De Matos *et al.* (1995) demonstrated that cysteamine supplementation of IVM medium increased the intracellular content of GSH in bovine oocytes. In a subsequent paper by De Matos *et al.* (1996a,b), a significant difference was recorded in embryonic development after freezing between day-6 embryos from oocytes matured with or without cysteamine; it was also recorded that significantly more embryos reached the blastocyst stage at days 6 and 7 than in controls. The authors concluded that it would be useful to examine the freezing sensitivity of bovine oocytes matured in media designed to increase GSH levels.

Work by Agca *et al.* (1997) in Indiana demonstrated that the permeability of cattle oocytes

is significantly higher for water and cryoprotectant after IVM; the permeability of the oocyte was also found to be higher for DMSO than for EG. The authors suggested that such results might provide a biophysical basis for developing more suitable protocols regarding cooling rates, as well as the addition and removal of cryoprotectants.

Freezing of bovine oocytes has usually been either at the GV or the MII stages of meiosis; limited success in the early years suggested that freezing at both stages probably led to chromosomal abnormalities or reduced developmental potential. Studies by Le Gal *et al.* (1994), using goat oocytes as a model, suggested that there are differences in actin organization between the two types of oocytes, probably at the cortical level. In the primary oocyte, it is believed that destruction of gap junctions between cumulus cells and the oocyte usually occurred; when the secondary oocyte was frozen, it was believed that microtubules were often damaged, resulting in chromosome scattering. Premature release of cortical granules in the cryopreserved secondary oocyte was also thought to occur. A study by Barnes *et al.* (1997) reported evidence suggesting that oocytes cooled at the germinal-vesicle breakdown (GVBD) stage cleaved and developed into blastocysts at higher rates than oocytes cooled at the GV or MII stage. However, cleavage rate (17%) and blastocyst yield (7%) were far below those of controls (78 and 28%, respectively).

In Korea, Lim *et al.* (1999b) reported on the development of IVM bovine oocytes after freezing with different cryoprotectants (glycerol, DMSO, propylene glycol); the percentage of oocytes developing to the eight-cell or morula stage was significantly higher with propylene glycol than with the other two agents. In Japan, the effect of different cryoprotectants (DMSO, EG, propylene glycol) on the survival of IVM cattle oocytes was studied by Tsuzuki *et al.* (2000a,b); cleavage rates in all treatment groups were significantly lower than those in control groups. Other work in that country, reported by Asada and Fukui (2000), indicated that post-thaw 6 h repeat culture did not significantly improve the fertilizability and embryonic development of cattle oocytes cultured for 18 h or 24 h before freezing; it had been thought that the repeat-culture period might allow meiotic spindles to recover from possible chilling injury. Experiments reported by Isachenko *et al.* (2000) tested protocols for vitrification of GV sheep oocytes that involved three cooling/warming regimes: slow cooling/quick thawing, quick cooling/slow thawing and quick cooling/quick thawing. They demonstrated that the OPS method, in combination with elevated rates of cooling and thawing (20,000°C/min–16,000°C/min) was the most effective protocol; evaluation by fluorescent microscopy showed that 35% of the oocytes reached MII after 24 h maturation, in contrast to zero percentage in the other two regimes.

Ultrastructural evaluation

A paper by Schmidt *et al.* (1995) reported on the ultrastructure of IVM cattle oocytes after controlled freezing in 10% glycerol; in more than half of the oocytes, the oolemma was ruptured and the perivitelline space was occupied by ooplasmic content. It was clear that severe ultrastructual damage was associated with such freezing protocols.

8.7.3. Vitrification

In Israel, Arav and Zeron (1997) reported on the vitrification of bovine oocytes, using a modified minimum-drop-size technique on electron-microscope grids; MII oocytes equilibrated in their vitrification solution (5.5 M EG, trehalose and 0.4% BSA in HEPES-buffered TALP medium) in liquid nitrogen yielded a cleavage rate (48%) comparable to that of controls (52%). Vitrification was employed by Kuchenmeister and Kuwayama (1997) as the method of cryopreserving GV bovine oocytes; their vitrification solution, which contained 40% EG and 1 M sucrose, permitted limited cleavage (23–42%) and a small yield of blastocysts (1–6%). In Japan, Hochi *et al.* (1998) examined how far the stage of meiosis influenced the outcome of vitrification; initiating vitrification at 12 h (just beyond the GVBD stage) gave the best results. It is believed that the cytoskeleton of the first meiotic division in immature oocytes is particularly susceptible to damage. Further studies by this group in Japan dealt with vitrification of matured cattle oocytes in open-pulled glass capillaries of differing diameters (Hochi *et al.*, 2000a). What was described as a simple, efficient method for

vitrifying cattle blastocysts was providing by Park, S.P. *et al.* (1999), using electron-microscope grids.

A significant development of the late 1990s was the work of Vajta *et al.* (1998a), who showed that chilling injury and osmotic damage to embryos and oocytes could be minimized by providing very high cooling and warming rates (> 20,000°C/min) and a reduced period of contact with cryoprotective additives (see Fig. 8.5); of 184 vitrified oocytes, 25% developed to the blastocyst stage after fertilization and culture for 7 days. The authors reported pregnancies after transfer of embryos derived from such oocytes, confirming the normality of events after the OPS method of vitrification. The results of studies in the vitrification of buffalo oocytes are to be found in the papers of Dhali *et al.* (2000a,b).

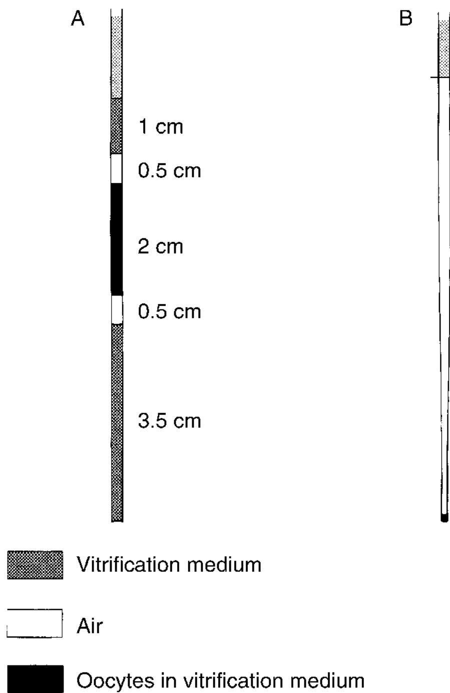

Fig. 8.5. Conventional straw vitrification vs. open pulled straw (OPS). A. The 0.25 ml ministraw is loaded with 1 cm of vitrification medium, 0.5 ml of air, 2 cm of vitrification medium containing oocytes, 0.5 cm of air and 3.5 cm of vitrification medium using a syringe. B. The open pulled straw is loaded with vitrification solution (1–2 μl) containing oocytes by means of the capillary effect by a simple touch. (From Chen *et al.*, 2000a.)

In Belgium, Le Gal and Massip (1998) reported on the development of thawed oocytes fertilized *in vitro* after vitrification by the OPS method before or after IVM. In later studies, Le Gal *et al.* (2000) used the OPS method to vitrify partly denuded bovine oocytes either at the start or 6 h after the start of the maturation process; blastocyst yield was 3–4% for 0 h and 0.9% for the 6 h group. The same authors showed that pregnancies could be established after previously vitrified oocytes were again vitrified at the blastocyst stage, thawed and transferred. In the USA, Dinnyes *et al.* (2000a,b) vitrified matured oocytes in microdrops on a precooled (–150°C) metal surface (solid-surface vitrification (SSV)); cleavage rate and blastocyst yield were lower than those of controls. In Brazil, Brandao and Rumpf (2002) also evaluated the influence of oocyte maturational stage on the outcome of the OPS method of vitrification; significant degeneration of oocytes was evident at all maturational stages. In the same country, Siqueira *et al.* (2002) attempted vitrification by the OPS method of immature bovine oocytes in the presence of bovine follicular fluid; their results showed that a high proportion of such oocytes degenerated during IVM.

Factors influencing the survival of mature oocytes, using a technique based on minimizing the volume of the vitrified sample, were studied by Papis *et al.* (2000) in Poland; they concluded that gentle pre-equilibration of oocytes in a diluted 3% EG solution prior to a 30 s period of equilibration and vitrification in droplets in a VS14 solution (5.5 M EG + 1.0 M sucrose) improved the effectiveness of their procedure. They reported cleavage rates and blastocyst yields of 76% and 30%, respectively, compared with 89% and 42% in controls. Successful cryopreservation of a high percentage of bovine oocytes by vitrification was reported by Arav *et al.* (2000) in Israel and Dinnyes *et al.* (2000a,b) in the USA. The Israeli group suggested that an increase in the cooling rate at the temperature when chilling injury occurs (i.e. between 20 and 12°C) was responsible for their success; they recorded a cleavage rate of 74% and a blastocyst yield of 38%, no different from values achieved with control oocytes. The workers at Connecticut achieved their success by developing a novel SSV procedure. The SSV method was compared with the OPS procedure in studies of Yellow Cattle oocytes reported by Li, X. *et al.* (2001, 2002b);

they demonstrated that, although the OPS method (20% EG + 20% DMSO) resulted in higher developmental rates compared with SSV (35% EG + 5% PVP + 0.4 M trehalose), both methods significantly reduced embryonic developmental rates. Cleavage rates and blastocyst yields were 38.3% and 12.5% for OPS and 35.8% and 6.0% for SSV, respectively, differences that were not significant.

The biological protein usually added to vitrification solutions has been serum; however, serum may pose the risk of disease transmission and, for that reason, some workers have examined ways of eliminating this protein source. In Japan, Asada *et al.* (2002) investigated the possible use of polyvinyl alcohol (PVA) as an alternative to serum in a vitrification solution; they showed that 0.1% PVA supplementation in their vitrification solution resulted in a significantly higher rate of morula stage embryos than other levels of supplementation. The workers suggest that PVA could be substituted for FCS in vitrifying IVM bovine oocytes.

In Madison, Men *et al.* (2002a) examined DNA damage in mature cattle oocytes caused by various forms of cryopreservation; their results showed that both slow cooling and vitrification in 0.25 ml straws resulted in significant morphological damage to oocytes compared with OPS vitrification. It was evident, however, that all the cryopreserved oocytes showed intensive damage in comparison with controls. Further work in the same laboratory reported by Men *et al.* (2002b) recorded a significantly higher blastocyst yield from vitrified MII oocytes than from GVBD oocytes; evidently MII oocytes had greater resistance to cryopreservation. In Brazil, Vieira *et al.* (2002) reported studies on the vitrification by the OPS method of primary oocytes treated with cytochalasin D (CD), an agent known to modify the cellular microfilament network; such treatment did not improve the viability of the oocytes.

Previtrification additives

As noted in an earlier context, CD, which induces microfilament depolymerization, has been employed in cryopreservation studies. In Japan, Shimizu *et al.* (2000) subjected mature denuded cattle oocytes to CD treatment (2.5 μg/ml) for 10 min before transfer to three changes of their VA14 vitrification solution (5.5 M EG + 1.0 M sucrose) and plunging in droplets into liquid nitrogen; the CD treatment resulted in a blastocyst yield significantly greater than that of controls. In Brazil, Magnusson *et al.* (2002) evaluated the developmental competence of bovine oocytes exposed to two vitrification solutions (SV1, 30% EG; SV2, 40% EG), each supplemented with CD, at different stages of meiosis (0, 12 and 18 h of IVM) embryos were evaluated in terms of blastocyst yield and hatching rates. It was shown that oocytes exposed to SV1 after 0 and 12 h of maturation gave blastocyst yields similar to those of controls. Elsewhere in the same country, Mezzalira *et al.* (2002) found that CD had no beneficial effect on the survival or developmental potential of oocytes vitrified by the OPS method (blastocyst yields of 7.8–12.0% vs. 31.3% in controls).

Bovine vs. equine oocytes

The general experience is that MII oocytes are more resistant to cryopreservation than immature ones. In Colorado, Hurtt *et al.* (2000) found a trend towards higher survival rates for mature than for immature oocytes in both cattle and horses; they also showed that both primary and secondary equine oocytes had lower survival rates than cattle oocytes.

Ultrastructural evaluation

Cattle oocytes were vitrified at the GV or MII stage in DAP213 cryoprotectant solution in a study reported by Fuku *et al.* (1995); survival was assessed on the basis of fertilization and culture to the blastocyst stage. Vitrification caused profound ultrastructural modifications of microvilli, mitochondria, vesicle formation and ooplasm of GV oocytes; in matured oocytes, these structures were better preserved. The workers found that the integrity of organelles was relatively better maintained after a three-step than after a one-step procedure, in both GV and matured oocytes.

In Denmark, Hyttel *et al.* (2000a,b,c) reported on the ultrastructural consequences of vitrifying MII bovine oocytes with the OPS method; they found that vitrification had resulted in alterations in the oocyte after thawing (e.g. clustered cortical granules rather than

solitary cortical granules aligned along the oolemma), which were apparently reflected in subsequent fertilization and embryonic development.

8.8. Embryo Evaluation after Thawing

8.8.1. Evaluation of IVP embryos

IVP cattle embryos are characterized by increased chilling sensitivity and a much reduced ability to tolerate freezing compared with their *in vivo*-produced counterparts; after cryopreservation, the rate of re-expansion and hatching *in vitro* is high, but development after transfer remains low. It is believed that lack of viability after thawing and transfer is probably due to cellular damage or metabolic disturbances during the cryopreservation process. A study by Kaidi *et al.* (1998a) compared two co-culture systems to assess the survival of IVP bovine blastocysts after vitrification; they concluded that co-culture with buffalo–rat liver (BRL) cells under 20% oxygen was the best combination to evaluate blastocyst survival and quality after vitrification. A report by Vajta *et al.* (1999b,c) confirmed the need for serum rather than PVA supplementation of the post-thaw culture medium for the hatching of IVP cattle blastocysts after vitrification by the OPS method.

Using the vitrification method described by Donnay *et al.* (1998b), a study aimed at comparing the effects of conventional slow cooling and vitrification on *in vitro* development, morphology and metabolism of IVP embryos was reported by Kaidi *et al.* (2000); they evaluated embryos either immediately or 72 h after thawing. While it was found that both cryoprotection methods decreased survival and hatching rates, it appeared that cell alterations probably resulted from different mechanisms. Freezing affected more trophectoderm cells than vitrification and this may have led to a decrease in nutrient uptake by the embryos; it was also suggested that the stress response to cryopreservation measures by glycolytic activity might be more important after freezing.

In Poland, Gajda *et al.* (2000) studied the effect of equilibration time and developmental stage on the viability of vitrified cattle embryos: the higher the developmental stage, the higher the viability. They also showed that the viability of IVP embryos was lower than than found with *in vivo*-produced embryos. In a study in which IVP embryos were frozen by a controlled freezing method (10% glycerol), Khurana and Niemann (2000a,b,c) showed that embryos produced *in vitro* or pre-exposed to IVC exhibited greater sensitivity to cryo-injury; this sensitivity was affected by embryo quality and was reflected at the biochemical level. The authors suggested that the determination of oxidative metabolism offered a method of selecting viable embryos after freezing/thawing.

A study by Ferre *et al.* (2000a) in Argentina evaluated the effect of hypotaurine as an antioxidant on the development and post-thaw viability of IVP embryos; there was some evidence that a concentration of 1 mM could have a positive effect on post-thaw viability. A study reported by Markkula *et al.* (2001) attempted to find a reliable functional criterion for estimation of the viability of IVP embryos and to compare the proportion of proliferating cells in the embryos that had undergone two vitrification treatments (normal straw and OPS). Using proliferating cell nuclear antigen (PCNA) immunocytochemistry in combination with confocal microscopy, they were able to show that day-7 embryos tolerated cryopreservation treatments better than day-8 embryos; they also showed that vitrification in normal straws was especially detrimental to day-8 embryos. The authors concluded that the ratio of PCNA-positive nuclei can provide much useful information and numerical data about the developmental potential of IVP embryos after cryopreservation. In Ireland, Rizos *et al.* (2002b), culturing embryos for 72 h after thawing, showed that both bovine and ovine blastocysts showed significant decreases in blastocyst survival after vitrification, using the OPS method; they also showed, however, that the survival rate in sheep blastocysts was greatly superior to that in cattle (27.8% vs. 9.7%). It appeared that inherent species differences existed in embryo tolerance to the vitrification process.

9

Establishing Pregnancies with IVP Embryos

9.1. Introduction

Much of the information assembled over the years, particularly since the early 1970s, in dealing with *in vivo*-produced cattle embryos is directly relevant to the way in which *in vitro*-produced (IVP) embryos should be handled.

9.1.1. Historical

The first embryo transfer (ET) was recorded by Walter Heape, working in Cambridge with the rabbit (see Table 9.1). Many years were to pass before this novel animal-breeding technology was to be used on the farm in earnest. In the 30 years since cattle ET first appeared on the commercial scene, it has played an increasingly important role in the genetic improvement of cattle. In dairy cattle genetics, for example, Betteridge (1995) drew attention to the fact that the top 27.5% of cows and top 44% of bulls tested for productivity in the USA in 1990 were produced by the new technology.

Table 9.1. First records of successful embryo transfers.

Year	Species	Researcher(s)
1890	Rabbit	Heape
1932	Goat	Warwick and Berry
1933	Rat	Nicholas
1933	Sheep	Warwick *et al.*
1942	Mouse	Fekete and Little
1951	Cow	Willet *et al.*
1951	Pig	Kvansnickii
1974	Horse	Oguri and Tsutsumi
1978	Human	Steptoe and Edwards
1983	Buffalo	Drost *et al.*

Significant expansion of commercial cattle ET started with the introduction of non-surgical flushing in the early 1970s, and grew further with the introduction of simple non-surgical techniques towards the end of that decade. Although the surgical transfer of embryos, in suitably experienced hands, probably remains the method capable of achieving the highest pregnancy rates (70%), any thought of low-cost extensive use of cattle ET requires a non-surgical transfer technique essentially similar to that involved in routine artificial insemination (AI) (see Fig. 9.1). The animal welfare implications of using non-surgical procedures must also be kept very much in mind.

Work in Ireland and elsewhere showed that it is possible to establish pregnancies by a non-surgical procedure involving the use of the standard Cassou (Instruments de Médecine Vétérinaire (IMV)) inseminating instrument (Boland *et al.*, 1975; Sreenan, 1975). The embryo is loaded, held in a small volume of medium (e.g. phosphate buffered saline (PBS), supplemented with 15% bovine serum), into a plastic straw (usually 0.25 ml capacity). At transfer, the straw is inserted into the inseminating instrument ('gun') in the usual way and the same procedure followed as for AI, the main difference being that the embryo is deposited around the mid-horn position (ipsilateral horn);

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Fig. 9.1. Non-surgical embryo transfer in the cow. The development of non-surgical transfer methods in the mid-1970s was a major milestone in progress towards extensive application of embryo transfer technology in cattle. Those with skill and experience in artificial insemination could be trained to become competent in embryo transfer without difficulty.

before performing the transfer, the recipient is given an epidural anaesthetic. During the past 25 years, several variants of the standard inseminating instrument have been marketed, with appropriate modifications to take account of the need to ensure that the embryo is deposited safely.

9.1.2. Requirements for on-farm applications

Among factors affecting the success and economics of a commercial ET programme, the following would come to mind: (i) the effectiveness of the superovulation treatment; (ii) the skill and experience of the ET operator; (iii) the selection and management of recipient animals, which need to be cyclic, healthy and reproductively normal; (iv) the synchrony of oestrus between donor and recipient; (v) the quality of the embryos transferred; and (vi) the methods employed in embryo handling and ET on the farm. In measuring the success rate in cattle ET, the recipient pregnancy rate is usually the most crucial criterion. In general terms, using single embryos, the pregnancy rates that are achievable after non-surgical transfer should be broadly in line with those expected for the same category of recipient animal when bred normally (i.e. 50–70% pregnant to first service). Using frozen–thawed embryos in transfers is likely to result in pregnancy rates some 10% below those found with fresh embryos.

ET technology in cattle has relied heavily on the stimulation of selected donor animals to produce several embryos after the induction of superovulation by gonadotrophin treatment. However, attention has been drawn to the lack of progress in the control of superovulation over the past half-century and the need for a greater understanding of the mechanisms underlying follicular development (Jaskowski, 1999; Webb *et al.*, 1999). The advent of *in vitro* embryo production opened up an alternative approach to getting calves from genetically superior cattle; the need is to refine procedures so that a much greater yield of oocytes per ovum pick-up (OPU) recovery session is achieved. This avenue towards a ready supply of transferable embryos is likely to include the possibility of gender preselection, which can be achieved more readily by *in vitro* fertilization (IVF) rather than cervical insemination and even more so if intracytoplasmic sperm injection (ICSI) can be adopted as a regular routine. All such developments

must have recourse to expensive equipment and laboratory-type facilities to deal with the embryo production process, which contrasts sharply with requirements for conventional cattle ET.

From research to practice

Although ET has been used in research since the 1930s, usually with laboratory species such as the rabbit or the mouse, it was in cattle that the technique first arrived on the farm. In the UK, and the same would hold true for North America, ET was initially used commercially to facilitate the introduction of various breeds of Continental cattle (e.g. Charollais, Simmental, Limousin) and to boost the size of the population in the country. The emphasis subsequently changed from beef to dairy cattle and the uptake of the technology was largely in the hands of a generation of producers who had made good use of AI in their dairy breeding programmes since the1950s. Within several countries, large-scale multiple ovulation and embryo transfer (MOET) schemes were set up in the 1980s on the basis of suggestions made by Nicholas and Smith (1983). These special breeding schemes were characterized by the systematic use of super-ovulation, multiple ovulation (MO) and ET on young female dairy cattle.

The advantage of MOET schemes lies in their potential to increase the rate of genetic gain by reducing the length of the generation interval and increasing the number of calves from genetically superior cattle. Within the recent decade, moves to incorporate new techniques into MOET schemes have been reported from several countries (Kruip *et al*., 1994; Lange, 1995; McGuirk, 1997); these include the use of OPU of oocytes for *in vitro* embryo production, embryo sexing and embryo splitting. On the wider international front, cattle ET is now an important element in the trade of genetic material; this is facilitated by the fact that cattle embryos can readily be shipped from country to country, with minimal disease risks, to enhance the speed of genetic improvement.

Embryo transfer as a research tool

It is estimated that poor fertility costs the Irish dairy industry millions of euros each year, a large part of which is due to delayed pregnancy caused by early embryo loss. Dairy farmers in Ireland, the UK, the USA and elsewhere regard low fertility as one of the most serious problems they face. For such reasons, there is increasing research emphasis in Ireland and elsewhere on factors such as nutrition, which may influence the establishment of pregnancy and embryo development in cattle (Nolan *et al*., 1998a; Gath *et al*., 1999; O'Callaghan *et al*., 1999, 2000; Boland *et al*., 2001b); ET is often an important tool used in such investigations.

9.1.3. Welfare implications of using IVP embryos

The technique of AI in cattle, which is employed in many countries to breed millions of cattle annually, poses few welfare problems or ethical concerns. When carried out by trained inseminators or suitably experienced stockmen, AI can be regarded as an acceptable method of breeding cattle without imposing unacceptable stress on the animal. The use of IVP embryos, prepared in straws and transferred directly a week or so after breeding by way of a technique similar to AI, should not pose problems or ethical dilemmas that are essentially different from those involved in AI. There is, however, the question of whether an epidural anaesthetic is routinely required for recipient cattle. It is certain enough, due to the stage of the cycle, that the risk of discomfort in the cow is greater in non-surgical ET than with routine insemination; for that reason, recipients should routinely receive the benefit of an epidural anaesthetic. There is also the point that cows at the time of transfer are in the luteal phase of the oestrous cycle, when they are more susceptible to infection than during the heat period when they are inseminated; this means strict observance of hygiene during the transfer process. About a half-century ago, Cambridge workers drew attention to the fact that low-grade uterine infections could be readily established during the luteal phase of the cow's oestrous cycle, but that these could be controlled using appropriate antibiotic cover; in ET practice, this meant providing cover by incorporating penicillin/streptomycin in the transfer medium.

Although the use of a sedative in addition to the epidural analgesic at the time of transfer may not be essential, there are those who suggest that it may be advisable and necessary for the general welfare of the recipient, for operator comfort and to achieve good pregnancy rates. In the UK, appropriately trained ET technicians can administer epidural anaesthetic under a law introduced in 1992. Also established in that country is a suitable course of instruction for cattle ET technicians at Cambridge University and the publication of a Code of Practice for ET operatives approved by the Royal College of Veterinary Surgeons.

It is in the matter of the suitability of recipients for the embryos transferred that most genuine concern must be directed; this is true whether the embryos in question are laboratory-produced or recovered from superovulated donor cattle. In the UK, the Banner committee reported in 1995 on some of the implications of new breeding techniques, such as IVF, cloning, embryo sexing and genetic modification; among other things, the committee recommended that the farming industry should be sensitive to public concerns and open to debate with interested parties. No one would disagree with such sentiments and in the recent decade there has been no shortage of sensible articles dealing with animal welfare concerns.

9.2. Preparing Embryos for Transfer

9.2.1. Media employed

In the early Cambridge work on the recovery and transfer of cattle embryos, tissue-culture medium 199 (TCM-199) was commonly employed as the holding medium; the medium, however, does require a gas atmosphere of 5% carbon dioxide for its buffer system. During the second half of the 1970s, workers started using Dulbecco's PBS (see Table 9.2), supplemented either with glucose, sodium pyruvate and bovine serum albumin (BSA) or with fetal calf serum (FCS); this medium is widely recognized as being particularly effective. Commercially available FCS is usually added in volumes of 1% for flushing and 10–20% for storage; as a source of macromolecules, homologous or heterologous blood serum or its albumin fraction has been regarded as an essential component of culture media. In the early 1990s, however, the need to avoid possible diseases risks and the growth of IVP systems led to interest in alternatives to Dulbecco's PBS; in the USA, Nelson and Nelson (2001) note that the two most widely used alternatives have been the zwitterion-buffered Emcare medium and the HEPES-buffered Vigro Plus preparation.

Antibiotic/antimicrobial cover

It is standard practice to add antibiotics to media used in the collection, handling and transfer of cattle embryos (see Riddell *et al.*, 1995; Riddell and Stringfellow, 1998). Popular antibiotics to control bacteria include penicillin G (50–100 iu/ml), streptomycin sulphate (50–100 μg/ml) and kanamycin (100 μg/ml); to deal with fungal contamination, mycostatin (50 u/ml) or amphotericin B (0.25 μg/ml) can be used.

Serum and serum substitutes

There are, as noted elsewhere, persuasive animal-health reasons for not using serum or BSA in media used with cattle embryos, particularly embryos destined for international trade. A series of experiments was designed by Palasz *et al.* (1995) in Canada to examine the role of serum or serum substitutes in media used for

Table 9.2. Dulbecco's phosphate-buffered saline (D-PBS).

To make 10 l:		
$CaCl_2$-$2H_2O$	1.32 g	A
$MgSO_4$-$7H_2O$	1.21 g	A
NaCl	80 g	B
KCl	2 g	B
Na_2HPO_4	11.5 g	B
KH_2PO_4	2 g	B
Glucose	10 g	B
Streptomycin sulphate	0.5 g	B
Na pyruvate	0.36 g	B
Na penicillin G	1,000,000 units	B

A: May be weighed in advance and stored indefinitely in a sterile bottle under refrigeration.
B: May be weighed in advance and stored in sterile bottle under refrigeration for 1 month. Do not mix with $CaCl_2$ and $MgSO_4$ until just before use.

cattle ET procedures. These authors showed that biological sera or BSA could be replaced in cattle embryo collection, holding, culture and freeze–thawing media by the chemically defined surfactant VF5 (Vetrapharm). It was suggested that the surfactant properties of sera or BSA are particularly important in the handling of 7-day-old cattle embryos on a short-term basis.

Tropical environment

There is increasing use of cattle ET in the countries of Latin America and reports dealing with problems specific to such regions are available. A paper by Barros and Benyei (2000) dealt with work at an ET centre located in the semi-arid region of Brazil, where they compared zwitterion- and PBS-based embryo handling solutions; their results suggested that ET practitioners could use either solution for collecting and transferring fresh embryos in a tropical environment. It was noted, however, that zwitterion buffer-based solutions should be used for frozen–thawed embryos.

Handling cattle embryos

In dealing with the manipulation of cattle embryos destined for transfer, where they inevitably come in contact with glassware, Petri dishes, plastic straws and other objects, their possible exposure to toxic factors must always be a serious consideration; this is likely to be much more of a concern under commercial on-farm conditions than in the research laboratory. It is, for example, essential to ensure adequate aeration of straws after their sterilization with the powerful antimicrobial ethylene oxide. Various reports have dealt with the safety of the wide range of products employed in routine cattle ET operations; sound hygiene at all stages is necessary for success (see Mapletoft and Stookey, 1998).

9.2.3. Protecting the embryo

There are several problems associated with non-surgical embryo transfer in cattle which may influence the establishment of a pregnancy. As a result of its microscopic size, it is difficult to confirm that the embryo has been deposited in the uterine horn at a site appropriate for its subsequent development. Although in a research setting, a transfer straw can be inspected after removal to ensure that the embryo is not retained, this does not necessarily constitute proof of transfer; the embryo may have adhered to the exterior of the straw during withdrawal from the uterus and cervical canal.

Embryo encapsulation technology

There has been some research interest in encapsulating the bovine embryo in a semi-permeable and biodegradable material on the assumption that this might eliminate some of the transfer problems and improve the protection of the embryo. In the mid-1980s, German workers reported a method of encapsulating cattle embryos in microspheres measuring 3–4 mm in diameter; embryos were held in culture medium within the capsule without this apparently compromising their viability. A paper by Yaniz *et al.* (2000) reported the effect of sodium alginate encapsulation of day-4 IVP cattle embryos on rates of development and hatching; they concluded that the encapsulated embryos could develop to the blastocyst stage in co-culture with bovine oviduct epithelial cells at least as readily as controls. The same workers suggested that encapsulation offered better identification of embryos and reduced the effect of environmental factors.

Predicting embryo hatching

Hatching of the bovine blastocyst from the zona pellucida is a key event in embryo development; without hatching, pregnancy will not be established. A study reported by Schmoll *et al.* (1998) led them to conclude that zona pellucida diameter and thickness evaluated on day 7 could be used as a marker for the hatching of IVP blastocysts in their Charles Rosenkrans 1 (CR1) culture system.

9.2.4. Number of embryos transferred

More than three decades ago, Rowson in Cambridge showed that the surgical transfer of two embryos to recipient cattle could result in high pregnancy rates; similar evidence was apparent

from studies carried out elsewhere and there is now much in the literature to support the view that higher pregnancy rates can be achieved using two embryos rather than one. Work in France, using frozen–thawed embryos, clearly demonstrated how pregnancy rate was enhanced after transfer of two embryos rather than single or half-embryos (Table 9.3). Without attempting unnecessary comparisons, it is well established in human assisted reproduction that single embryo transfers usually result in unacceptably low pregnancy rates. How far improved pregnancy rates are due to factors associated with maternal recognition of the embryo remains uncertain. In cattle, it is possible to exploit possible connections between embryo survival and enhanced maternal–embryo recognition signals in various ways, including the use of trophoblastic vesicles (TVs). In Japan, Hashiyada *et al.* (2001) demonstrated that pregnancy rate was significantly enhanced (68% vs. 39%) when half-embryos were transferred with TVs rather than without; the authors concluded that the vesicles enhanced pregnancy rates by increasing the embryonic signal strength.

It may also be noted that some pregnancy maintenance strategies in pigs have involved the co-transfer of parthenogenetic embryos with fertilized embryos. In Scotland, King *et al.* (2002) showed for the first time that such a strategy may be a feasible alternative to hormonal treatment for supporting fertilized embryo development to term; they co-transferred three fertilized embryos with 55–60 parthenogenetic embryos to each of six recipients and recorded the birth of two live piglets from one of the host mothers.

9.3. Surgical and Non-surgical Transfers

9.3.1. Surgical transfers

The work of Rowson at Cambridge in the late 1960s marked an important turning-point in cattle ET prospects by showing that an acceptable pregnancy rate could be achieved, albeit by way of a surgical technique. The midventral laparotomy technique employed in these Cambridge studies involved general anaesthesia, with surgical preparation of the midline just anterior to the mammary gland of the recipient. Having brought the uterus to the site of incision and confirmed the location of the corpus luteum (CL), a small puncture was made in the uterine wall to provide access to the lumen of the uterine horn ipsilateral to the CL; the transfer pipette was introduced through the puncture and the embryo deposited. Although the midventral procedure could be carried out quickly on heifers, it was clearly not a procedure for use on the farm; as well as being both labour- and capital-intensive, the technique was not at all suitable for milking cows.

Where surgical transfers are now performed, most commercial ET operators have adopted the flank surgical approach, with the recipient standing sedated and under appropriate analgesia (paravertebral block). The site of incision is the sublumbar fossa and the embryo is transferred into the upper third of the uterine horn. Prior to surgery, the location of the CL is established, to identify the side on which the transfer should be made. In comparison with the midventral procedure, the flank approach is widely recognized as being a highly effective, practical and rapid means of performing surgical

Table 9.3. Pregnancy rates in recipients after transfer of half-embryos, embryos or two embryos (from Heyman and Chesne, 1984).

	Group 1	Group 2	Group 3
No. of embryos transferred	½	1	2
No. of recipients	20	36	22
No. presumed pregnant at day 21 (%)	10 (50)	24 (66.6)	17 (77.2)
No. pregnant (% in parentheses)			
at day 45	6 (30)	18 (50.0)	16 (72.7)*
at day 60	5 (25)	17 (47.2)	15 (68.2)*
at day 90	4 (20)	16 (44.4)	14 (63.6)

*Significantly greater than group 1 for each stage of pregnancy control ($P < 0.025$) by χ^2 analysis.

transfers with a minimum of equipment, facilities and labour.

As to the reasons for using the surgical approach, this is likely to be in an effort to give the recipient the greatest opportunity of becoming pregnant with the embryos that are available. According to some commercial operators, if the embryo has been frozen and thawed or is of second-grade quality, a 10% or so improvement in pregnancy rate may be achieved with the flank transfer approach, in comparison with non-surgical methods.

Endoscopy and tubal transfer of embryos

As shown in recent studies in Ireland, there is evidence strongly supporting the view that the quality of the artificially matured bovine oocyte determines the yield of IVP blastocysts; it is currently the conditions of embryo culture that determine the quality of these blastocysts (Enright *et al.*, 2000a,b; Lonergan *et al.*, 2001a,b,c, 2002; Gutierrez-Adan *et al.*, 2002; Rizos *et al.*, 2002a,b). Ample evidence exists to show that culture in the sheep oviduct results in cattle embryos that are more viable than those produced by any of the current *in vitro* culture systems. For such reasons, some workers have attempted to develop transfer procedures that enable IVP early-stage embryos to be placed in the oviduct of the recipient animal. In Austria, Besenfelder and Brem (1998) developed an endoscopically mediated transvaginal method for transferring early-stage embryos (two-cell to morula) to the oviducts of recipient cattle.

Although surgical procedures and endoscopic cannulation via the cow's paralumbar region are not acceptable for routine applications with IVP embryos, the procedure described by the Austrian workers is similar to the endoscopy technique used in the repeated recovery of oocytes for *in vitro* embryo production (Reichenbach *et al.*, 1994a,b).

9.3.2. Non-surgical transfers

Although numerous instruments designed specifically for the non-surgical transfer of cattle embryos were described in the literature during the 1960s and early 1970s, it was the successful application of the Cassou AI gun that eventually proved to be the solution to this particular problem.

Factors affecting success

There are a number of embryonic, maternal and environmental factors that may affect the pregnancy rates established in recipient cattle after non-surgical transfer (see Fig. 9.2). In many instances, such reports have dealt with transfer of IVP embryos rather than those produced *in vivo* (Avery *et al.*, 1994; Ling *et al.*, 1995; Lonergan *et al.*, 1995; Dayan *et al.*, 2002). Using heifers as recipients, there have been those reporting that in some 10% of such animals it is difficult, if not impossible, to carry out ET via the cervix. The embryo should always be transferred to the uterine horn associated with the ovulating ovary (ipsilateral horn); this is in the light of evidence showing that pregnancy rates are higher when single transfers are made to the ipsilateral rather than the contralateral horn. It is believed that maternal recognition of the embryo is more positive when it is in the ipsilateral horn. In Japan, Cerbito *et al.* (1994) found evidence that progesterone level and its distribution in the uterus are dependent on luteal function and CL location; this may be a factor influencing the survival of the embryo in early pregnancy.

In vivo *embryos*

The single most important factor affecting the success of transfers is embryo quality, according to Janowitz (1994) in an analysis carried out on 2478 transfers of fresh or frozen *in vivo*-produced embryos in Germany. Other work in the same country by Piturru (1994) recorded a pregnancy rate of 59% after transfer of 292 fresh embryos in Piedmont cattle. A review by Dawson (2000) has described the non-surgical technique for ET used in the UK and discussed factors affecting its success, including management factors, the collection and handling of embryos and the transfer itself; the article presents a timetable for a typical ET programme and the legal requirements for the technique.

In vitro *embryos*

Pregnancy rates recorded in commercial ET programmes have been lower for IVP embryos than for those normally achieved with *in vivo* embryos (Looney *et al.*, 1994; Van Wagtendonk-De Leeuw *et al.*, 1998; Bousquet *et al.*, 1999; Hasler, 2000a,b). An example of such rates is given in Table 9.4, which refers to pregnancy rates in Friesian heifer recipients over a 4-year period. The highest rate was achieved with grade 1, fresh day-7 *in vivo* embryos; significantly lower pregnancy rates were evident, in descending order, by *in vivo* frozen day-7, fresh day-7 IVP, fresh day-8 IVP, frozen day-7 IVP and, finally, frozen day-8 IVP embryos.

Operator skill

One important factor influencing the pregnancy rate is likely to be the skill and experience of the transfer operator, especially in the matter of avoiding trauma to the endometrium. For optimal results, according to Hasler (2002), the embryo should be placed halfway up the uterine horn ipsilateral to the CL; elsewhere, and based

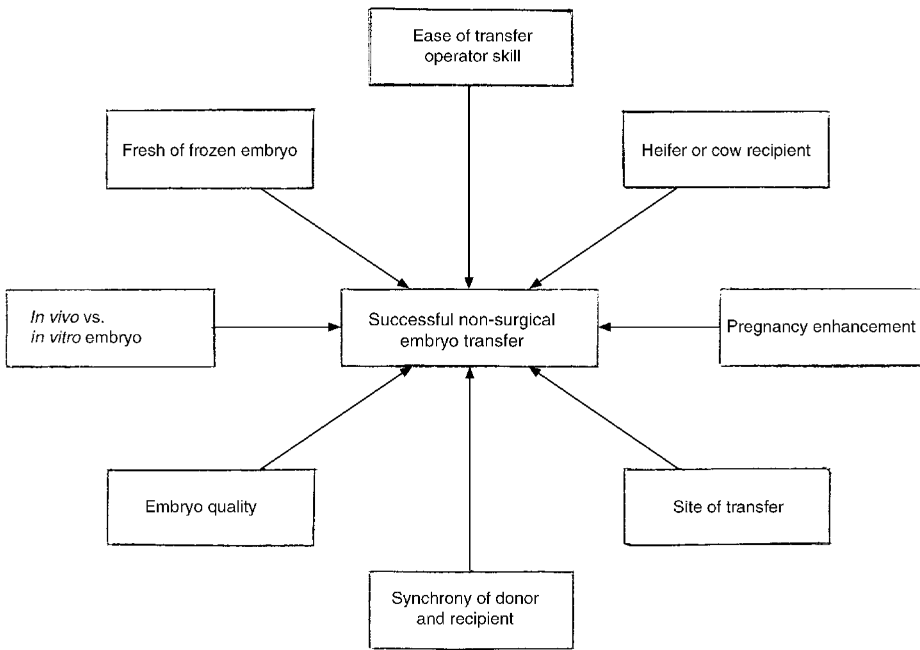

Fig. 9.2. Factors affecting success of non-surgical embryo transfer in cattle.

Table 9.4. Pregnancy rates after transfer of fresh or frozen *in vivo* and IVP embryos into Friesian heifer recipients at one location (from Hasler, 1998).

Embryo	Embryo age (days)	No. of transfers	Percentage pregnant: Embryo grade 1	Embryo grade 2	Embryo grade 3	All grades
IVF – fresh	7	4606	56[a]	41[a]	–	54[a]
IVF – fresh	8	462	48[b]	30[b]	–	43[b]
IVF – fresh	9	22	41	–	–	41
IVF – frozen	7	67	42[b]	–	–	42[b]
IVF – frozen	8	30	20[c]	–	–	20[c]
In vivo-fresh	7	599	76[d]	67[c]	56	67[d]
In vivo-frozen	7	517	64[e]	–	–	64[d]

[a,b,c,d,e]Values within a column with different superscripts differ ($P < 0.05$).

on more than 2000 transfers, Callesen *et al.* (1994) reported that pregnancy rates were lower for the basal third of the horn than for the middle or apical regions of the horn. There are indications that ET to the horn of the cow's previous pregnancy may affect the outcome of transfer; Callesen *et al.* (1998) recorded a 39% pregnancy rate for ipsilateral vs. 45% for contralateral transfer. The importance of avoiding damage to the endometrium or otherwise traumatizing the uterus during the transfer process cannot be overemphasized. Introducing the transfer instrument for some distance up the uterine horn, however, clearly increases the likelihood of such trauma unless appropriate care is taken by the operator. There is evidence showing that good pregnancy rates are achieved when transfers take place quickly and smoothly; some operators may be too slow, as well as being less careful than those that are suitably skilled. In Portugal, Chagas e Silva *et al.* (1999) recorded that the difficulty of transfer significantly affected pregnancy rate in their ET programme (53% for easy vs. 35% for difficult transfers).

In identifying the ipsilateral horn, it is necessary for the operator to palpate the CL *per rectum* before carrying out the transfer. Only a 5% difference in pregnancy rates has been found between recipients evaluated as possessing a good rather than a poor CL. Although the size and consistency of the CL may bear little relationship to the probability of establishing a pregnancy, palpating the ovaries and detecting the CL, which is occasionally well buried in the ovary rather than clearly protruding, calls for skill and experience. The importance of adequate restraint of the recipient animal during rectal palpation and the transfer procedure, to avoid unexpected movements, should always be appreciated; the epidural injection should eliminate rectal contractions. The dose of local anaesthetic should be such as to ensure that the recipient remains standing throughout the transfer procedure.

9.4. Donor–Recipient Synchrony

A considerable body of evidence has accumulated over the years on the importance of synchrony between donor and recipient in terms of stage of the cycle. Exact synchrony should be the aim, but recipients out of phase by ± 1 day can be regarded as acceptable; this means that a 7-day embryo can be transferred into a recipient that showed heat 6–8 days earlier. Recipients that are out of synchrony by as much as 2 days would not normally be used because of the reduced pregnancy rates. There are, however, some circumstances in which exact synchronization may not be so essential for embryo survival. A report by Hashiyada *et al.* (2002) in Japan suggested that day-14 bovine conceptuses have the ability to develop in asynchronous uterine conditions; they showed the wide adaptability of the day-14 conceptus by establishing pregnancies in day-7 to day-14 recipients. It is apparent that the day-14 conceptus secretes signals capable of maintaining pregnancy even in asynchronous uterine conditions.

9.4.1. Importance of synchronization

Rowson at Cambridge in the late 1960s was one of the first to show that exact synchronization between embryo age and recipient cycle stage resulted in the optimum pregnancy rates and that a degree of variation between donor and recipient of ± 1 day could be tolerated. In the light of cattle ET experience over the years since that Cambridge work, the aim would still be to have exact synchrony (Hasler, 2002). Such close synchrony must be regarded as essential because the embryo and the maternal uterine tissues form a complex communication system involving secretions from the embryo as well as from the uterine endometrium; such secretions stimulate and mediate changes during early pregnancy in the cow. In Germany, in an analysis of 2478 transfers, it was shown by Janowitz (1994), that when the occurrence of oestrus in recipients deviated by −48, −24, 0, +24 or +48 h (the minus sign indicating that oestrus in the recipient preceded that in the donor), pregnancy rate was 23.8%, 52.2%, 58.2%, 49.5% and 44%, respectively. In the USA, in a large-scale trial with beef cattle, Spell *et al.* (2001) concluded that there was no need to deviate from the normal practice of carrying out synchronous transfers where possible; they found that asynchrony of 24 h between

recipient and donor did not decrease pregnancy rate. In Arkansas, Rorie *et al.* (2002) have pointed to the advantage of using electronic oestrus detection (HeatWatch) in being able to determine the exact synchrony between embryo donors and recipients. Over a 3-year period, they used monitors to determine exact (± 0.5 h) synchrony; results (see Table 9.5) showed that pregnancy rate was significantly higher for synchrony of ± 0 to 12 h than for synchrony of ± 12 to 24 h (63% vs. 50%, respectively).

Accuracy of oestrus detection

A paper by Lester *et al.* (1999) had observed that, when embryo recipients and donor cows are observed twice daily for oestrus, a difference of several hours can occur between the actual and 'detected' onset of ostrus; they used the electronic oestrus detection system (Heat Watch) mentioned above to continuously monitor recipient animals that had been synchronized with prostaglandin (PG). As with the later data of Rorie *et al.* (2002), a higher pregnancy rate was apparent with closer synchrony (± 0–12 h) between embryo and recipient (58% vs. 48%).

9.4.2. Synchronization in the IVP embryo context

As a result of laboratory embryo production, day-7 blastocysts can be made available for transfer to recipients that are at day 7 of their oestrous cycle. This would be regarded as exact synchrony between donor (embryo age/stage) and recipient. It should also be noted that blastocysts can develop over a period of several days (from day 7 to day 10 and even beyond these limits). However, it is generally recognized that IVP embryos that reach the blastocyst stage at 7 days post-insemination are of higher quality than those forming blastocysts later. The aim with laboratory embryo production should be to produce blastocysts for transfer to day-7 recipients. However, it should be emphasized that retarded development of an IVP blastocyst may be symptomatic of defects that cannot be overcome simply by an adjustment in embryo/recipient synchrony; in other words, *in vitro* blastocysts should not be transferred at all if they are too far out of phase (stage for age) with what would be regarded as normal for *in vivo* embryos.

Table 9.5. Effect of embryo–recipient synchrony on pregnancy rates (from Rorie *et al.*, 2002).

Oestrus synchrony category (h)	Number of embryo transfers	Pregnancy rate (mean ± SEM)
–12 to –24	37	51.4 ± 8.2
0 to –12	67	58.2 ± 6.1
0	9	66.7 ± 16.6
0 to +12	78	61.5 ± 5.6
+12 to +24	37	48.6 ± 8.2
± 0 to 12	126	62.7 ± 4.4[b]
± 12 to 24	102	50.0 ± 4.9[a]

[a,b]Numbers within columns with different superscripts differ ($P = 0.054$).
SEM, standard error of the mean.

9.5. Oestrus Synchronization Techniques

Oestrus control in recipient animals is an essential element of cattle ET technology; by its application, a number of recipients can receive embryos at the same time, rather than at the time decided by their oestrous cycle. There has been some controversy as to whether an induced oestrus prior to transfer is less suitable than a spontaneous one (Callesen *et al.*, 1996). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been one of the most commonly used treatments and oestrus has been synchronized with progesterone or progestogens, contained in a variety of intravaginal devices or ear implants. As well as the synchronizing treatment, there is also the crucial need to detect oestrus accurately when it does occur. Of the various difficulties in dealing with cattle that are to be inseminated or are to receive an embryo, the single most important one may be poor detection of oestrus. In the most recent decade, new possibilities for oestrus sychronization in recipient cattle without the need for oestrus detection have been examined.

9.5.1. Protocols for synchronizing oestrus

Synchronization of oestrus in recipient cattle may be achieved by several means, including

the use of PGs (single or double injection regimens), progestogen ear implants (variously marketed as Synchromate-B or Crestar) and intravaginal devices, such as the progesterone-releasing intravaginal device (PRID) or the controlled internal drug release (CIDR); an injectable microsphere formulation of progesterone has been a more recent addition to the range of products available (Whisnant and Burns, 2002). A review by Bo *et al.* (2002) has dealt with recent protocols designed to control luteal and follicular function with ET in recipient cattle without the need for oestrus detection.

Studies in the 1970s showed that the maturity of the CL at the time of $PGF_{2\alpha}$ treatment influenced the luteolytic response and that the PG did not effectively induce luteolysis in the first 5 or 6 days of the oestrous cycle. Where oestrus was induced, however, it was often exhibited over several days, effectively ruling out fixed-time ET. It is now known that the interval from PG treatment to oestrus and ovulation is determined by the stage of development of the dominant follicle at the time of PG administration. If PG is given when the dominant follicle is in the late growing or early static phase, ovulation will occur within 3 or 4 days. If treatment is initiated when the dominant follicle is in the mid- to late-static phase (i.e. when it is no longer viable), this will result result in ovulation of the dominant follicle from the next follicular wave, 5–7 days later. The duration of the interval between PG and the expression of oestrus is a reflection of the time required for the dominant follicle of the new follicular wave to develop to the preovulatory stage; it is clear that control both of luteal function and of follicular development is necessary to achieve precise oestrus control.

Ovsynch

Among protocols developed in recent years is one that uses an initial gonadotrophin-releasing hormone (GH-RH) injection (100 μg) to induce ovulation of a dominant follicle and the formation of a CL. In cyclic dairy cattle, it can be expected that about 60% of animals given GH-RH, depending on their stage of cycle, will ovulate a follicle in response to the luteinizing hormone (LH) surge initiated by the releasing hormone. Following this induced ovulation, a new wave of follicles emerges in the ovaries within 48 h, from which a new dominant follicle develops. Seven days after injection of GH-RH, $PGF_{2\alpha}$ is given to induce luteolysis in all the CL (those present at the time of the GH-RH injection and those formed after its administration). In the course of the next 48 h, the new dominant follicle matures and a second dose of GH-RH is given 2 days after the PG, to induce LH release and synchronize ovulation of the new dominant follicle. Cows destined for insemination are bred either when they show oestrus or at a fixed time interval of 16 h after GH-RH (Geary *et al.*, 1998; Pursley *et al.*, 1998).

The Ovsynch protocol has also been employed to synchronize ovulation in the recipients of embryos produced *in vivo* (Hinshaw, 1999; Baruselli *et al.*, 2000, 2001; Zanenga *et al.*, 2000) and *in vitro* (Ambrose *et al.*, 1999) embryos. Elsewhere, researchers have conducted field trials with sizeable numbers (1637 recipients) treated with an Ovsynch in combination with a preliminary Synchromate-B or CIDR device without oestrus detection (Fig. 9.3). Such studies have been reviewed by Bo *et al.* (2002), who noted that acceptable pregnancy rates can be achieved when embryos are transferred to recipients without the need for oestrus detection.

Progesterone/progestogen

Attempts to control oestrus in the cow date back to those of Casida and colleagues in Wisconsin a half-century ago; most of the early studies were with the natural steroid, progesterone, and it became clear that, although oestrus and ovulation could be controlled with some degree of accuracy, the conception rate was usually unacceptably low. At this point in time, it is well accepted that long-term progesterone/progestogen treatments of greater than 12 days in length and those started late in the oestrous cycle result in high oestrous responses but poor pregnancy rates. More than three decades ago, Wiltbank and associates showed that the duration of progestogen treatment could be shortened to about 9 days if oestradiol was administered at the time of initiating the synchronization treatment; the oestrogen injection induced luteal regression. Although there have been reports of satisfactory pregnancy rates in recipients synchronized with progesterone/progestogen devices (Looney *et al.*, 1999), there have also been those reporting

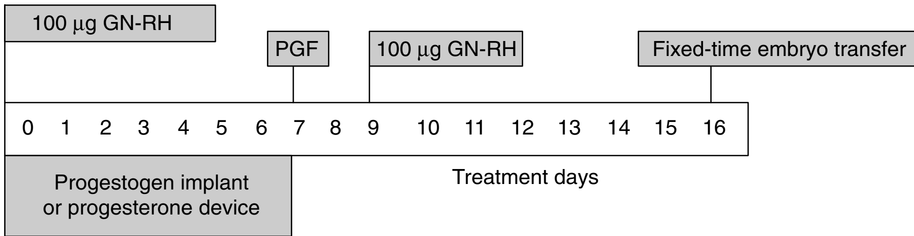

Fig. 9.3. Treatment protocol for fixed-time embryo transfer in cattle with an Ovsynch programme or Ovsynch plus a progestogen/progesterone-releasing device (Ovsynch + P). The Ovsynch treatment consisted of an injection of GH-RH on day 0, followed by prostaglandin $F_{2\alpha}$ (PGF) on day 7 and a second injection of GH-RH on day 9. In the Ovsynch + P treatment, a progestogen ear implant or a progesterone-releasing device is placed on day 0 and removed on day 7. Oestrus is not observed, and embryo transfer is performed on day 16; recipients with a CL > 12 mm receive an embryo. (From Bo *et al.*, 2002.)

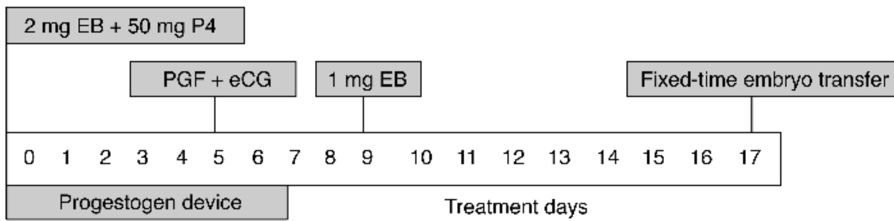

Fig. 9.4. Fixed-time cattle embryo transfer protocol using progesterone device. Treatment consisted of insertion of a progesterone-releasing device and intramuscular administration of oestradiol benzoate (EB) plus progesterone (P4) on day 0, prostaglandin $F_{2\alpha}$ (PGF) and 400 iu eCG on day 5, device removal on day 8 and EB on day 9. Oestrus is not observed, and embryo transfer is performed on day 17; recipients with a CL > 10 mm receive an embryo. (From Bo *et al.*, 2002.)

reduced rates (Tribulo *et al.*, 1995). One of the protocols employing a progesterone-releasing device in conjunction with other treatments is shown in Fig. 9.4.

Conventional cattle ET programmes, utilizing PG treatment and oestrus detection in recipients, with selection of those suitable for transfer a week later, are often faced with high animal costs. According to Tribulo *et al.* (2002), such programmes involve considerable time in oestrus detection and the proportion of recipients used over those actually treated rarely exceeds 50%. The authors reported an experiment to evaluate whether treatment with a low dose (400 iu) of pregnant mare serum gonadotrophin (PMSG) (equine chorionic gonadotrophin(eCG)) on day 5 in cows treated with progesterone-releasing devices (DIV-B, Syntex, Argentina) and oestradiol benzoate would increase pregnancy rates (see Table 9.6). Although treatment with PMSG did not result in multiple ovulations (2% with two CLs), the mean diameters of CLs were significantly larger than in those recipients not receiving gonadotrophin; treatment resulted in a significant enhancement of the pregnancy rate (58% vs. 42%). The authors suggest that gonadotrophin treatment may have led to an increase in the plasma progesterone concentration.

Table 9.6. Effect of low-dose eCG (PMSG) treatment on pregnancy rates in embryo recipients (from Tribulo *et al.*, 2002).

Group	*n*	Transferred/ treated (%)	Pregnant/ transferred (%)	Pregnant/ treated (%)
Control	156	127/156 (81.4%)[a]	53/127 (41.7%)[a]	53/156 (33.9%)[a]
eCG	156	132/156 (84.6%)[a]	76/132 (57.6%)[b]	76/156 (48.7%)[b]

[ab]Proportions with different superscripts are different ($P < 0.02$).

A further study by the group in Brazil, reported by Moreno *et al.* (2002), evaluated the effect of advancing the time of PG administration in their progesterone–oestradiol synchronization protocol as a means of increasing the number of suitable recipients. They employed the same basic protocol as that shown in Fig. 9.4. Recipient Angus cows received a progesterone-releasing device (DIV-B) and 2 mg oestradiol benzoate plus 50 mg progesterone on day 0 and PG on day 4; they received 1 mg oestradiol benzoate on day 9, and day 10 was regarded as the day of oestrus. Suitable recipients were selected on day 16 and ET was carried out on day 17. The rationale of treatment was that early luteolysis would result in a low progesterone level, leading to a high LH pulse frequency, which would stimulate the growth of the dominant follicle and form a larger CL after ovulation. Although the treatment with PG on day 4 did not increase CL size, it did increase the proportion of recipients selected for ET and the overall pregnancy rate (see Table 9.7).

Table 9.7. Effect of timing of PG dose in progesterone-synchronized recipients on pregnancy rate (from Moreno *et al.*, 2002).

Group	*n*	Transferred/ treated (%)	Pregnant/ transferred (%)	Pregnant/ treated (%)
PGF day 4	95	67/95 (70.5)[a]	39/67 (58.2)[a]	39/95 (41.1)[a]
PGF day 8	93	49/93 (52.7)[b]	20/49 (40.8)[a]	20/93 (21.5)[b]

[a,b]Proportions with different superscripts are different ($P < 0.02$).

Not all treatments leading to increased luteal function have been followed by improvements in pregnancy rates. Experiments by Nogueira *et al.* (2002) in Brazil have shown that high levels of progesterone, induced by PMSG, decreased pregnancy rates in recipient heifers; these authors used doses of PMSG ranging from 200 to 600 iu to increase progesterone levels but found that pregnancy rates decreased rather than increased at the highest progesterone concentrations. The authors selected their recipients on the basis of possessing multiple CLs or having a single CL larger than 15 mm in diameter.

9.6. Selection and Management of Recipients

9.6.1. Heifers versus cows

Heifers are usually preferred as recipients in conventional cattle ET operations (Callesen *et al.*, 1996); quite apart from being free of problems arising from previous pregnancies (e.g. endometritis), such animals are likely to be easier to acquire than cows and to achieve higher pregnancy rates. However, in terms of ease of transfer through the cervix, the parous cow may have an advantage; at the other end of pregnancy, taking calf survival and viability into consideration, there may be no great difference from heifers in the final outcome (Callesen *et al.*, 1994). In Portugal, Chagas e Silva *et al.* (1999) showed that heifers were good recipients for both fresh and frozen embryos while cows only showed acceptable pregnancy rates with fresh embryos. A subsequent report by Chagas e Silva *et al.* (2002) showed that, in lactating dairy cattle, low progesterone levels on day 7 could adversely affect embryo survival, an effect not evident in heifer recipients; probably as a result of the lower competence of the CL at day 7, lactating cows were more prone to embryo loss than heifers, especially when frozen–thawed embryos were used. It is recognized that, in the dairy cow, luteal function can be negatively affected by nutritional and metabolic factors (Lamming and Darwash, 1995).

A paper by Jaskowski and Zbylut (1999) dealt with the selection of cows and the qualities required of recipients. In France, Ponsart *et al.* (2000) conducted a field survey to investigate the sources of variation in pregnancy rates after transfer of frozen–thawed embryos in dairy cattle; they concluded that the use of ethylene glycol rather than glycerol + sucrose had improved the success of transfer, but the choice of recipient was still an important cause of variation in pregnancy rates. They recorded rates of 52% in heifers, 51% in primiparous cows and a significantly lower 40% in multiparous cows. They also found that minor asynchrony between donor and recipients, the type of oestrus (spontaneous or induced) before transfer and paternal origin of embryos did not significantly affect results.

In Denmark, Jacobsen *et al.* (2000a,b), taking note of a slight increase in birth weight and the incidence of dystocia with IVP-derived calves in their studies, suggested that choosing cows as recipients should be considered.

9.6.2. Factors affecting recipient suitability

The success of ET depends on factors associated with the embryo, the recipient or an interaction among factors associated with the embryo and recipient. A paper by Garcia and Salaheddine (2000) dealt with the ultrasonic morphology of CL and central luteal cavities during the selection of recipients for ET. Using transrectal ultrasonography to evaluate ovarian structures, Spell *et al.* (2001) concluded that the best assessment of the suitability of a potential ET recipient is an observed oestrus and a palpable CL, regardless of size or quality; they did not find progesterone levels at the time of transfer predictive of pregnancy rates. In Italy, a study by Veronesi *et al.* (2002) in Friesian dairy cows led them to conclude that the ultrasonographic appearance of tissue was a more reliable method for assessing the mid-cycle CL than measuring its diameter.

Recipient hormone levels

The relationship between plasma progesterone profiles and pregnancy rate in heifers is controversial. In Portugal, a study by Chagas e Silva *et al.* (1999) recorded a highly significant correlation between progesterone levels on day 7 and day 21 in pregnant animals. They concluded that viable embryos stimulate luteal function and suggested that either a failure in CL stimulation by an embryo of poor viability or the presence of a non-competent CL will probably lead to pregnancy failure.

Plasma urea nitrogen

Nutrition is seen by many to be a key factor in recipient management and one that affects all aspects of cattle reproduction. It has been suggested that the protein and energy balance of the diet can affect the fertility of cows and that its plasma urea nitrogen (PUN) content may be an indicator of reproductive ability (Park, S.B. *et al.*, 2000); on the basis of results presented, there is need for much more evidence before such a test can be accepted as a useful indicator of recipient fertility.

Repeated transfers

Recipients that fail to become pregnant after ET can be reused one or several times before being finally excluded from a transfer programme. In some studies, it has been shown, with both cows and heifers, that the pregnancy rate achieved with repeat transfers does not differ from that following initial transfers (Callesen *et al.*, 1994). It is believed that non-pregnancy after ET may just be a matter of chance; there appears to be no evidence that an unsuccessful transfer impairs the ability of the recipient to conceive.

Role of the major histocompatibility complex (MHC)

A paper by Aguilar *et al.* (1997) discussed the role of the MHC in reproduction and presented results from their experiments on compatability vs. incompatability between embryo and recipient in ET in cattle; they showed that MHC antigens are selectively recognized by the immune system of the recipient and suggest that they can influence the outcome of pregnancy. It was found that fully compatible combinations tended to be less successful than incompatible ones; if such results are borne out in further work, there may be a case for typing recipients and embryos as a possible route towards influencing pregnancy rate.

9.6.3. Minimizing stress in recipients

Stress is revealed by the inability of the cow to cope with its environment (Dobson and Smith, 2000). The importance of minimizing stress in recipient animals is rightly emphasized in various reports (Dobson *et al.*, 2001). It was shown by Lowman *et al.* (1994) that cattle subjected to a 5-mile walk over a hill 2 days after insemination were less fertile than non-stressed herd-mates: not exactly a likely scenario for most recipients, but there are probably more subtle causes of stress that need to be recognized. Any routine treatment (e.g.

antiparasitic) should take place at least 3 weeks prior to transfer; changes in the feeding regimen should be prohibited for 3–4 weeks before and after transfer. Recipients should be located where they can be easily and quietly handled on the day of transfer. Dobson *et al.* (2001) note that members of ET teams are likely to be strangers to donor and recipient cows; in lessening such effects, the authors observe that animals associate positively with people who offer feed rewards, pat them and speak in a pleasant voice. The same authors stress the need for ET teams to examine their procedures to reduce stress and improve the welfare of all animals involved in their activities.

Tranquillization

Japanese workers reported on Friesian heifer cattle given 20 mg xylazine before embryo transfer (Nishigai *et al.*, 1997); pregnancy rate was higher in the xylazine group than in controls. They also showed that the percentage of recipients requiring 5 or more minutes for completion of ET was lower (10% vs. 19%) and that the percentage of recipients showing evidence of transfer difficulty was lower (13% vs. 22%).

Welfare concerns

The use of certain categories of maiden heifers as recipients may pose very real welfare problems. In the UK, for example, the use of beef-type heifers as recipients for embryos from large dairy breeds (e.g. Friesian) and double-muscled beef breeds has occasionally resulted in a proportion requiring surgery to deliver the fetus. It is clearly undesirable for unsuitable embryos to be transferred to recipients. Legislation has been enacted in several countries to prohibit transfer of embryos likely to produce large calves (Dobson *et al.*, 2001).

9.6.4. Detecting early pregnancy in recipients

The aim of ET is to establish pregnancy in the recipient; for various good practical reasons, the sooner it can be established that this has been achieved, the better (see Table 9.8). Using ultrasonography in cows, it is difficult to detect pregnancy with accuracy earlier than day 25 after breeding. A report by Dovenski *et al.* (1999) demonstrated reasonable accuracy (84.3%) using ultrasonics to visualize the ovaries of the cow on day 21 after AI. In a further report, Dovenski *et al.* (1999) examined the possibility of predicting pregnancy 2 weeks after ET (day 21 post-oestrus) on the basis of measurements of ovarian structures by ultrasonography. Criteria for positive diagnosis included CLs with 'good echogenicity' and at least 22 mm in diameter and ovarian follicles no larger than 13 mm. Data demonstrated the high accuracy of the test, with the ability to detect pregnancy and non-pregnancy with 84.2% and 100% accuracy, respectively. The authors concluded that their method could be a suitable and promising test for practical application in the ET industry. In Hungary, Szenci *et al.* (2000) noted that, although the bovine embryonic disc in the early stages of pregnancy (10–20 days) is usually round, in some proportion of animals (27%) it can be oblong; under field conditions, they found that acceptable results with ultrasonics (5–7.5 MHz

Table 9.8. Methods of pregnancy diagnosis and times at which they can be used during pregnancy.

Length of Gestation	Method
18–24 days	Failure to return to oestrus
18–24 days	Persistence of the corpus luteum
22–26 days	Milk or plasma progesterone assay
30 days	Scanning by real-time ultrasonics
30–65 days	Palpation of the amniotic vesicle
35–90 days	Disparity in size of horn
35–90 days	Palpation of the allantochorion (membrane slip)
70 days to term	Palpation of caruncles
90 days to term	Fremitus in middle uterine artery of gravid horn
105 days to term	Oestrone sulphate in milk assay
150 days to term	Fremitus in middle uterine artery of non-gravid horn

transducer) could only be achieved from day 26–27 after breeding.

9.7. Enhancing Pregnancy Rates in Recipients

As a result of increasing information and the development of new techniques, there are several ways in which it may be possible to enhance pregnancy rates in recipient cattle (see Fig. 9.5). However, it should be emphasized that much of the evidence in this field is so variable that it is neither easy nor desirable to formulate precise recommendations on some of these possibilities (see Binelli *et al.*, 2001). There is also much yet to be learnt about the routes by which hormonal treatments exert their effects; local progesterone concentrations may greatly exceed systemic levels. In practical terms, using a treatment that stimulates the animal's own CL to secrete more progesterone may prove more effective than using intravaginal devices to add progesterone to the general circulation (see Macmillan *et al.*, 2001).

9.7.1. Progesterone supplementation

Progesterone is the hormone of pregnancy and many attempts have been made to influence the cow's ability to become pregnant by giving additional amounts of the steroid in the early luteal phase of the cycle. At this point in time, none of these attempts have led to a genuine enhancement of fertility in the normal animal. The literature contains various reports indicating that cows failing to conceive may have lower progesterone concentrations than those that become pregnant. There is also evidence that the bovine embryo's ability to develop and secrete interferon-tau (IFN-τ) may be related to progesterone levels (Kerbler *et al.*, 1994; Mann *et al.*, 1999). However, the use of supplementary progesterone in attempts to enhance pregnancy rates in recipient animals has resulted in inconsistent results. Insertion of a CIDR-B device at time of ET in a study by Macmillan *et al.* (1994) led to a 12.8% increase in pregnancy rates in cyclic recipient milking cattle. Such a response, however, was not confirmed with inseminated cows in subsequent studies involving several thousand cattle (Macmillan *et al.*, 2001).

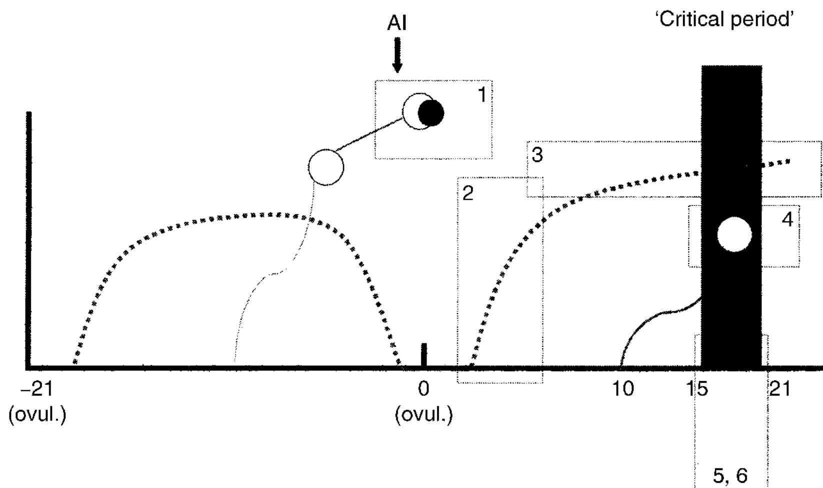

Fig. 9.5. Possible antiluteolytic strategies to enhance pregnancy rates in cattle. Strategies are numbered 1 to 6 across an interval of time representing an oestrous cycle followed by artificial insemination (AI) and pregnancy. Ovulations (ovul.) are indicated at days –21 and 0. Progesterone concentrations are represented by dotted lines, dominant follicles by open circles, dominant ovulatory follicle by two superimposed circles (open and closed) and the critical period by a grey box. Strategies are: (1) increase size of preovulatory follicle to generate larger CL, (2) increase rate of growth of CL, (3) increase luteal-phase progesterone, (4) decrease effect of a dominant follicle on the critical period, (5) increase antiluteolytic stimulation–conceptus unit and (6) decrease luteolytic response–maternal unit. (From Binelli *et al.*, 2001.)

Elsewhere, a study by Tribulo *et al.* (1997) failed to find any beneficial effect of CIDR-B insertion in cows and heifers in Brazil. At a later date in the same country, a study by Marques *et al.* (2002) found that insertion of a CIDR-B device from day 7 to day 20 did not result in a significant increase in progesterone levels in heifers.

In England, Mann *et al.* (2001) found that, despite the adoption of a supplementation regime with CIDRs that achieved a controlled progesterone increase (from days 10 to 17) in dairy cows targeted as being at risk of pregnancy failure (no conception at first service), there was no improvement in pregnancy rate. In Illinois, Steckler *et al.* (2002) attempted to improve pregnancy rate in post-partum beef cow recipients by using a CIDR device for a 7-day period (−9 to −2 days) during the Ovsynch treatment that preceded transfer of frozen–thawed embryos; they recorded a higher pregnancy rate in the CIDR-treated recipients than in the controls (61% vs. 50%).

9.7.2. Hormonal therapy in early dioestrus

Alternative routes to increasing plasma progesterone levels have involved the use of human chorionic gonadotrophin (hCG) and PMSG (eCG). In the study reported by Marques *et al.* (2002), treatment with hCG on day 7 increased progesterone concentrations in zebu recipients. A study by Fuentes and de la Fuente (1997) took another approach to increasing progesterone concentrations by inducing multiple ovulations by PMSG during a synchronization treatment. Friesian heifers treated with a PRID for 6.5 days, with progesterone and oestradiol on day 0 and 1000 iu PMSG on day 4 (expected time of follicular wave emergence), responded with additional ovulations (two to five per ovary); this led to more recipients being selected and a higher pregnancy rate than in controls.

A further approach to progesterone supplementation during early dioestrus was evaluated by Schmitt *et al.* (1996); it was shown that a GH-RH analogue or hCG injected 5 days after oestrus could induce ovulation of the dominant follicle in almost every instance. The accessory CL increased plasma progesterone levels, with the greater increase being observed with hCG; when the hCG treatment was applied to dairy heifers and heat-stressed milking cattle, there was no increase in conception rate. In Scotland, Smith and Grimmer (2002) unsuccessfully employed synthetic GH-RH at the time of transfer of grade 2 (*in vivo*-derived) embryos in an attempt to enhance pregnancy rate; they suggested that the timing of the treatment may have contributed to the negative effect. In Japan, a significant improvement in pregnancy rate (67.5% vs. 45% in controls) in Japanese black cows was achieved by the injection of hCG (1500 iu) 6 days after oestrus (Nishigai *et al.*, 2002); these cattle had received frozen–thawed *in vivo*-derived embryos 7 days after oestrus.

9.7.3. Hormonal therapy in late dioestrus

It has been estimated that more than 3000 analogues of GH-RH, containing various structural modifications, have been synthesized; some of these analogues are 50–100 times more potent than the parent hormone and may be used to influence the reproductive process. The literature contains many reports on the use of GH-RH analogues between 11 and 14 days after insemination to improve pregnancy rates in cows (e.g. Drew and Peters, 1994); unfortunately, results have been far from consistent. In an attempt to make sense of conflicting reports, a paper by Peters *et al.* (2000) investigated whether a consolidated estimate of the response could be quantified. The authors conducted a meta-analysis of data available at that time – 19 studies from 14 published papers – on the effect of GH-RH analogues. They showed that response to GH-RH varied with cow type (beef or dairy), age (cow or heifer), oestrus synchronization (synchronized or natural), pregnancy diagnosis (method and time) and the effect of the individual study. When all these variables were taken into account, only six of the 14 publications could be analysed, representing 2541 instead of 10,945 cattle. In this limited subgroup of data, a significant improvement in pregnancy rate was evident among treated cattle. The authors concluded that, although GH-RH may produce significant benefits in certain circumstances, there was need for further research to

identify factors operating at cow or farm level that influence responsiveness.

9.7.4. Use of trophoblastic vesicles

It is now well established that pregnancy-specific proteins, including IFN-τ are secreted by the elongating bovine embryo; these proteins play a crucial role in the early establishment of pregnancy in the cow (Bazer *et al.*, 1994). Structures known as trophoblastic vesicles (TVs), which are similar in many ways to blastocysts without the embryonic disc, can be produced by cutting the elongated blastocyst (13–14 days old) into several pieces and culturing these *in vitro* for 24 h or so until they re-form into vesicles. Studies reported by Stojkovic *et al.* (1995a) demonstrated that the bovine TVs produced in their *in vitro* culture system (B2 + buffalo–rat liver (BRL) cells) were capable of secreting IFN-τ.

In Ireland, bovine TVs, derived from day-13/14 *in vivo*-produced embryos and frozen with glycerol as a cryoprotectant, were transferred to the uterus of cows artificially inseminated 5–7 days earlier (Ryan *et al.*, 1994); in early post-partum Friesian cows (those calved less than 55 days previously) pregnancy rate was increased by 13% but not in those at later post-partum stages. It may be of some relevance to note that some workers have found ethylene glycol to be less toxic to bovine TVs than glycerol (Lester *et al.*, 1994).

The use of additional embryonic tissue to enhance pregnancy rates may be particularly relevant in dealing with recipient cattle; the TV could be co-transferred with the embryo to augment the embryonic signal. In the report of Stojkovic *et al.* (1995a), they mentioned that trials were in progress to test that possibility; in Canada, Johnson *et al.* (1995) reported studies in which quarter-embryos were co-transferred with fresh TVs, resulting in twin pregnancies in three of four recipients, some part of this success being possibly due to the TVs.

9.7.5. Prostaglandin inhibitors

There is evidence that elevated uterine concentrations of $PGF_{2\alpha}$ may be a negative factor in the establishment of pregnancy in the cow. In some laboratories, workers have recorded improved pregnancy rates after ET when an inhibitor of cyclo-oxygenases was administered at the time of ET (Schrick *et al.*, 2001). A subsequent study by Scenna *et al.* (2002) showed that culturing *in vivo*-derived morulae in media containing $PGF_{2\alpha}$, although not compromising development to the blastocyst stage, did have a direct negative effect on hatching rates. The authors suggest that this may help to explain improvements in pregnancy rates noted after using compounds that inhibit $PGF_{2\alpha}$ production.

In Australia, McNaughtan *et al.* (2002) noted that manipulation of the reproductive tract may result in increased levels of PG, which may decrease pregnancy rates, particularly in heifers that often require additional manipulation. These authors produced some evidence suggesting that heifer recipients had a greater chance of becoming pregnant when given a PG inhibitor (flunixin meglumine) prior to transfer.

In Italy, Elli *et al.* (2001) investigated the use of ibuprofen lysinate (5 mg/kg body weight) 1 h before ET as a means of enhancing pregnancy rates in recipient cattle; they recorded a significant improvement in treated cattle (82% vs. 56%). Although they noted that ibuprofen may be an effective treatment, the authors suggest the need for further studies to clarify whether the beneficial effect is associated with a reduction of cyclo-oxygenase enzyme isoforms during ET or whether other mechanisms are involved.

9.7.6. Oral treatment with propylene glycol

Workers in Spain have used the oral administration of propylene glycol, a gluconeogenic precursor, in an attempt to influence progesterone level and CL quality. Propylene glycol is known to increase the molar percentage of ruminal propionate, which is transformed into pyruvate, and eventually glucose, in the liver. Hidalgo *et al.* (2002b) fed heifer recipients a daily dose of 250 ml propylene glycol for 20 days and found evidence of a significant increase in progesterone level and pregnancy rate (see Table 9.10); it was suggested that the increased pregnancy rate might be partly due to a better-quality CL.

Table 9.9. Progesterone levels and pregnancy rates in recipients orally dosed with propylene glycol (from Hidalgo *et al.*, 2002b).

Serum progesterone concentrations (P4; ng/ml) and corpus luteum (CL) quality values on day 7 of all recipient heifers.

Treatment	*n*	P4	CL	*n* selected for ET (%)
Propylene glycol	75	5.87 ± 0.36^{x}	1.46 ± 0.08^{x}	$52 (69)^{x}$
Untreated	74	4.47 ± 0.35^{y}	2.11 ± 0.10^{y}	$28 (38)^{y}$

Day 7 serum progesterone concentrations (*P*4; ng/ml) and corpus luteum (CL) quality values, and day-60 pregnancy rates (PR) in heifers transferred with a frozen embryo.

Treatment	*n*	P4	CL	PR (%)
Propylene glycol	52	6.16 ± 0.41	1.27 ± 0.06	65 ± 6.6^{a}
Untreated	28	5.76 ± 0.51	1.10 ± 0.06	46 ± 9.6^{b}

Values in the same columns with different superscripts differ significantly.

9.7.7. Re-synchrony of non-pregnant recipients

The number of recipients available to ET operators is one of the factors limiting the effectiveness of this technique. The return to oestrus of non-pregnant recipients after ET may be irregular and may only be determined after pregnancy diagnosis; this may mean that many recipients are not available for reuse for at least 6 weeks or so after transfer. As a means of improving the efficiency of ET operations, it is important to improve rates of oestrus detection among non-pregnant recipients.

Although intravaginal devices inserted during the period of late dioestrus may not increase pregnancy rates, removing the device on day 21 after the previous heat period can resynchronize the return to service interval. The various treatment protocols for resynchronizing previously inseminated cattle have been described and discussed by Macmillan *et al.* (2001) and are applicable to recipient animals used in ET programmes. They note that combining the use of oestradiol dibenzoate (ODB) at progesterone device insertion with ODB at device withdrawal can be used to resynchronize returns to service as well as increasing the submission rates (percentage of animals detected in oestrus) among cows that had previously failed to conceive. In Brazil, Barreto and McManus (2002) evaluated the use of the CIDR device when used from day 14 to day 23 on pregnancy rate of recipient cattle; they found that most non-pregnant recipients showed heat after CIDR removal and were available to receive new embryos.

10

Embryos and Oocytes in Research and Commerce

10.1. Introduction

The world of animal production has undergone many changes in the course of the past 50 years, thanks to the efforts of researchers and others in many lands. In the course of the next 25 years, global output of the livestock sector is expected to double; it is important that such increased production can be achieved by way of greater efficiency rather than by increasing animal numbers (Cunningham, 1999). The relentless growth of the human population and the urgent need to sustain reliable food supplies, at reasonable cost and with due regard to animal welfare and the environment, make it essential to maintain a highly motivated and technically based livestock industry. Those in agricultural research have a responsibility to ensure that the various forms of reproductive biotechnology now being developed are capable of being integrated into livestock production systems in a way that has the support of those who work and live on the land and of society at large.

The development of *in vitro* techniques for the laboratory production of cattle embryos has proved to be useful, both for research and for agricultural and non-agricultural applications. The technology developed during the past two decades now permits the low-cost production of large numbers of cattle embryos, available for study or use at specific stages of development; this, in turn, has had an effect on the accumulation of knowledge on early embryonic development in this species. The same technology now utilizes the oocytes present in the ovaries of the cow in a way that would not have been readily visualized 20 years ago. There are those who believe that in view of the similarity between cattle and humans in the rate of early embryonic development, the bovine model may supplant the mouse as the model for early mammalian development (Niemann and Wrenzycki, 2000).

Within 3 years of the birth of 'Dolly', cloned by way of nuclear transfer (NT) from a somatic cell, several laboratories had demonstrated the feasibility of this new technique in cattle. Currently, there are probably more laboratories working worldwide on cattle cloning than in all other species combined. The success of the various groups in cloning cattle is the result not only of numerous research programmes focused on NT, but also of the enormous base of knowledge that has developed over the past three decades in the technology of assisted reproduction in cattle. Successful and repeatable procedures for *in vitro* oocyte maturation and *in vitro* embryo culture are now well established in cattle; each of these represents a key step in the cloning process. Commercial exploitation of cloning possibilities will depend upon optimizing procedures for NT; the need is for a comprehensive understanding of how factors in the ooplasm act upon the DNA of the transferred nucleus to regulate gene expression.

Although commercial interest in cattle cloning initially centred on the production of large numbers of genetically élite animals for agricultural purposes, this particular avenue quickly lost its appeal in the light of emerging

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problems. Several companies in the USA and elsewhere financed ambitious research and development programmes only to face problems of dramatically low pregnancy rates, increased birth weights, neonatal anomalies and poor survivability of cloned calves. There are certainly those in the embryo biotechnology industry who currently believe that livestock cloning may never become profitable. At this point in time, most commercial interest in cloning lies in commercializing proteins produced in the milk of genetically modified cows rather than for agricultural purposes. The production of transgenic animals is a process not necessarily limited by current inefficiencies in the NT technique; it is clear that gene targeting can be carried out efficiently in somatic cells and that viable animals can be produced by NT.

On the farming front, genetic engineering of livestock is likely to be eventually employed to produce cattle with altered traits such as milk composition and disease resistance; on the biomedical front, the same technology is likely to bring benefits to society at large, in terms of producing animals for pharmaceutical protein production. As noted by Eyestone and Campbell (1999), one interesting transgenic possibility is that genes coding for the major proteins in cow's milk could be 'silenced' and replaced by their human counterparts; cow's milk containing a human milk protein could be more nutritious for human infants and prove more suitable for infant formula manufacture than ordinary milk. Whether for farming or otherwise, it is essential that the introduction of such novel biotechnologies should be validated by studies into their effects on animal welfare, within the guidelines of a comprehensive welfare protocol (see Broom, 1998; Kruip and van Reenen, 2000).

10.1.1. From research to commercial application

Less than two decades ago, it would have appeared unlikely that large numbers of viable cattle embryos could be produced from oocytes collected indiscriminately at the local abattoir. None the less, a study based on such abattoir material and involving almost 900,000 oocytes showed an average of 2.32 good-quality embryos per ovary (Lu and Polge, 1992). The decade since then has seen the rise of an alternative source of cattle oocytes, this time from the living rather than the dead animal. The introduction of ovum pick-up (OPU) technology now means that *in vitro* embryo production technology can be utilized by many of those in the cattle embryo transfer (ET) industry, rather than being limited to large-scale commercial concerns; there are, however, questions of cost as well as technical efficiency to be considered.

According to Hasler (2000b), with many years experience in the commercial cattle ET industry, current low efficiencies in OPU–*in vitro* production (IVP) technology and high costs make the technology uneconomic in comparison with conventional cattle ET procedures for most donors. The author, who operated a commercial *in vitro* fertilization (IVF) unit from 1992 in Pennsylvania, mainly dealing with aged infertile cows, found that there was demand for the service for a few years, but this decreased markedly when word of various problems with IVF-derived pregnancy started to circulate throughout the cattle industry; by 2000, demand was at a very modest level and almost exclusively confined to infertile donors. The same author concluded that wider acceptance of IVF embryos by the cattle industry is dependent on an improvement in the normality of pregnancies, even if this is achieved at the expense of embryo production and pregnancy rates. In Brazil, the use of IVP embryos at this time is similarly confined to problem cows – those that fail to yield embryos by conventional ET protocols.

Some of the potential research and commercial applications of *in vitro* embryo production relevant to cattle are provided in summary form in Table 10.1. In such applications, it should be remembered that it is cattle embryo production, linked to cattle ET technology, that will eventually enable advantage to be taken of the considerable advances that are currently taking place in cellular and molecular biology.

10.1.2. Cattle products and human health

Advances in reproductive technology as applied to cattle and the other farm animals can be viewed as potentially valuable in increasing the

Table 10.1. Potential applications of cattle IVP technology in commercial practice and research.

Commercial applications	Research applications
Embryos	
Twins in beef and dairy cattle	R & D in developing sperm separation by flow cytometry
Singleton beef calves from dairy cattle	Development of new embryo sexing methods
Producing sexed calves for beef and dairy herds	Improving IVF methods
Heifer calves for once-bred heifer beef systems	Developing a bull fertility test
Using IVF as part of normal cattle ET operations	Research in all aspects of freezing
Producing cattle hybrids for the tropics	Studies in embryo growth and development
	Research in the production of heat-tolerant embryos
Unfertilized oocytes	
Production of uniform calves and carcasses for the meat trade	Research in all aspects of cloning in cattle
	Studies in oocyte freezing
Providing genetically superior and sexed calves for beef and dairy herds	Testing effectiveness of embryonic stem cells
Pronucleate oocytes	
Transgenic cattle for the production of medically important proteins in milk	Development of gene injection procedures
	Studies in freezing
Producing dairy cattle with novel milk proteins for the dairy industry	
Trophoblastic vesicles	
Use of enhanced pregnancy rates in recipient cattle	Research in all aspects of maternal recognition of pregnancy

R & D, research and development.

overall efficiency of animal production and in improving the quality of the products available for human consumption. As the pressures mount in the developed countries to move away from increased production using high inputs to greater efficiency with reduced inputs, there will be an ever-increasing need to find ways of utilizing the genetically superior farm animals, both male and female, to advantage. Although much of the concern about food in the developed countries relates to health problems that arise from its abundance rather than its scarcity, there needs to be a constant awareness of those whose daily experience is to be hungry. It is towards the developing countries, therefore, that much greater emphasis must be turned in getting new forms of animal and plant technology applied for the benefit of the human population; the same order of priority also needs to be directed towards the control of human fertility in these countries.

Population growth and food resources

Advances in medical science, which now prevents and cures diseases that formerly regulated the human population, have dramatically decreased infant mortality and increased life expectancy; it has been estimated that the proportion of the world's population above the age of 60 will increase from 9.5% today to 20% in 2050 and 27% by 2100 (Lutz *et al.*, 1997). A thousand years ago, the world population was about 300 million. The first 1000 million was reached around 1800, the second (2000 million) about 1927. Currently, the population stands at 5800 million and is increasing by about 90 million a year, a statistic that should influence every action that bears upon the balance between food supply and human need. Were the world population to continue growing at the present rate up to AD 2100, it would reach 55,000 million at that time and double again in a further 35 years.

However, despite the religious, commercial and political pressures that currently restrict adequate fertility-control measures, estimates by the World Health Organization (WHO) and other bodies suggest that different countries are likely to reach a net reproduction rate of 1.0 at various times between the years 2010 and 2050. Equilibrium population is forecast at about 10,400 million and this should be reached around AD 2100; to meet nutritional needs, as well as those arising from population growth, it has been estimated that food supplies would have to be trebled between now and AD 2100. The required increase in food production, however, would be greater in the initial period, in view of the slow rate of achieving zero population growth. Zero population growth within the lifetime of our children is achievable, but only if action is taken without delay. It certainly cannot be achieved by science and technology alone; political will and appropriate policy formulation are equally important. The fact that the proportion of elderly people is predicted to grow strongly in the 21st century has led some to forecast that the focus of public, political and scientific concern is likely to shift increasingly from global population growth to population ageing (Lutz *et al.*, 1997).

The crucial point is to provide for the needs of the malnourished in the developing countries, using the most appropriate forms of technology; in the developed countries, the need is to provide the quality of food that is most appropriate for the maintenance of good health. In regard to the latter consideration, it is possible that emerging forms of technology will eventually be the means of yielding cattle capable of producing leaner meat and milk of more acceptable composition; such developments may have significant health benefits for the consumer.

10.2. Embryo Production Technology: Problems

Statistics presented by Thibier (2001a) estimated that almost 42,000 IVP cattle embryos were transferred in 2000, the figures showing an increase of about 50% over the previous year. Although such numbers represent less than 8% of the total embryos transferred in that year, they are indicative of an increasing use of embryo production technology by the cattle ET industry. In Europe, the number of IVP embryos was estimated at 26,520, roughly similar numbers being produced from abattoir material (12,441) and live cattle (14,079).

Authors have reported experiences with IVP embryos from many countries, including Argentina (Iudica *et al.*, 1995a,b, 1998; Ferre *et al.*, 2000a,b, 2002), Belgium (Dehareng *et al.*, 1999), Brazil (de Oliveira *et al.*, 1994), Canada (Massicotte and Sirard, 2001), China (Bou, 1998), France (Mermillod *et al.*, 1995; Nibart *et al.*, 1995a,b, 1998), Hungary (Seregi *et al.*, 1995), Italy (Galli *et al.*, 1994, 1997a,b; Mantovani *et al.*, 1998, 1999a,b), Korea (Lee *et al.*, 1994; Yang, B.S. *et al.*, 1995) and Spain (Pascual *et al.*, 1998). As with many new forms of technology, cattle embryo production is influenced by a wide range of factors, including human factors, such as skill of technicians (Yang, X. *et al.*, 1995) and many animal factors. Clearly, the increasing use of embryo production technology has to be based on the premises that most of the calves born will be normal and healthy, with no unexpected effects appearing in later life or in the young that they themselves produce.

Inevitably, whether in cattle or humans, naturally occurring or artificially induced, a low proportion of births will show evidence of an abnormality, some minor, some more serious. Although it is clearly essential to keep any risks associated with embryo production constantly under review, it is also necessary to put such risks in a realistic context. Human nature being what it is, there is a tendency for alarmist reports to be given greater prominence than those that report that all is well. Problems and prospects for IVP in cattle have been reviewed by authors in recent years (Hasler, 1994, 1998; Callesen *et al.*, 1998; Hill *et al.*, 1998a: Kruip, 1998; McEvoy and Staines, 1998; Boland *et al.*, 1999; Leibfried-Rutledge, 1999; Kruip *et al.*, 2000; Lonergan *et al.*, 2001c). It is clear from such reviews that a clean bill of health cannot be given to IVP cattle embryos for commercial use until such time as methods are available for minimizing or entirely eliminating the possibility of abnormalities such as the large-offspring syndrome (LOS). As part of the crucial body of evidence required to deal with the LOS problem, it is likely that the knowledge necessary to effectively cryopreserve the IVP embryo will also be forthcoming.

10.2.1. Differences between IVP- and *in vivo*-derived offspring

Many reports for IVP-derived calves have presented evidence of increased birth weights; workers have examined the developing fetus for evidence of changes in development that may explain this effect. Others have examined the progress of calves after their birth. In the USA, Hasler *et al.* (1995a,b) reported that 1% of their pregnant IVP recipients had hydro-allantois, which was higher than the normal rate of 1 out of 7500 pregnancies. In Scotland, McEvoy *et al.* (1998a,b,c,d) demonstrated birth-weight-related developmental effects on postnatal organ weights in Simmental IVP-derived calves at 13 months of age. In the USA, Blondin *et al.* (1999) found no difference from controls in insulin-like growth factor II (IGF-II) mRNA levels in the skeletal muscles of IVP fetuses. In the same laboratory, however, a study by Crosier *et al.* (2000a,b) found that skeletal muscles at day 222 in embryos produced *in vitro* had a decreased total fibre area density and an increased ratio of type II to type I fibre number compared with fetuses from embryos produced *in vivo*.

In the UK, Byrne *et al.* (1999a,b) proposed that perturbed apoptotic rates may be involved in IGF-II-related fetal oversize. In Japan, Iwata *et al.* (1998a,b) recorded that the birth weights of Hyogo bull calves were significantly heavier than those of *in vivo* calves but without gestation length being affected. Elsewhere in the same country, Numabe *et al.* (1999, 2000a,b, 2001a) recorded clear evidence that Japanese Black IVP calves were heavier at birth than their *in vivo*-derived counterparts but that this was not necessarily associated with a longer gestation period (see Table 10.2).

In the Netherlands, De Roos *et al.* (2000) reported higher birth weights for IVP-derived calves that had been produced in serum co-culture systems than for calves cultured in synthetic oviductal fluid (SOF) without serum; they also found certain differences in blood parameters (cortisol, thyroxine, insulin levels) that may justify further investigation.

Although clear differences in birth weights between IVP calves and controls have been found in some studies, differences in the subsequent growth rate of animals has not been evident (De Ruigh *et al.*, 2000); increased birth weight was already normalized at 1 month after birth. According to a report by Van Wagtendonk-De Leeuw *et al.* (2000), IVP-derived calves differed from controls in several physiological parameters (e.g. blood oxygen saturation level, heartbeat frequency). In California, Bertolini and Anderson (2002) and Bertolini *et al.* (2002a) showed that *in vitro*-derived pregnancies sustained larger conceptuses towards the end of the second trimester of pregnancy and demonstrated an increased transport of D-xylose from the maternal circulation to the fetus; D-xylose is a slowly metabolized pentose that enters cells via the D-glucose transporter system. A further study by Bertolini *et al.* (2002b) was designed to characterize growth and development of *in vivo*- and *in vitro*-derived conceptuses by measuring changes in fetal and placental development from early pregnancy until full term; they concluded that high birth weights may be the result of aberrant placental development due to disruption of the placental restraint on fetal growth towards the end of pregnancy.

Table 10.2. Birth weights and gestation lengths in Japanese Black cattle after transfer of IVP embryos or after artificial insemination (from Numabe *et al.*, 1999).

Production	No. of calves	BW (kg)	Pregnancy duration (days)	Bull	No. of calves	BW (kg)	Pregnancy duration (days)
IVF co-culture	307	30.0 ± 0.3[a]	288.3 ± 0.3[a]	A	97	30.3 ± 0.4[ab]	291.3 ± 0.5[a]
				B	210	29.7 ± 0.3[b]	285.2 ± 0.3[c]
IVF non-co-culture	41	31.0 ± 0.7[a]	289.2 ± 0.7[a]	A	21	32.0 ± 0.9[a]	292.2 ± 1.1[a]
				B	20	30.0 ± 0.9[abc]	286.2 ± 1.1[bc]
AI	1493	27.8 ± 0.1[b]	286.6 ± 0.2[b]	A	1211	27.3 ± 0.1[d]	286.0 ± 0.1[c]
				B	282	28.2 ± 0.2[c]	287.2 ± 0.3[b]

[a–d]Values within columns with different superscripts differ ($P < 0.05$).
BW, body weight.

10.2.2. The large-offspring syndrome (LOS)

As observed by Barnes (2000), the enigma of LOS has often been presented as a problem of *in vitro* embryo production, although its initial discovery had little, if any, connection with such technology; the author noted that the syndrome was first observed in NT calves produced at Granada Genetics in the years of 1978–1988, prior to the introduction of *in vitro* embryo technology. Ironically, the syndrome was first reported in NT calves that were produced using donor cells from *in vivo*-derived embryos, recipient cytoplasm from *in vivo*-matured oocytes and *in vivo* culture to the blastocyst stage in the sheep oviduct. For those engaged in *in vitro* embryo production in the late 1980s, there was little to indicate that this form of technology was likely to encounter the problems thrown up by NT; such workers could look back on many years in which sheep and cattle embryos had been exposed to numerous *in vitro* culture (IVC) and *in vivo* (rabbit oviduct) culture systems without problems of growth and development arising in the young that were born after ET.

Cloning by NT, involving the reprogramming of the donor nucleus, was clearly, in physiological and biochemical terms, in a category far removed from that of IVF; an understanding of the complexities of cell nuclear reprogramming was very limited. There was, on the other hand, much evidence from work covering more than a decade in human assisted reproduction that IVF and culture of human embryos did not bring unanticipated problems in the later development of the fetus. At that point in time, around 1990, it should be remembered that countless thousands of cattle embryos had been manipulated, frozen, thawed and exposed to a variety of culture conditions before transfer, all apparently without mention of the LOS. To those workers who had dealt with cattle and sheep embryos since the early 1950s in ET research, knowing that such embryos were often exposed to a great variety of culture conditions and environmental effects, it would have appeared surprising that evidence had been so long surfacing about the effect of culture on abnormalities in embryonic and fetal development.

None the less, it is now clear that certain culture conditions do give rise to problems that affect the survival of the embryo and fetus during prenatal life and can seriously impair its survival around the time of calving (see Farin *et al.*, 1994, 2001; Leibfried-Rutledge, 1999). In Belgium, Massip *et al.* (1996) reporting on calving outcome after transfer of IVP embryos, recorded that birth weights and gestation lengths were in the normal range for 190 of the 195 calves born; five calves (2.6%) were exceptionally large. In Scotland, two unusually large calves were recorded in the studies of Sinclair and Broadbent (1996), who speculated that this might be related to their *in vitro* origins. It is now well established that NT procedures may give rise to a proportion of calves being heavier than normal (Wilson *et al.*, 1995) and some calves showing evidence of defects in metabolic regulation (Adams *et al.*, 1994; Garry *et al.*, 1996). Although, in cattle, the effect of IVC *per se* on fetal development is much less evident, with authors generally finding most calf weights to be within the normal range (Hasler *et al.*, 1995a,b; Penny *et al.*, 1995), there have been reports dealing with a variety of anomalies that have been evident in calves around the time of calving. In sheep, there has been a growing number of reports drawing attention to the effect of exposure of the early embryo to hormonal and other influences on subsequent development (Holm *et al.*, 1994a,b; Kleemann *et al.*, 1994; McEvoy *et al.*, 1995).

Placental abnormalities

Reports by Hasler *et al.* (1995a,b) and van Wagtendonk-De Leeuw *et al.* (1998) have recorded an increased incidence of hydroallantois in IVP calves, a problem that is associated with placental abnormalities. According to Peterson and McMillan (1998), allantoic aplasia is also associated with anomalies in the development of the hind-gut region. There were also indications in a report by Farin and Farin (1995) that IVP embryos may develop fewer placentomes than *in vivo* embryos; further studies in the same laboratory found evidence of heavier placentas in association with larger fetuses and reduced caruncular surface area and villous density (Farin *et al.*, 2000). In a further report by Van Wagtendonk-De Leeuw *et al.* (2000), they recorded that 3.7% of calves born after IVP co-culture showed congenital malformations (e.g.

hydro-allantois, abnormal limbs and spinal cords) as compared with an incidence in controls of 0.8%. A review by Thompson and Peterson (2000) has dealt with *in vitro* development of cattle embryos and the pathology of post-transfer loss of early fetuses; they concluded that allantoic malformation may be a major cause of fetal loss in IVP embryos. According to Peterson *et al.* (2000), high and sustained embryo and early fetal loss in their experience resulted in only about 30% of transferred IVP embryos surviving to term; they recorded an incidence of allantoic malformation ranging from 20 to 25% over days 22–34, reducing to some 10% by day 70.

There is also evidence suggesting that placental dysfunction may be a factor affecting survival in NT conceptuses (Garry *et al.*, 1996; Stice *et al.*, 1996; Hill *et al.*, 2000a,b,c). In sheep, De Sousa *et al.* (2001) reported that NT conceptuses may show both fetal and placental defects, including fetal liver enlargement, dermal haemorrhage and lack of placental vascular development; these authors suggest that the developmental transition from yolk sac to allantoic-derived nutrition represented an ideal focal point on which to base future studies of fetal failure. In a study reported by Bertolini and Anderson (2002), differences existed in the distributions of placentomes and cotyledonary diameters between IVP and control pregnancies; fetal membranes from IVP calves contained 21% fewer cotyledons than controls but had a larger cotyledonary surface area in the pregnant horn. The authors suggested that morphological and functional differences between IVP and *in vivo* derived pregnancies could affect fetal growth and calf birth weight; it remains to be determined whether large calves and associated anomalies resulting from IVC and those from NT result from the same developmental defects. A study by Crosier *et al.* (2002) led them to conclude that IVP of embryos resulted in fetuses with altered development of skeletal muscle fibres; myostatin was identified as the candidate gene whose expression may contribute to such changes in muscle development.

Gene expression

It is believed that at least some of the problems associated with the IVC of ruminant embryos may result from disruptions in imprinted genes (see Young *et al.*, 1998, 1999; Young and Fairburn, 2000; Surani, 2002); several imprinted genes (i.e. genes that express only the maternal or paternal allele) are known to affect fetal size and survival. In attempts to resolve the problem of dramatically heavy birth weights in ruminants after cloning or culture of embryos *in vitro*, workers in Scotland have found that sheep fetuses developing after IVC have abnormally low levels of expression of the imprinted gene coding for IGF-II receptor and that this is associated with reduced DNA methylation (Young *et al.*, 2001). Elsewhere, several papers have now been published demonstrating aberrant methylation in cloned as compared with normal embryos (Bourc'his *et al.*, 2001; Dean *et al.*, 2001; Kang *et al.*, 2001), suggesting that there may be widespread impairment of epigenetic reprogramming. According to Wilmut (2001), this may indicate that various other imprinted genes contribute to the abnormal development of cloned embryos and points to the need to examine the expression of such genes in cloned and normally created embryos.

A study reported by Lazzari *et al.* (2002b) used cattle blastocysts to analyse cellular parameters (e.g. number of cells in day-7 blastocysts; size of day-12 elongating blastocysts) and molecular parameters (e.g. the relative abundance of developmentally important genes). Blastocysts produced by IVC (SOF medium supplemented with bovine serum albumin (BSA) or human serum) or by *in vivo* culture (sheep oviduct) were compared with those recovered from superovulated donors. The results from the early embryo tests were related to a representative number of calves obtained from each production system and from artificial insemination (AI). The results support the view that cellular and molecular deviations in the early cattle embryo are causally involved in the incidence of LOS, in particular in increased birth weights. It is suggested that the parameters analysed in the study could be considered early markers of LOS in cattle.

IVP embryo laboratories and LOS

Not all laboratories appear to have reported the LOS, despite performing many transfers of IVP embryos. In Ireland Bourke *et al.* (1995) and in Scotland Penny *et al.* (1995) fail to mention fetal oversize in IVP-derived calves as a significant

problem in their studies with cattle. In Denmark, control calves obtained from inseminated cows were compared with IVP calves derived from the production protocol described by Avery *et al.* (1998a,b); calves were studied in considerable detail, in terms of physiological factors (e.g. blood pH, glucose, cortisol, respiration rate), anatomical features (e.g. body dimensions and organ weights) and clinical parameters (e.g. uterine contractions and ease of calving). The results of such studies, reported in several papers (Jacobsen *et al.*, 1998, 1999, 2000a,b; Sangild *et al.*, 1999, 2000; Schmidt *et al.*, 1999a,b; Hyttel *et al.*, 2000a,b,c) indicated that the deviations recorded in the literature, including embryonic and fetal survival, placental development (e.g. caruncular size), pregnancy length, labour intensity, ease of calving, proportion of large offspring, perinatal viability amd calf physiology and anatomy, were apparently not provoked with the IVP technique employed in their laboratories (culturing embryos with or without serum and oviduct cells). At the same time, the authors acknowledged that certain batches of oocytes or certain IVP systems may provoke such defects; obviously, it is important to identify the IVP systems that are producing abnormal offspring and try to understand the factors involved.

Some laboratories report defects in IVP embryos that result in severe losses during pregnancy but without recording evidence of LOS in those calves that reach full term. In New Zealand, McMillan *et al.* (1999a,b,c,d) found that fetal oversize was not a feature of cattle embryos in their laboratory (see Table 10.3); they speculated that the higher loss rate of early pregnancies which they experienced had eliminated pregnancies with a potential to develop abnormally from mid-pregnancy.

10.2.3. Large-offspring syndrome: human implications?

The awareness that *in vitro* embryo culture conditions could result in minor or major defects in the offspring of cattle and sheep has triggered several papers dealing with the possible implications of such findings for human assisted reproduction (Leese *et al.*, 1998; Boerjan *et al.*, 2000; McEvoy *et al.*, 2000a,b,c,d; Sinclair *et al.*, 2000a,b), although it needs to be emphasized that the abnormalities described in sheep and cattle have not been described for children resulting from IVF (Barnes, 2000). While it is known that certain problems may arise as a consequence of human IVF, their origin appears to be a secondary effect due to the transfer of multiple embryos rather than to problems in the embryos themselves; multiples in humans, as in cattle, lead to significant reductions in birth weights, and it is the low birth weight, rather than IVF *per se* that is the cause of serious concern.

Seamark and Robinson (1995) noted that the multiple pregnancy rate in New Zealand and Australia after IVF stood at 16%, compared with the usual figure of 2.7%; they drew attention to the data showing an eightfold increase in the risk of cerebral palsy with twins and a 47-fold increase with triplets. Data reported by Fall and Barker (1998) in the UK showed that men and women with a birth weight of 5.5 lb. or less are 1.5–2 times more likely to die of coronary heart disease (CHD) than those weighing more than 9.5 lb. at birth. It is believed that many human fetuses may adapt to a limited supply of nutrients in mid- to late pregnancy by reprogramming their physiology and metabolism (the 'fetal reprogramming' hypothesis); although such

Table 10.3. Fetal, placental and other measures of IVP-derived and AI calves. Unadjusted mean fetal, placental and uterine weight and total fluid volume between day 126 and day 254 of pregnancy in cattle pregnant following either *in vitro* embryo transfer or AI. (From McMillan *et al.*, 1999c.)

Day of pregnancy	Fetal weight (g)		Placental weight (g)		Uterine weight (g)		Fluid volume (ml)	
	IVP	AI	IVP	AI	IVP	AI	IVP	AI
Day 126	1,208	1,226	605	651	1,361	1,308	3,704	3,256
Day 161		3,965		1,201		2,562		3,973
Day 187	7,458	8,042	1,529	1,901	4,105	4,016	7,470	5,193
Day 226		18,356		2,016		6,875		6,325
Day 254	23,267	28,560	3,386	3,730	6,726	7,871	7,250	8,200

adaptations enable survival in prenatal life, they do so at the expense of life-shortening conditions in adult life. These are questions of serious concern to workers in human assisted reproduction; clearly there is a great need to concentrate attention on achieving acceptable pregnancy rates with single rather than multiple embryos to avoid the medical, financial and psychological consequences involved in multiple births. Focusing attention on single embryo transfer as the way of minimizing multiple births in human IVF is likely to involve extended IVC (to enable the transfer of blastocysts rather than early-cleavage stage embryos) and it is in this regard that careful attention should be paid to data available from farm animal studies.

10.3. Embryo Production Technology: Prospects

Breeding improvement in cattle in developed countries has made much progress over several decades due to the large-scale field data derived from many herds based on the progeny testing of males. AI has facilitated such progeny testing of bulls and permitted the subsequent use of those identified as being of superior genetic merit. It can be claimed, with every justification, that AI has revolutionized dairy cattle breeding; where once one bull was kept to breed 30–40 cows, it is now possible to think in terms of an outstanding dairy bull siring 100,000 calves in a year, with his semen being used in several countries simultaneously and for years after his demise. In economic terms, the widespread application of AI in countries such as the USA, has resulted in a steady improvement in the genetic quality of dairy animals and a doubling of milk yields during the past three decades (Cunningham, 1999). ET has an important part to play in cattle-breeding improvement, building on the excellent foundations laid by the cattle AI industry.

10.3.1. Animal-health considerations

Although initially limited to national or international movements of live animals or semen, the shipment of cattle embryos from country to country is now commonplace. It is vitally important to all in the cattle ET industry to ensure that embryos are produced without associated pathogens (see Bielanski and Dubuc, 1994; Booth *et al.*, 1994, 1998b; Zurovac *et al.*, 1994; Modl *et al.*, 1995; Wrathall, 1995; Vanroose *et al.*, 1996, 1997a,b, 1998a,b, 1999a,b; Bielanski, 1997, 1998, 2000; Brownlie *et al.*, 1997; Guerin *et al.*, 1997; Stringfellow *et al.*, 1997, 1999, 2000; Givens *et al.*, 1998, 2000; Kafi *et al.*, 1998; LeBourhis *et al.*, 1998; Marquant-Le Guienne *et al.*, 1998, 2000; Langston *et al.*, 1999; Tsuboi and Imada, 1998; Tsuboi *et al.*, 1998; Shin *et al.*, 2000; Stringfellow and Givens, 2000a,b; Thibier, 2001b). As shown in Fig. 10.1, the transmission of an infectious agent through ET requires the uninterrupted occurrence of five events; each link in the 'chain of infection' must be intact for an infection to be transmitted, and transmission is prevented if any link in the chain is broken.

In the UK, it has to be remembered that foot-and-mouth outbreaks led to the destruction of 1.2 million farm animals in 2001 and 'mad cow disease' (bovine spongiform encephalopathy) has led to the slaughter of millions of cattle since the mid-1990s; such catastrophic disease losses have had severe economic, social and animal-welfare effects. In passing, it may be noted that a comprehensive study reported by Wrathall *et al.* (2002) led them to conclude that embryos are unlikely to carry BSE infectivity, even if collected at the end stage of the disease when the risk of maternal transmission is believed to be greatest. While there has been much reassuring evidence on disease risks in relation to *in vivo*-derived embryos (see Table 10.4), the production of IVP embryos has raised a new set of problems that must be satisfactorily resolved (see Van Wagtendonk-De Leeuw *et al.*, 2000). Routine quality-control measures in bovine embryo production must include appropriate screening of materials for possible contaminants (Starvaggi Cucuzza *et al.*, 2002). As noted by Givens *et al.* (2002a,b), quality-control measures may present challenges because cumulus–oocyte complexes (COCs) and other materials may be collected and cultured at laboratories separate from those in which embryo production is completed.

As noted by McEvoy and Staines (1998), the International Embryo Transfer Society (IETS)

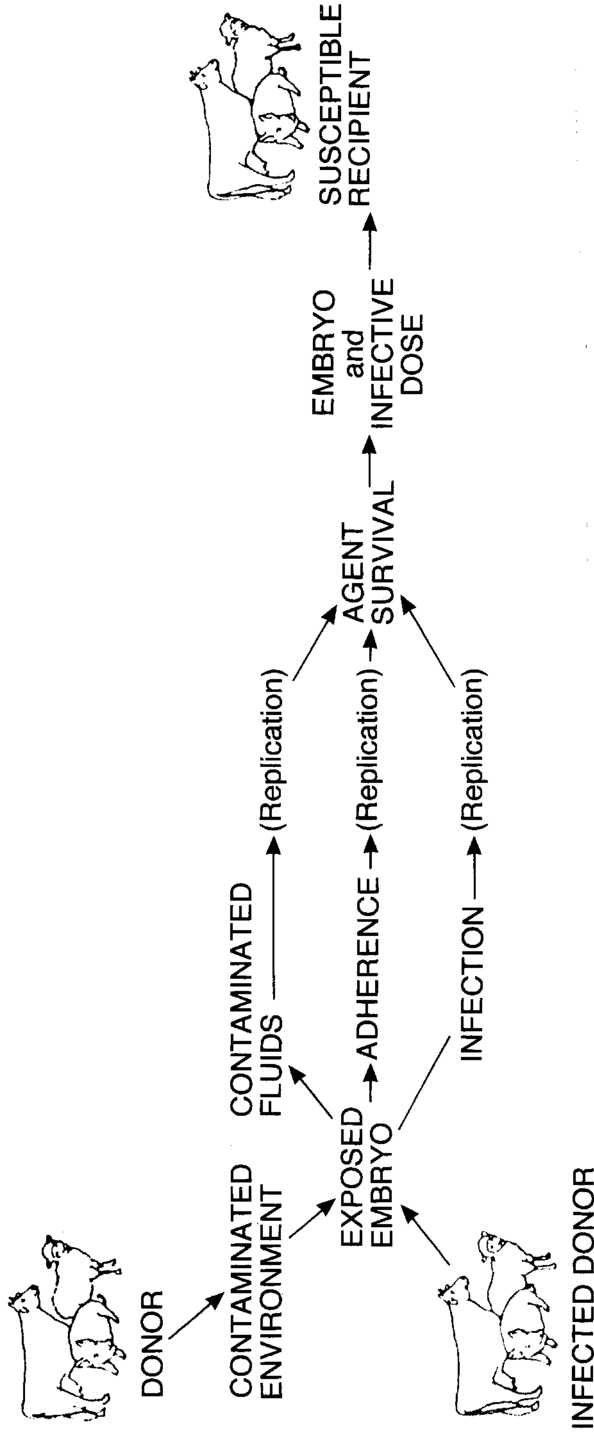

Fig. 10.1. Hypothetical chain of infection in embryo transfer. For an infectious agent to be transmitted through embryo transfer, every link of the chain must be intact. If a single link is broken, transmission is prevented. (From Stringfellow *et al.*, 1991.)

Table 10.4. Assessment of risk of transmission of infectious diseases by *in vivo*-derived cattle embryos. Categorization of infectious diseases apropos the risk of their transmission through *in vivo*-derived embryos. Based on the 2001 review of available information by the Research Subcommittee of the IETS Import/Export (now Health and Safety Advisory) Committee. (From Wrathall, 2002.)

Category 1	Diseases or disease agents for which sufficient evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer: Aujeszky's disease (pseudorabies) (pigs) – trypsin treatment required; bluetongue (cattle); *Brucella abortus* (cattle); enzootic bovine leucosis; foot-and-mouth disease (cattle); infectious bovine rhinotracheitis – trypsin treatment required
Category 2	Diseases or disease agents for which substantial evidence has accrued to show that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer, but for which additional experimental data are required to verify existing data: bluetongue (sheep); classical swine fever (hog cholera); scrapie (sheep)
Category 3	Diseases or disease agents for which preliminary evidence indicates that the risk of transmission is negligible provided that the embryos are properly handled between collection and transfer, but for which additional *in vitro* and *in vivo* experimental data are required to substantiate the preliminary findings: bovine immunodeficiency virus; bovine spongiform encephalopathy (goats); bovine viral diarrhoea; *Campylobacter fetus* (sheep); foot-and-mouth disease (pigs, sheep, goats); *Haemophilus somnus* (cattle); sheep pulmonary adenomatosis; rinderpest (cattle); swine vesicular disease

operates a responsible approach to the certification of IVP embryos in the light of the information now available about them and how they differ from *in vivo*-produced embryos (see Bielanski, 1998). In a study reported by Vanroose *et al.* (2000a,b,c), it was concluded that the intact zona pellucida of the cattle oocyte and embryo is constructed in such a way that bovine viral diarrhoea virus (BVDV) and bovine herpes virus 1 (BHV-1) should not be able to traverse the zona and reach the embryonic cells, but the risk remains that viral particles can be trapped in the outer layers of the zona. In considering disease risks associated with IVP embryos, factors in the production process itself are also relevant, in view of the fact that many systems continue to employ animal-derived components (e.g. cells, serum, albumin). It is clear that the risks associated with IVF embryos are more difficult to manage than those associated with embryos from live animals (see Le Tallec *et al.*, 2001).

Contaminated semen

Various reports have dealt with disease risk problems that may arise with bull sperm in the production of cattle embryos. In Canada, Bielanski *et al.* (2000a,b) presented results showing that mycoplasmas in semen can be transmitted through the IVF system and infect embryos; they also showed that supplementation of culture media with standard antibiotics and washing embryos as recommended by the IETS were not effective in rendering IVF embryos free from *Mycoplasma bovis* and *Mycoplasma bovigenitalium*.

Problems posed by IVP cattle embryos

It is now well established that there are differences between *in vivo*-derived and IVP cattle embryos that have important animal health implications (see Matthews *et al.*, 1998). It is believed that differences in the properties of the zona pellucida between *in vivo* and *in vitro* embryos may be, at least in part, due to the deposition of glycoprotein on to the zona while the *in vivo* embryos travel down the oviducts. Regardless of whether embryos are produced *in vivo* or *in vitro*, they should be free from bacterial and viral infections and steps should be taken to ensure that the integrity of the zona pellucida is preserved (see Fig. 10.2). It is important to differentiate between the different IVP systems that are in use and to take precautions appropriate to the source of oocytes (abattoir or obtained from live animals by OPU). Systems using only

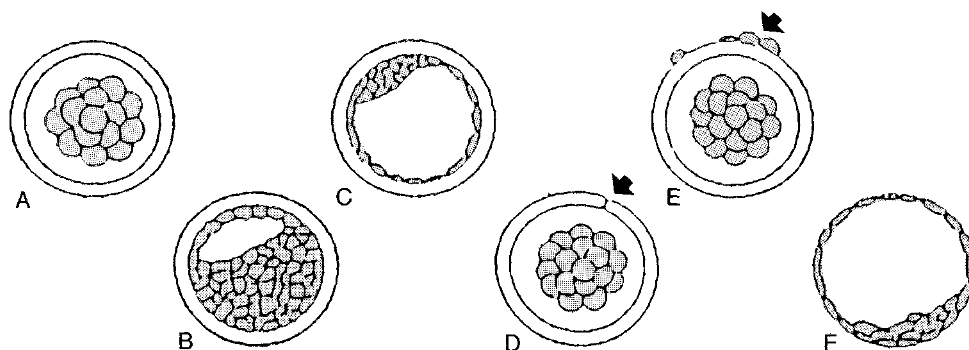

Fig. 10.2. Acceptable and unacceptable embryos in terms of disease control in cattle ET. The zona pellucida is an effective pathogen barrier; for that reason, it is essential that all cattle embryos possess an intact zona pellucida and are free from any adherent material. A, B and C are acceptable embryos, D, E and F are unacceptable embryos; D has a break in the zona pellucida; E has debris present on the zona pellucida; F has no zona (hatched embryo).

cumulus cells from the COCs of an approved donor cow for the co-culture of embryos pose a small disease risk compared with systems employing oviductal or granulosa cells from abattoir materials. Other differences include the source of the protein used in the various steps of maturation, fertilization and early embryo culture; where serum is used, then there must be assurance that it comes from a supplier that can guarantee it to be free of specific pathogens. Ideally, an IVP system based on a defined medium and free of cells will answer many of these concerns in future years.

In Canada, Bielanski *et al.* (2000a,b) examined the effect of bovine leukaemia virus (BLV) on embryonic development and its association with IVP embryos; they concluded from their data that IVP embryos generated in the presence of BLV did not appear to be associated with infectious BLV.

Detection of viruses

It is important that sensitive methods are available for the rapid identification of pathogen-free oocytes and IVP embryos. A sensitive and rapid assay reported by Givens *et al.* (2001) is believed to provide such evidence for embryos infected with BVDV.

Reducing infectivity associated with IVP embryos

Studies by Dinkins *et al.* (1999, 2001) sought to extend the potential for virus photoinactivation to IVP cattle embryos; earlier work had shown that photosensitive chemicals, in combination with light, could completely inactivate BHV-1 and BVDV associated with *in vivo*-produced embryos without adversely affecting embryonic development (Givens *et al.*, 1999a,b). The workers in Georgia were able to demonstrate that photoinactivation could be employed to eliminate epizootic haemorrhagic disease virus 2 (EHDV-2) from IVP embryos experimentally exposed to this virus. It is known that BHV-1 remains associated with IVP embryos after their exposure to the virus, even when embryos are washed or treated with trypsin according to IETS recommendations for *in vivo*-produced embryos. Studies reported by Edens *et al.* (2002) provided some preliminary evidence that trypsin treatment might effectively prevent infection of recipients when individual day-7 exposed embryos were transferred. In a study reported by Givens *et al.* (2002a,b), novel antiviral agents were evaluated for their ability to inhibit replication of BVDV without affecting embryonic development; they showed that bovine embryo culture medium might be safely supplemented with antiviral agents.

10.3.2. IVP embryos in breeding-improvement programmes

MOET schemes

Using multiple ovulation and ET (MOET) in combination with AI was first advanced as a

concept in the late 1970s. MOET schemes use ET technology to achieve more rapid gains in genetic quality than are provided by conventional progeny testing. The main feature of such schemes is that superovulation and ET are carried out on all the heifers in the MOET herd before they have commenced their first lactations (termed the juvenile method); subsequent modifications to the scheme enable heifers to complete one lactation before ET is attempted (adult method). As currently used, MOET breeding schemes are characterized by the formation of a nucleus herd, with extensive use of superovulation of donors selected among heifers and young cows. In the context of MOET breeding schemes, the use of IVP technology may be valuable, either as a means of permitting embryos to be produced from prepubertal cattle or as a means of increasing the efficiency of *in vitro* embryo production in selected donors (i.e. via repeated transvaginal ultrasonic-guided oocyte recovery). Such applications of IVP technology in MOET programmes have been described by Reinders and Van Wagtendonk-De Leeuw (1996).

In Australia, Montaldo *et al.* (1998) made a comparison of the selection response expected over a 15-year period in nucleus herds using MOET, *in vitro* embryo production and sexed semen with that for an efficient progeny testing scheme. They concluded that sexed semen allowed a reduction in the total numbers of ETs per year and that using IVP technology to obtain more embryos per donor would increase the rate of genetic gain in MOET nucleus herds. Data from a MOET programme operating in a semi-arid tropical region of Brazil, reported by Benyei and Barros (2000), indicated that under high ambient temperature conditions the superovulatory response of milking cows was adversely affected; the repeated application of OPU–IVP embryo production technology applied to such cows during the winter period may be an alternative approach to producing embryos for the programme.

Open-nucleus breeding scheme

A scheme that may be particularly appropriate for use in developing countries is the method known as the 'open-nucleus breeding scheme' (ONBS), which, combined with MOET, may offer the potential for greater progress in genetic improvement in cattle than the traditional or well-established selection methods. The genetically superior nucleus herd is established under controlled conditions where testing and genetic selection can be carried out; this test group is first established by screening the base population for outstanding females. The outstanding animals in the herd are then recorded individually and the élite females among them are used, by MOET with superior sires, to produce embryos that are carried by recipients from the base population. The offspring are reared and recorded and the males among them are evaluated genetically, using the performance of their sibs and paternal half-sibs and their own performance where they exhibit the trait. From these animals, an élite group of bulls with high breeding value is selected to be used in the base population for genetic improvement by natural service or AI.

The ONBS/MOET combination may be the most appropriate route for channelling new developments in reproductive technology into livestock improvement programmes. In establishing the required support facilities for operating the combination, there may be merit in including those required for OPU and *in vitro* embryo production, as well as for conventional ET purposes.

Reducing the generation interval

A key element in cattle breeding programmes is the interval between generations; as noted elsewhere (Section 3.3.8), producing viable embryos from prepubertal cattle could be of considerable value to breeding-improvement schemes. In Argentina, Brogliatti *et al.* (1999) determined the feasibility of producing embryos, pregnancies and calves from prepubertal Brangus calves; they demonstrated that a combination of IVP technology applied to 8–12-month-old calves, and conventional embryo collection at an early age could markedly increase the annual rate of genetic gain in beef cattle. Elsewhere in South America, and as a means of accelerating progress in the Nellore breeding genetic programme, Fernandes *et al.* (2002a,b) produced IVP embryos from peripubertal (9–14-month-old) Nellore heifers of high genetic merit.

Post-mortem use of valuable genetic material

One possibility of making *in vitro* embryo production technology useful to dairy farmers may be by using the ovaries of the genetically superior cow after its demise. The value of a laboratory-produced embryo, whether from dairy or beef animals, will be determined by the animal's genetic merit, the IVP costs and the charges arising from transfer of the embryo. *In vitro* embryo production technology would only come into play upon the slaughter of the cow at the end of its productive life. The appropriate genetically superior dairy bull would be used in providing sperm for IVF; the use of sexed semen could be an additional feature to provide heifer replacements. In beef cattle, there are also opportunities for making use of animals after their demise. In South Africa, Arlotto *et al.* (2001) noted that cattle IVF has become a useful breeding tool in most of the developed world and has been usefully employed commercially in their country to produce live calves from slaughtered Bovelder beef cattle. In British Columbia, Giritharan and Rajamahendran (2001) removed ovaries from culled Friesian cows (using a surgical technique) and were able to obtain an average of six to eight transferable embryos from each FSH-treated cow.

Future developments

The identification of genetically superior cattle, no matter how it is conducted, is inevitably both costly and time-consuming. A paper by Bulfield (2000) reviewed the state of emerging technologies at that time and their potential impact on animal breeding; genome analysis was seen to be one of the technologies that will be developed for use in animal breeding. Malhomme *et al.* (2000) noted that progress in the field of quantitative genetics and *in vitro* embryo production may be the means of developing selection schemes that would greatly ease such problems of cost and time; they suggest that selection could be based partly on markers linked to quantitative trait loci (QTL), genes of economic interest (marker-assisted selection (MAS)). Such markers could be identified in embryos by taking a biopsy sample before the time of transfer. As described in a review by Bodo *et al.* (2001), technical advances in preimplantation genetic diagnosis (PGD) in cattle embryos have already enabled its integration as a part of MOET schemes; the authors note the considerable economic potential of PGD in selecting embryos according to their sex and valuable production traits, using MAS.

Studies by Chrenek *et al.* (2001) demonstrated the possibility of determining the genotype of different loci with the use of a single blastomere and subsequent primer extension by preamplification polymerase chain reaction (PCR); this could be done within a relatively short period of time (30 h). The authors concluded that PGD in cattle opens up new possibilities for practical application of MAS as used in conjunction with OPU–IVP–ET technology. In human assisted reproduction, where PGD represents an alternative to invasive prenatal diagnostic procedures, such as chorionic villus sampling or amniocentesis, Handyside *et al.* (1998) described new approaches to PGD then under development, including the use of lasers to facilitate the biopsy of embryos. Selection of the early bovine embryo for QTL is likely to require the development of methods capable of analysing tens of loci from a small biopsy sample; taking a sample of two to three blastomeres at the eight- to 16-cell stage during IVC would allow sufficient time for analyses to be completed before ET. As noted by Virta *et al.* (2002), who used a fast, nested, multiplex preamplification PCR method and fluorogenic probes (to eliminate the need for time-consuming electrophoresis) in sexing early cattle embryos, similar technology should provide cattle breeders with a powerful tool for selecting genetically superior embryos.

10.3.3. Beef calves from dairy cows

The question of making greater use of dairy cattle in the production of high-quality beef calves has been examined in various ways by different countries over the years. In New Zealand, Vetharaniam and McCall (1999) discussed the use of ET technologies to produce pure-bred beef animals from dairy animals. They carried out a simulation study to predict the size and composition of the New Zealand beef herd under differing levels of ET adoption. In another report from the same country, Tian *et al.* (1999) presented a

quantitative analysis of the potential benefits of using IVP embryos and ET technologies for beef production from spring-calving dairy herds. Their computer simulation study demonstrated that sex-controlled embryo technology was able to capitalize on the significant beef production potential from dairy herds. The authors concluded that profit opportunities for the dairy farmer would be significant if beef IVP and ET costs are no more than AI costs and conception rates for the ET technology are at least 50%.

10.3.4. Twinning by embryo transfer

The incidence of twin births in cattle varies between breeds and according to factors such as age and environment. In the UK and Ireland, the incidence recorded in surveys usually falls somewhere between 2 and 3% (see Table 10.5). Elsewhere, in some reports, the twinning incidence is almost negligible; in others, a twin birth rate as high as 10% has been recorded. Unlike the ewe, in which ovulation rate can be well controlled by active immunization (Fray *et al*., 1994), using such technology in cattle has proved much more difficult (see Gordon, 1996). The only method of achieving consistent and predictable twinning rates in the cow is by way of ET.

Mechanisms controlling double ovulations

Worthy of note is the fact that the treatment of dairy cows in the USA and elsewhere with recombinant bovine somatotrophin (r-BST) has usually been followed by a marked increase in the birth of twins. In a review of mechanisms involved in the ovulation rate in lactating dairy cows, Wiltbank *et al*. (2000) proposed high steroid metabolism as the critical link between high milk production and twin ovulations; these workers believe that high milk production increases steroid metabolism due to increased blood flow to the digestive tract and subsequently to the liver. The liver represents the primary source of steroid metabolism and blood entering the organ is cleared of steroids. At the time of selection of the dominant follicle, the normal increase in circulating oestradiol concentrations and subsequent decrease in FSH is lessened due to oestradiol metabolism; this results in FSH levels remaining elevated sufficiently long to allow two follicles rather than one to undergo the physiological changes necessary to proceed to ovulation.

Elsewhere, Cushman *et al*. (2000) in North Carolina studied the ovaries of cattle selected for twin births; they concluded that such cows were able to maintain more growing follicles at the secondary and subsequent stages of development. At Clay Center, Kappes *et al*. (2000) conducted genomic scans with 183 markers on 181 sires from a cattle population selected for increased twinning rate; their results suggested the location of the region containing the gene(s) involved in follicular recruitment and development. The effect of spontaneously occurring double ovulations on fertility in dairy cows was the subject of a study by Kaim and Bor (2000) in Israel; their results indicated an effect of season on the incidence of twin ovulations and that its type (unilateral or bilateral) may affect fertility. In one herd studied, summer conception rates in cows with unilateral double ovulations were significantly below those in cows with single ovulations; bilateral double ovulations, on the other hand, markedly increased conception rate, in both summer and winter.

Table 10.5. Twin ovulations and twin births in British and Irish cattle (from Gordon, 1996).

	Gordon *et al.*	Scanlon *et al.*
Total no. of calvings	3826	2323
No. with twins	108 (2.82%)	64 (2.76%)
Total no. of cattle examined	436	3136
No. with twin ovulations	18 (4.13%)	107[a] (3.41%)

[a]Includes four animals with more than two ovulations. Data from studies conducted in Wales and Ireland.

Embryo transfer

Improving the biological and economic efficiency of production systems is a matter of concern to workers undertaking research and development work in the beef industry; it has been estimated that twinning would increase the biological and economic efficiency of beef production by 20–25%. In Aberdeen, Sinclair *et al*. (1995) examined pregnancy, twinning and embryo

survival rates of recipient cattle in which twin pregnancies were induced by various combinations of embryo source and transfer method and compared them with animals inseminated or not prior to ET; based on the results, the twinning method adopted at Aberdeen involved the cervical transfer to the contralateral uterine horn of an *in vivo*-produced embryo to recipients that had previously been artificially inseminated. A review of literature by Gong and Webb (1996) led them to conclude at that time that ET was the only reliable approach to the induction of twins in cattle; the same conclusion would probably be true today.

A field-scale test in Ireland to examine cow reproductive performance, twinning rate and overall calf output following the direct transfer of IVP embryos was reported by Bourke *et al.* (1995); the outcome of this work was mentioned in an earlier chapter (see Table 1.2). Of 469 cows that calved after receiving a single IVP embryo a week after breeding by AI, 34% gave birth to twins. The twinning technique was based on research conducted over the previous 30 years in Ireland and which had been reported on by various authors (see Table 10.6). In New Zealand, McMillan (1996) summarized the calving performance of beef cattle induced to calve twins by ET, using data from 30 published studies; the pooled data showed that a higher proportion of cows that received *in vivo*-derived embryos produced twin calves (52 vs. 39%) than those receiving IVP embryos. Since that time, however, the efficiency of embryo production technology is likely to have improved and the gap between the survivability of *in vivo*- and *in vitro*-derived embryos may well have decreased.

In Russia, Vorobyov (1997), using *in vivo*-derived embryos, reported on the effect of twin pregnancy induced by transfer to the contralateral horn of the uterus on birth weights of calves; twins born to cows had a better survival rate and higher birth weight than those born to heifers. In a field trial, Fabaro *et al.* (1998) recorded data which suggested that twinning is feasible provided that IVP embryos are transferred to the contralateral uterine horn of inseminated cattle and that recipients are treated with progesterone (day 5 to day 28) to promote early embryonic development. In Brazil, Capovilla *et al.* (2000) examined the economic viability of twin calf production in Nellore cattle by the transfer of an *in vivo*-derived embryo to the contralateral horn of animals inseminated a week previously; about 40% of cows carried twins and it was concluded that twin production under the conditions described was technically and economically feasible.

In Japan, Numabe *et al.* (2000a,b) examined the production efficiency of Japanese Black beef calves after transfer of IVP cattle embryos; they showed that calves could be produced by Friesian recipients after twin transfers using fresh IVP embryos. The authors calculated that the cost of producing one IVP embryo was about 10% that of producing one *in vivo*-derived embryos; they also estimated that the value of a Japanese Black calf was about ten times more valuable than a Friesian bull calf. Taking all costs into account, it was estimated that the calf birth rate should exceed 40% for this production system to be acceptable. In Brazil, Tomita *et al.* (2002) evaluated the twinning rate obtained by way of IVP embryos; their best results were achieved by

Table 10.6. Twinning by embryo transfer to bred recipient cattle. Pregnancy and twinning rates in cattle after transfer of one embryo to the contralateral horn of the bred recipient's uterus.

		Other reports					
	Boland *et al.* (1975) Ir	Boland *et al.* (1979) Ir	Sreenan and McDonagh (1979) Ir	Renard *et al.* (1979) Fr	Sreenan *et al.* (1981) Ir	Holy *et al.* (1981) Cz	Total of 'other reports'
Cattle	24	52	25	63	84	95	319
Pregnant first service	15 (62%)	34 (65%)	15 (60%)	36 (57%)	49 (58%)	58 (62%)	192 (60%)
Pregnant with twins	6 (40%)	18 (53%)	9 (60%)	16 (44%)	20 (41%)	28 (48%)	91 (47%)

Ir, Ireland; Fr, France; Cz, Czech Republic.

the transfer of an additional embryo into an inseminated recipient.

Feasibility of twinning in farming practice

According to Parminter and Smeaton (1999), social research in 1995 in New Zealand showed that farmers wanted information about twinning technology and its interactions with sheep production and lamb finishing, This led to a 3-year systems trial to examine these issues at Whatawhata Research Centre; the trial showed that it may be profitable for farmers to produce twin calves and rear them to 18 months of age provided they could manage calving. A mentor group was initiated at the start of the trial, including farmers, scientists and a veterinarian; this group was involved in the management of the trial over the 3-year period of its existence. In Canada, Small *et al.* (2000) simulated twin-calf rearing by the adoption of a second calf born within 24 h of the birth of the dam's natural calf; their study suggested there were large potential benefits to beef cows raising two calves, as opposed to one. In New Zealand, Knight *et al.* (2001) reported calf survival from IVP-embryo-induced twinning in dairy-beef cows; they showed that continuous supervision over the calving period achieved perinatal survival rates for twins similar to those for singles.

As noted by Rutledge (2002), one useful application of IVP cattle embryos might be with the twinner breed of beef cattle developed over many years by selective breeding at the Meat Animal Research Centre in Nebraska. Farmers with twinner beef animals are well aware of the important feeding and management aspects of dealing with twin-bearing cows. It is suggested that twinner cows could be checked, a week after breeding, to determine those with a single ovulation; those presumed to be pregnant could be provided with an additional IVP beef embryo, transferred to the contralateral horn of the uterus, to augment the twin-bearing population.

10.3.5. Preserving genetic diversity

There is much interest in the establishment of programmes for the preservation of livestock breeds that may be in danger of extinction, in both developed and developing countries (see Falge *et al.*, 1996; Blackburn *et al.*, 1998; Da Mariante and Fernandez-Boca., 1998; Wells *et al.*, 1999a; Patterson, 2000; Solti *et al.*, 2000; Alderson and Ramsey, 2002; Da Mariante and Egito, 2002). There has been an increasing awareness in recent decades of the importance of domestic breeds for world biodiversity; it is recognized that certain genes and gene combinations may be useful for agriculture in the future. It is also realized that the progress and future development of animal production systems will be dependent on the genetic variability between and within breeds; there is the need for the maintenance of maximum diversity in the genetic pool of each breed to protect against genetic loss, as diversity may be required for the development of sustainable production systems. Changes in the environment or society may require breeds other than those in common use today. It is not easy to predict what genetic traits are likely to be required in the future; for that reason, as many as possible should be preserved.

As a means of safeguarding against the disappearance of local cattle breeds around the world, the Food and Agriculture Organization of the United Nations (FAO) established contacts in 1987 with a view to installing regional animal gene banks in countries around the world. The FAO's mandate to establish an international programme for conserving domestic animal diversity and its sustainable use also led to a database and training information being made available to interested parties by means of the Internet.

It is possible to save male gametes from bulls by low-temperature preservation, although obviously this only preserves half the genetic material. What is needed is a system in which oocytes from female cattle are collected and preserved; this is already feasible to some extent using existing technology. For research purposes, *in vitro* embryo production technology has already been used in studies with wild bovids. In the USA, Johnston *et al.* (1994) sought to determine whether IVP technology as developed for domestic cattle (*Bos taurus*) could be used with a wild bovid, the gaur (*Bos gaurus*); they succeeded in producing embryos and establishing a pregnancy that resulted in a live-born gaur calf. Their work was the first to demonstrate the value of IVP technology in salvaging rare genetic material from animals. A paper by Loi

et al. (2001) dealt with the genetic rescue of an endangered mammal by cross-species NT using post-mortem somatic cells.

Using immature sperm cells

The literature of the recent decade has carried reports dealing with the use of immature male gametes in human assisted reproduction; there is ample evidence from such work that elongated spermatids are cells with the potential to fertilize mature oocytes and produce viable embryos when used in sperm injection (see Tesarik and Mendoza, 1996; Tesarik *et al.*, 1998). Immature gametes may become a consideration when cattle of high genetic value or those in danger of extinction have suddenly died, because such cells maintain their viability for several hours after the animal's demise. In Japan, Goto *et al.* (1996) were the first to demonstrate that bovine oocytes injected with spermatids or testicular sperm were capable of developing to the blastocyst stage (see Table 10.7). A report by Martins *et al.* (2002) in Brazil indicated that the intracytoplasmic injection of refrigerated spermatids may potentially be a useful tool for the proliferation of genes of endangered species.

10.3.6. Embryos for tropical/ subtropical regions

The use of IVP embryos as a means of upgrading the quality of cattle in developing countries is worthy of consideration. It is clear from many reports that cross-breeding native cattle with exotic breeds may create serious problems. Cross-breeding programmes introduced to India in the 1960s showed that neither 100% nor less than 50% *B. taurus* genotype proved to be really useful in that country's animal agriculture. Several studies have shown that *B. taurus* × *Bos indicus* cross-breds may have particular merit in combining the qualities of productivity with the ability to withstand the rigours of the tropical and subtropical environment (Teodoro *et al.*, 1996). It is known that Brahman embryos show superior thermotolerance to Angus or Friesian embryos and that the oocyte plays a more important role in conferring such thermotolerance than the sperm (Block *et al.*, 2002).

The production of first crosses between European breeds and native stock in countries such as India may be one area in which *in vitro* embryo production may prove valuable. Although it may be desirable to maintain the milking cow population at 50% *B. taurus* : 50% *B. indicus* to exploit heterosis, such a population cannot be replaced under existing breeding programmes. The use of IVP technology to produce cross-bred embryos from European dairy cows (e.g. Friesian, Jersey) and native Indian (e.g. Red Sindhi, Sahiwal, Tharparkar) may be one solution to this problem. It would not be difficult to use ovaries from European dairy cows and fertilize oocytes with semen imported from the zebu bulls (see Hutton *et al.*, 1997), although the greater thermotolerance of the zebu oocyte might be an argument for using the reciprocal cross.

The feasibility of producing Brahman × Friesian cross-bred cattle in the tropics by combining US-based IVP technology with direct ET in Venezuela was explored by Hernandez-Fonseca *et al.* (2002); Friesian oocytes obtained from abattoir ovaries were matured and fertilized by Brahman sperm. ETs were carried out early or late in the day to avoid conditions of highest humidity and heat; the work demonstrated that it was possible to produce healthy F1 calves under tropical conditions.

10.3.7. Bypassing heat-stress problems

Among the commercial possibilities worth exploring is the use of IVP technology and ET to bybass the adverse effects of heat stress in dairy cattle; much attention to problems of heat stress in dairy cows has been shown by workers in Florida and Israel. Pregnancy rates from AI or natural service can fall dramatically due to heat stress during periods of hot weather, particularly in high-yielding milking cows (see Roth *et al.*, 2000; Wolfenson *et al.*, 2000); although modern cooling systems are used on dairy farms, fertility may remain unacceptably low. It is well documented that lactating animals are more adversely affected than heifers (see Fig. 10.3), presumably due to their much greater internal heat production. In Australia, McGowan *et al.* (2000) developed a bovine *in vitro* model to

Table 10.7. Development of bovine oocytes injected by various types of male gametes (from Goto *et al.*, 1996).

Type of gamete	No. of oocytes injected	No. (%) reaching		
		2-to 8-cell	Morula	Blastocyst
Spermatocyte[1]	46	16 (34.8)	6 (13.0)	4 (8.7)[a]
Spermatid	42	15 (35.7)	5 (11.9)	3 (7.1)[ab]
Testicular sperm	44	16 (36.4)	8 (18.2)	5 (11.4)[a]
Epididymal sperm				
Caput	45	17 (37.8)	8 (17.8)	4 (8.9)[a]
Corpus	42	13 (31.0)	5 (11.9)	3 (7.1)[ab]
Cauda	45	15 (33.3)	5 (11.1)	2 (4.4)[ab]
Ejaculated sperm	75	24 (32.0)	9 (12.0)	5 (6.7)[ab]
Control[2]	50	11 (22.0)	0 (0)[b]	0 (0)[b]

[a,b]Significantly different, $P < 0.05$ (χ^2 analysis).
[1]Used for injection after meiotic division *in vitro*.
[2]Control = sham injection.

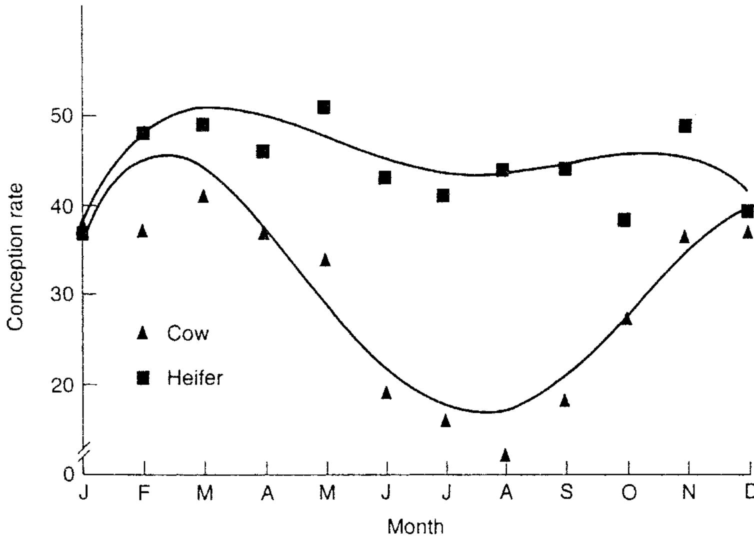

Fig. 10.3. Effect of environment on conception rates in cows and heifers. Least squares means for monthly conception rates (%) in heifers and cows, unadjusted for environmental effects. (From Thatcher and Collier, 1986.)

study the direct effects of hyperthermia on gamete and embryo development; their *in vitro* findings were consistent with reports from the field, showing a negative correlation between environmental temperature on the day of oestrus and subsequent conception rate. Seasonal effects on the development of IVP cattle embryos in a subtropical environment were reported by Rivera *et al.* (2000). Studies by Al-Katanani *et al.* (2001) led them to conclude that there was a summer depression in oocyte quality in non-lactating Friesian cattle in their region of the USA.

Papers by workers in Israel presented data suggesting that hot summer conditions may lead to changes in the membrane properties of oocytes that decrease their developmental competence (Zeron *et al.*, 2001; Zeron and Arav, 2002). Examination of granulosa cells and oocytes indicated a higher content of saturated fatty acids during the summer; in winter, monounsaturated and polyunsaturated fatty acids were higher. A

study by Rivera and Hansen (2001) sought to determine whether exposing bovine oocytes and embryos to temperatures characteristic of body temperatures of heat-stressed cows would affect embryonic development *in vitro*; they showed that embryonic development could be disrupted by a short-term severe (41°C) or prolonged mild (40°C) heat shock. Further work in the same laboratory reported by Rivera *et al.* (2002) demonstrated that exposure of two-cell IVP cattle embryos to elevated temperatures could disrupt embryonic development by causing alterations in the cytoskeleton, cytoplasm, nucleus and mitochondria; some of these changes occurred at temperatures experienced by heat-stressed cows. Studies by Al-Katanani and Hansen (2002) led them to conclude that bovine two-cell embryos may lack the capacity for induced thermotolerance; this may explain in part the increased sensitivity of such embryos to heat shock compared with embryos at later stages of development.

Reports by researchers in Florida (Ambrose *et al.*, 1999; Drost *et al.*, 1999), working with lactating Friesian cattle during the summer, demonstrated that the transfer of *in vivo*-produced embryos collected from non-heat-stressed superovulated donors significantly increased conception rates in heat-stressed cows in comparison with inseminated cattle. A subsequent review by Rutledge (2001) dealt with encouraging results recorded on the possibility of using fresh late-cleavage stage IVP embryos to give acceptable pregnancy rates; as yet, however, the use of frozen–thawed or vitrified IVP embryos has not matched the results achieved with fresh embryos (Al-Katanani *et al.*, 2002c). In due course, given quality IVP embryos that can be successfully cryopreserved, this form of reproductive assistance may have commercial relevance in tropical and subtropical dairy enterprises. The use of sexed semen in the production of embryos would be an added advantage to this approach.

There may be the possibility of preparing IVP cattle embryos to show greater than usual tolerance, by stimulating the production of heat-shock proteins by controlled exposure to sublethal temperatures in the laboratory (Howell and Hansen, 1995; Edwards and Hansen, 1997; Edwards, J.L. *et al.*, 1997). A report by Chandolia *et al.* (1999b) dealt with transcriptional control of development, protein synthesis and heat-induced heat-shock protein-70 synthesis in two-cell cattle embryos; they concluded that embryos can undergo transcription in response to heat shock as early as the two-cell stage and that transcription is essential for early embryonic development.

10.3.8. Dealing with repeat breeders

The developmental competence of oocytes recovered from the ovaries of repeat-breeding cows after *in vitro* embryo production has been noted by various authors. In Japan, Tanaka *et al.* (1995) collected an average of 45 oocytes from eight repeat-breeder cows after slaughter and produced an average of five IVP blastocysts from them; transfer to recipient animals established nine pregnancies and the birth of eight normal calves. There may be occasions other than those arising from environmental causes, such as heat stress, when ET rather than AI may prove effective in establishing pregnancy in high-yielding dairy cattle. In Brazil, Negrao *et al.* (2002) demonstrated that ET in high-producing Friesian dairy cows could be a method of increasing reproductive performance. The animals in question were cows that had not become pregnant after three AIs; ET pregnancy rates (winter/spring, 45%; summer/autumn, 41%) were significantly higher than with AIs (winter/spring, 31%; summer/autumn, 24%). Although such results were achieved with *in vivo*-produced embryos, there is no reason to believe that IVP embryos could not eventually be used for the same purpose.

Infertile cows

Ultrasound-guided aspiration of oocytes in combination with *in vitro* embryo production can be a useful tool in producing embryos from cows failing to respond to superovulation and/or suffering from reproductive disorders. In an English commercial programme, Green and McGuirk (1996) averaged 46 oocytes recovered and nine embryos produced per donor in more than 100 instances of chronically ill, injured and senile cows. In France, a paper by Lacaze *et al.* (1998) describes embryo production from a cow whose fertility was impaired by chronic pyometra; the cow was submitted to OPU once weekly for

10 weeks; this resulted in 19 IVP blastocysts being transferred to host mothers and the birth of ten calves to the cows that became pregnant. In South Africa, Shaw *et al.* (1999), dealing with an infertile Friesian cow of high genetic merit, described events leading to the production of IVP embryos and and a live birth. In Brazil, Avelino *et al.* (2002) reported work with cows from six different breeds and suffering from various forms of acquired infertility (e.g. tubal obstruction, ovarian adhesions); they concluded that *in vitro* embryo production was an alternative that could be employed with cows of high genetic merit unable to produce embryos by conventional ET. Such reports have been a regular feature in cattle ET literature for more than a decade (Palma and Brem, 1995b).

10.3.9. Cattle embryos and oocytes for research

In an age which has seen increasing resistance by animal welfare groups to the use of live animals in research, there are obvious advantages to techniques that enable farm animal embryos and oocytes to be made available for research without this necessarily involving live animals. It is clear, from the many reports appearing in the literature in the past decade, that large numbers of cattle oocytes and embryos are being used by laboratories around the world for research purposes. Among reports in the literature are those dealing with maternal contributions to cattle fertility (McMillan *et al.*, 1998), gossypol effects (Lin *et al.*, 1994; Rivera and Hansen, 1997), heterologous fertilization (Dopp *et al.*, 1995; Kelk *et al.*, 1995; McHugh and Rutledge, 1998), Robertsonian translocations (Geshi *et al.*, 1996), the effect of swainsonine, a plant alkaloid (Holyoak *et al.*, 1998, 1999; ultraviolet-irradiated sperm (Bordignon and Smith, 1999), heterosis in cattle embryos (Fischer *et al.*, 2000), the production of haploid, diploid and tetraploid embryos (Curnow *et al.*, 2000), pyriform-shaped sperm and sperm with proximal droplets (Thundathil *et al.*, 2000a, 2001), uniparental cattle embryos (Chang *et al.*, 2001), interferon-tau (IFN-τ) secretion by embryos (Kubisch *et al.*, 2001a,b) and the effects of scrotal insulation on semen quality (Walters *et al.*, 2002a).

Interspecies nuclear transfer

There is interest in using interspecies NT for practical purposes as well as for basic research. The ready availability of cattle oocytes makes them an attractive source of material for many novel NT studies (Nguyen *et al.*, 2000a); there is some evidence that bovine cytoplasm may be used as a universal recipient in NT. Although failing to produce young after transfer to appropriate surrogate animals, studies reported by Dominko *et al.* (1998, 1999a,b,c, 2000) and Mitalipova *et al.* (1998) in Madison showed that bovine oocyte cytoplasm supported the development of embryos produced by NT of somatic cell nuclei from several species. In Thailand, mindful of the low efficiency of embryo production in buffaloes and the limited availability of buffalo oocytes, Kitiyanant *et al.* (2000) attempted to use bovine oocytes as recipients for buffalo somatic cell NT; using fetal fibroblasts and oviductal cells as sources of donor nuclei, they were able to produce morulae and blastocysts. In subsequent papers from the same laboratory, Saikhun *et al.* (2002a,b) demonstrated that nuclei from buffalo fetal cells could be successfully programmed to develop to the blastocyst stage in bovine cytoplasm at a rate higher than that of nuclei from adult cells.

In Texas, Choi *et al.* (2002a) presented evidence showing that bovine oocytes could be successfully fertilized with equine sperm via intracytoplasmic sperm injection (ICSI); they suggest that bovine oocytes could be a possible alternative source of oocytes for research on stallion sperm nuclei transformation mechanisms, in view of the difficulty in obtaining equine oocytes. In Louisiana, Sansinena *et al.* (2002) also drew attention to the fact that oocyte availability was a major limitation in conducting NT studies in many species, including the horse; they showed that the bovine oocyte was capable of activating mRNA after transfer of equine fibroblast cells. The same workers suggested that the interspecies model could be used as a tool for cell-line screening, which could be useful in species where the limited availability of oocytes precluded prolonged experiments to determine the most suitable cell lines for use in NT.

In the USA, working with an endangered species, the mountain bongo antelope (*Tragelaphus eurycerus isaaci*), Lee, B. *et al.* (2002)

attempted to produce bongo embryos by the transfer of bongo somatic cells to bovine oocytes; blastocyst yields of 15–18% were recorded and the authors concluded that the interspecific NT technique could be used to increase the bongo population. In Japan, Saeki *et al.* (2002) examined early embryo development and gene expression in reconstructed embryos following fusion of bovine enucleated oocytes with cattle, rodent and avian somatic cells; their data indicated that embryos produced with foreign somatic cells may not develop beyond the eight-cell stage.

Identifying toxicants

It is known that early embryonic and fetal development in mammals is sensitive to deficiencies and excesses of specific nutrients and toxicants. In Denmark, Avery and Schmidt (1995) tested whether oocytes and embryos exposed to the antiparasitic drug ivermectin would be affected; they reported evidence of a pronounced toxic effect at 100 μg/ml. A review by McEvoy *et al.* (2001) dealt with threats posed by feed and forage toxicants to the developing embryo and their impact on early programming of fetal development. The authors note that certain toxicant-induced abnormalities are similar to genetic defects, which raises the possibility of a common causal mechanism operating at the gene level. Such evidence would suggest that oocytes and embryos are exquisitely sensitive to alterations in their environment and that disruption of that environment may have long-lasting and unexpected developmental consequences; it is not clear how this view is compatible with evidence from ruminant ETs over several decades which apparently failed to reveal problems that could be traced to embryo culture methods.

Environmental pollutants

There have been reports drawing attention to the fact that environmental oestrogens and other potential hormone-disrupting compounds are widespread and persistent in the environment and may be responsible for some disorders in human fertility and abnormalities found in wildlife. In Germany, Alm *et al.* (1995) examined the effect of different concentrations of widely used pesticides (methoxychlor (MXC); hexochlorcyclohexane (HCH); and dichlorodiphenyltrichloroethane (DDT) on *in vitro* maturation (IVM) of cattle oocytes; they reported increased chromatin degeneration with increasing concentrations of added pesticides. The environmental oestrogenic chemical Aroclor-1254 (a polychlorinated biphenyl (PCB)) was investigated by Pocar *et al.* (1999, 2000) in Milan; they found that the PCB affected bovine oocyte maturation and embryonic development *in vitro* at concentrations many times lower than concentrations found in human follicular fluid and serum.

10.4. Sex Control by Sperm Separation

Gender preselection has enormous implications for both farm animals and humans. Many authors have commented on the advantages of using sexed semen in cattle production (e.g. Hohenboken, 1999; Seidel, 1999a,b; Renaville *et al.*, 2001). Although numerous studies have been performed to separate X- and Y-chromosome-bearing cells, very few of those claiming to be successful have proved to be repeatable. However, in both cattle and humans, it is now clear that sperm may be separated with relatively low rates of contamination using flow cytometry. As in many other mammals, including humans, the Y sex chromosome in cattle is smaller than the X-chromosome; separation is possible on the basis of their relative DNA content. Flow cytometry permits separation, after measuring DNA content, based on the fluorescence emission intensity after staining with a DNA-specific fluorochrome (Hoechst 33342). A schematic diagram of a typical flow cytometer system is given in Fig. 10.4.

Although there are those who advise caution in applying such technology, if there are problems related to DNA staining or laser use in the sperm separation process, they have not been evident in the data already available in pigs, rabbits, cattle and sheep (Johnson and Schulman, 1994) and similar reassuring information comes from work with human sperm (Watkins *et al.*, 1996). The separation technology, which has become increasingly efficient, may be relevant at some stage in lessening serious human concerns. As observed by Edwards and Beard (1995), use of separation technology could one day help in

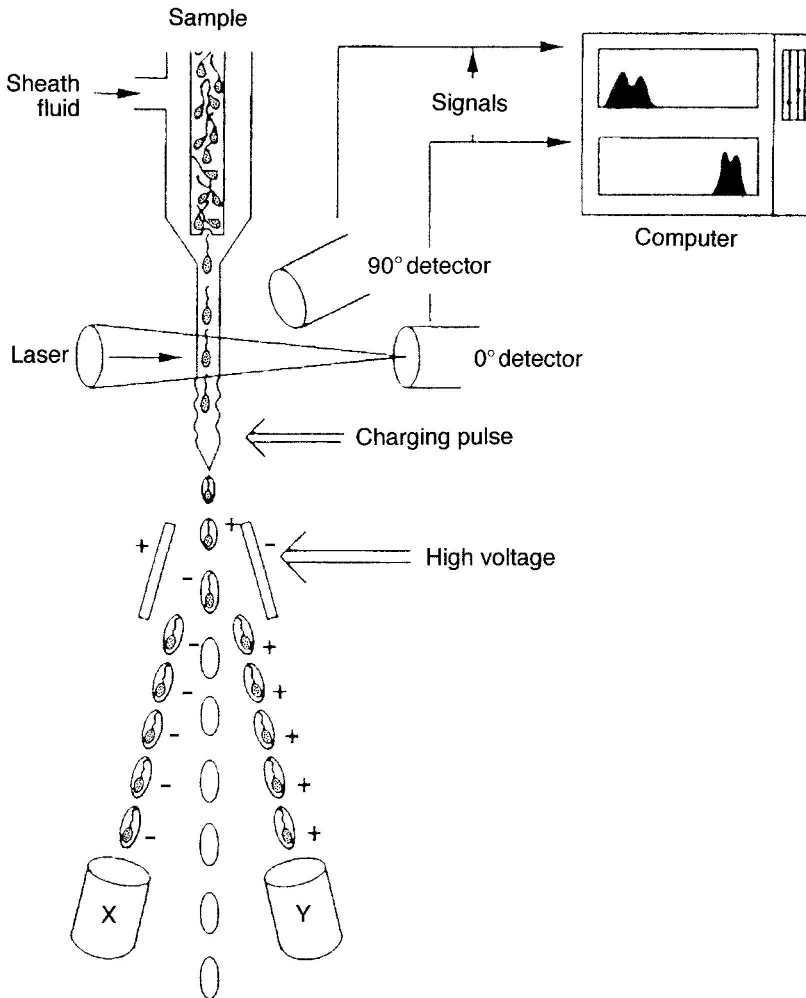

Fig. 10.4. Schematic diagram of main components of a typical flow cytometer.

reducing selective abortion of female fetuses or infanticide, which is currently reported from some societies.

It should be noted that there are situations in which bull sperm can give unexpected sex ratios without any form of technology being involved. A report by Chandler *et al.* (1998) showed a marked variation in the percentage of Y-chromosome-bearing sperm between ejaculates collected from a particular bull; the percentage of bull calves produced after AI with sperm from this bull confirmed the findings with the sperm. Such information led Chandler *et al.* (2002) to design experiments to evaluate the effect of different collection frequency regimes on the variation of the Y-chromosome percentage in successive ejaculates taken from several bulls; they found that different collection-frequency regimes made a significant contribution to the variation in Y-chromosome content in successive ejaculates from individual bulls. There appeared to be some association between sexual rest and a large amount of variability in Y-chromosome content, implying some epididymal function. The authors suggest that it may be possible to devise bull management routines that would maximize the observed variation in Y-chromosome content and eventually lead to a method of manipulating the sex ratio of calves on the farm.

10.4.1. The case for semen sexing

Considerable progress has been made in semen sexing in recent years; one British cattle-breeding company (Cogent) already makes limited supplies of sexed semen commercially available to farmers in the UK and Ireland. This semen sexing is based on the technology devised by Johnson and associates at Beltsville in the USA (Johnson, 1994, 1998, 2000; Johnson and Schulman, 1994; Johnson *et al.*, 1994, 1996, 1997, 1998, 1999, 2000; Johnson, M.L. *et al.*, 1997; Rens *et al.*, 1997, 1999; Gurnsey and Johnson, 1998; Johnson and Welch, 1999).

Of historical interest is the fact that the first calves born from semen sexed by the Beltsville technique were derived from IVP cattle embryos (Cran *et al.*, 1993a,b, 1995); clearly, far fewer sperm are required for fertilization when IVF rather than AI is employed, although account had to be taken in initial studies of the reduced motility and viability of sorted sperm. In some of the initial studies, it was estimated that sufficient sperm for 100 oocytes could be sorted in about 1 h (Cran *et al.*, 1994). In due course, sexed semen will be available to the cattle ET industry; this may involve bull sperm being sorted at a central facility for use by ET practitioners. For some in the cattle ET industry, there may be attractions other than in providing sorted semen for cattle IVF; sexed semen may have a role to play in breeding donor animals after conventional superovulation treatments. Workers in Colorado recorded the recovery of cattle embryos from superovulated beef heifers inseminated with low doses of sexed sperm; 93% of embryos recovered were of the intended sex (Chung *et al.*, 1998).

The possible role of semen sorting in cattle farming, on the wider front of both AI and ET, is summarized in Table 10.8. In an age of heightened animal-welfare concern, the commercial availability of sexed semen should improve animal welfare as well as returns on the dairy farm. Dairy farmers will be able to avoid the birth of dairy bull calves, which may otherwise be destined for early slaughter. For selective breeding purposes, the availability of sexed semen will enable young dairy sires to be proved much more efficiently, using their sperm to produce a preponderance of heifer calves (Seidel, 1998a,b; Jacobsen, 1999; Reichert, 1999; Seidel and Johnson, 1999).

Table 10.8. Possible value of sex control in cattle farming (from Foote and Miller, 1971).

Dairy cattle
More heifer progeny from good cows as herd replacements for milk production, or getting heifers from better cows
More bull progeny for meat, especially from ‘cull’ cows
Ensuring birth of bulls as potential sires from top cow × sire
Ensuring birth of heifers when progeny-testing young bulls
Avoiding freemartins in multiple births
Beef cattle
More bull calves for meat via beef rearing
Ensuring bull progeny as potential sires from top cow × sire
Ensuring heifers from next-best cows × top sires as future brood cows
Avoiding birth of freemartins in multiple births

There are those who believe that dairy breeds such as the Ayrshire, Jersey and Guernsey may become increasingly important as crossing animals in UK dairy farming; they visualize a scenario in which cross-bred cows are produced to take advantage of hybrid fertility and long-term health benefits. In this, it should be noted that in several countries, including the UK and Ireland, there has been a well-documented decrease in Friesian herd fertility (O’Farrell and Crilly, 1999; McEvoy, 2000); the hybrid vigour of first-cross cows (e.g. Ayrshire × Friesian) might be one way of dealing with that problem. The advent of semen sexing in the country should make it feasible for dairy farmers to countenance having relatively high numbers of first-cross dairy animals in their herds.

For those engaged in beef production, the availability of sexed semen could bring benefits in the returns from steer rather than heifer cattle. Possible advantages of sexed semen in relation to the American beef- and dairy-cattle scene have been discussed by Seidel (1998a,b, 1999a,b). On the wider agricultural front, there are likely to be distinct commercial advantages in the use of semen sexing in the pig world. Producing pigs of a single sex (male) could mean that half the pigs would be some 15% more efficient; it would also avoid the need for split-sex feeding and enable nutritional requirements to be met more accurately, resulting in a more uniform end-product.

10.4.2. Semen-sexing technology

Beltsville sexing technology

The Beltsville semen-sexing technology is based on separating sperm according to their chromosomal content. The autosomes carried by X and Y spermatozoa have identical DNA content; the difference in DNA mass between male and female gametes depends on the sex chromosomes. The X-chromosome is larger and carries more DNA than the smaller Y-chromosome in all mammals; the DNA differences between sperm of domestic livestock vary from 3.5 to 4.2% (Johnson, 1994). Although the difficulties of commercializing semen sexing by flow cytometry should not be underestimated, there are reasonable grounds for believing that they can be overcome within a reasonable time span (see Amann, 1999, 2000; Welch and Johnson, 1999; Johnson and Welch, 1999; Johnson, 2000). The speed of sperm-sorters has been increased considerably in recent years, which means that larger sperm doses can be employed with modified insemination routines. A review by Cran (1999) noted a 30-fold increase in sperm-sorting rates using current technology. In the early 1990s, before the advent of high-speed sorters (MoFlo-Cytomation, Inc.) and other technical improvements, the sorting rate was only 0.3 million/h; the improved sorting rate was 10 million/h. Reviews by Hunter (2000, 2001) have discussed deep insemination into the ipsilateral uterine horn, which might be useful in dealing with limited numbers of sex-selected cells.

Validation of the Beltsville sorting technology has been provided by sort reanalysis in the laboratory and by the birth of calves on the farm (Kawarasaki *et al.*, 1998; Welch and Johnson, 1999). Sort purities ranging from 85 to 98% in boar and bull semen were shown by way of such reanalysis (Welch and Johnson, 1999). Data gathered by Cran *et al.* (1995) and Seidel *et al.* (1999) showed the gender of calves on the farm to be markedly skewed in favour of the required sex. Referring to trials in the UK, Cran (2000) noted that embryonic death in the first 2 months after using sexed semen was similar to that of controls and there was no increased incidence of abortion between 2 months and term.

In the USA, Johnson, L.A. *et al.* (1997) determined the characteristic sorting pattern of semen from 40 young bulls (38 Friesian, one Guernsey and one Jersey); only one bull was classified as unsortable using the Beltsville sexing technology. Other work at the US Department of Agriculture (USDA) centre examined embryo development after IVF with sexed bull sperm and the effect of oocyte maturational status at the time of insemination (Beyhan *et al.*, 1998); results indicated a dimorphic pattern of development in male and female embryos during fertilization and first cleavage with little evidence of an oocyte effect. A further Beltsville paper by Penfold *et al.* (1998) recorded significant differences between X and Y sperm in certain motion parameters.

Other sorting studies

Almost all current reports on bovine sperm sorting by flow cytometry relate to work in the USA using the Beltsville technology. Exceptions include a paper by Gurnsey *et al.* (1994) in New Zealand, who showed that sorted bull sperm could be transported for considerable distances before and after sorting and could give acceptable fertilization and cleavage rates when used in embryo production. In the same country, Arlotto *et al.* (1996a,b) reported on their IVF studies; the greatest yield of early embryos and blastocysts was achieved at a ratio of some 500–600 sperm per oocyte. As noted elsewhere (see Section 6.9.4), the ultimate in utilizing a genetically superior bull would be by ICSI. Already, in Japan, single sperm heads from a Y-chromosome-rich population have been injected by Hamano *et al.* (1999a) into IVM oocytes; 6.9% of oocytes developed to the blastocyst stage and ten normal calves (eight males and two females) were born after transfer to recipient cattle.

In the Netherlands, the work of Merton *et al.* (1997) involved sorted sperm from a single bull in cattle IVF; they recorded a marked reduction in blastocyst yield using stained sperm and a further decrease using stained/sorted sperm. Other studies in the same country by Stap *et al.* (1998) examined the effect of preparing frozen–thawed semen for flow cytometry by removing the diluent's egg-yolk and staining the sperm with Hoechst 33342; they used a Percoll density gradient to reduce the fluorescence of dead sperm, which enabled such cells to be excluded from the sorting window. In Italy, Balduzzi *et al.* (2000) reported findings on sexing frozen–thawed bull

sperm by flow cytometry for the production of IVP embryos of high genetic merit; the fertilization rates quoted (35–40%) clearly left room for improvement.

Sperm-membrane changes in sorted sperm

In Germany, Medvedev *et al.* (1997) found evidence suggesting that frozen–thawed flow-sorted sperm may be as effective in activating bovine oocytes as non-sorted cells; this might indicate that sorting adversely affected characteristics such as motility rather than the fertilizing capacity of the spermatozoon when introduced into the oocyte by microinjection.

As to the nature of other defects occurring in bull sperm after staining and flow sorting, a Beltsville study by McNutt and Johnson (1996a,b) indicated that sperm-membrane proteins undergo changes that may affect sperm function and sperm–oocyte interactions. There is probably a good case for examining a range of factors, genetic, nutritional or otherwise, that may influence the nature and function of the sperm membrane. As mentioned earlier, the report of Doyle *et al.* (1999) indicating that certain bulls tolerate sperm sorting better than others may be relevant to sperm-membrane considerations.

A study reported by Lu *et al.* (2001) sought to determine differences among bulls in fertilization rates and embryo yields; one of the bulls was associated with a very low yield of embryos with sorted sperm (6%) but a normal yield with unsorted sperm (26%). Studies reported by Garner and Suh (2002) also found that bulls differed in the susceptibility of their sperm to the mechanical stresses of sorting and centrifugation, laser illumination, Hoechst 33342 staining and exposure to both laser and stain; the most damaging step in the sorting process was mechanical stress. Other studies in the same laboratory indicated that sperm viability was partially decreased during sexing by sorting due to plasma membrane damage and loss of mitochondria function (Caballero *et al.*, 2002); the variation found among bulls in their tolerance to the sexing procedure suggested that the sperm from bulls of good fertility produced sperm with a higher tolerance to sorting than did bulls of low fertility.

In the USA, workers examined the way in which seminal plasma may protect the viability of diluted bull sperm; it is well established in the cattle AI industry that dilution of bull semen below 20–30 million sperm/0.5 ml can lead to a loss of viability in the frozen–thawed sperm. It is also known that extreme dilution of mammalian sperm may result in what is termed the ‘dilution effect’, a phenomenon characterized by loss of motility, metabolic activity and fertilizing capacity. A paper by Garner *et al.* (1999) reported evidence that the addition of seminal plasma may prove beneficial to sperm diluted below the 30 million/0.5 level; such data may be relevant to those engaged in semen sorting. In Argentina, Cattaneo *et al.* (2002) found evidence suggesting that the seminal plasma/diluent ratio could affect the staining of sperm and the effectiveness of the sorting process; it may prove possible to keep this variable constant by centrifuging the raw semen.

In sheep, working in Australia, Maxwell *et al.* (1999) found that treatment of frozen-thawed ram semen with seminal plasma markedly improved fertility after AI and surmised that this may due to its decapacitation effect combined with a substantial improvement in sperm transport; further reports on such work have appeared (Evans *et al.*, 2000; Maxwell *et al.*, 1999; McPhie *et al.*, 2000; Maxwell and Evans, 2000). Clearly worth exploring is the possibility that seminal plasma may help bull sperm through the trauma of cell sorting.

Effect of Fert Plus peptide

There is evidence that the exposure of bull sperm to a synthetic peptide (Fert Plus, Biopore Inc., State College, Pennsylvania) was capable of effecting a marked increase in the pregnancy rate of subfertile bulls (Amann *et al.*, 1999). A subsequent study reported by Seidel and Maclellan (2001) exposed sperm destined for use in cattle IVF to a 15 min period of incubation with the synthetic peptide. Results showed a significant and beneficial effect on cleavage rate in comparison with controls (Table 10.9); the authors suggested that the peptide might be useful when sperm numbers are limited. It might also be possible that such a peptide has relevance to increasing the fertility of sexed semen.

Frozen sexed semen

Some early trials with sorted semen involved shipping sperm long distances, stored at ambient

temperature or 5°C (Seidel *et al.*, 1996). However, for any serious thought of commercial application, there is an obvious need to ensure that sexed semen, after it has been sorted, needs to be stored in the frozen state without any appreciable loss of fertilizing capacity (Schenk *et al.*, 1999). In Colorado, early encouraging results with frozen–thawed semen in Angus heifers were reported by Seidel *et al.* (1997). Studies that included the use of frozen sorted semen in lactating Angus cows were reported by Doyle *et al.* (1999), who drew attention to the fact that fertility of sexed semen was much higher with certain bulls; they noted that commercial application of the sexing technology may require selecting those bulls whose sperm tolerate the stress, particularly when low sperm doses are inseminated. As mentioned by Jacobsen (1999), trials are in progress in the UK using frozen sexed semen and a newly developed round-headed, side-delivery insemination 'gun' capable of delivering semen deep into the body of the uterus. In Argentina, Brogliatti *et al.* (2002), using sorted semen in three different breeds of cattle under field conditions reported on the feasibility of using frozen–thawed sperm doses of 3 million; they concluded from their preliminary results that commercialization of frozen sexed semen should be possible in the near future (Table 10.10).

Table 10.9. Effects of Fert Plus on cleavage and blastocyst production in cattle (from Seidel and Maclellan, 2001).

Treatment	*n*	Cleavage (%)	Blastocysts (% Cl)
0.0 nM peptide	499	272/499 (55)[a]	134/272 (49)
3.3 nM peptide	488	323/488 (66)[b]	170/323 (53)
10.0 nM peptide	486	335/486 (69)[b]	157/335 (47)
30.0 nM peptide	442	230/442 (52)[a]	112/230 (49)

[a,b]Values without common superscript differ ($P < 0.05$, χ^2).
CL, cleaved embryos.

In vitro *fertilization with sorted bull sperm*

Already mentioned is the fact that the first calves born from semen sexed by the Beltsville technology were produced by IVF in the laboratory and subsequent ET in the UK to host mothers (Cran *et al.*, 1993a,b, 1995). IVF could be the most efficient avenue for sexed embryo production for the ET industry; clearly, far fewer sperm are required for fertilization *in vitro* than for conventional AI (Hernandez-Fonseca *et al.*, 2001). Studies in Colorado by Lu, K.H. *et al.* (1999) have shown that, although fertilization rates with sorted and unsorted sperm were similar, the blastocyst yield was lower with the sexed cells. In subsequent reports, the workers in Colorado have shown that low concentrations of insulin (0.12 iu/ml) added to complete defined medium 1 (CDM-1) improve embryo quality and accelerates embryo development after fertilization with sorted sperm (Lu and Seidel, 2000; Olson and Seidel, 2000a,b). There was also evidence from the Colorado laboratory indicating that non-essential amino acids (NEAA) played a role in the early cleavage of embryos and in achieving higher blastocyst yields.

Work by Lu and Seidel (2002) established a two-step culture system for embryo production in which oocytes were cultured in CDM-1 medium containing NEAA for 72 h and then cultured in CDM-2 containing both NEAA and essential amino acids from day 4 to day 7, combined with insulin addition on day 5. The system

Table 10.10. Pregnancy rates using sexed and unsexed frozen–thawed semen. Pregnancy rates using control frozen unsexed semen (20 million sperm per straw) and frozen sexed semen (3 million sperm per straw) in three different breeds. (From Brogliatti *et al.*, 2002.)

	Hereford *n* = 111	Friesian *n* = 137	Angus *n* = 60	Overall total *n* = 308
Group 1, control (20 million)	43/66 (65%)	36/47 (76%)[a]	15/32 (47%)	94/145 (65%)[a]
Group 2, sexed semen (3 million)	22/45 (49%)	48/90 (53%)[b]	11/28 (37%)	81/163 (50%)[b]

Values with different superscripts in the same column differ ($P < 0.05$).

resulted in a cleavage and day-7 blastocyst yield with sorted sperm that reached the level achieved with unsorted cells (78.4%, 25.7% vs. 83%, 26.6%, respectively); a paper by Seidel *et al.* (2002) confirmed the normality of the calves derived from sperm sexed by this procedure.

10.4.3. Alternatives to sexing by flow cytometry

Immunological approach

Not all commercial concerns have been enthusiastic about sexing by flow cytometry; there were those who initially might have taken the view that the high cost and low pregnancy rates associated with the sorting technique made the procedure practically irrelevant. Some concerns have supported work that sought to detect sex-specific differences in sperm surface antigenicity in cattle; findings reported by Howes *et al.* (1997) suggested that this approach to semen sexing may not necessarily be fruitful. Similar views have been expressed by workers in the Netherlands (Hendriksen *et al.*, 1996; Hendriksen, 1999).

Sexed-semen research has been conducted by a Canadian biotechnology company (Gensel Biotechnologies). The Gensel technique was apparently based on the assumption that bull and boar sperm have sex-specific proteins on their surface that can be separated using appropriate antibodies; the hope was that addition of male antibody would permit X-chromosome-bearing gametes to be filtered out, without causing cell damage, enabling the sperm to be used either fresh (pigs) or frozen (cattle) in the normal way. Although a paper by Blecher (1996) reported the development of a non-invasive embryo sexing method, using antibodies to target sex-specific proteins on the outer cell surface of embryos and a subsequent paper held out the hope that a viable immunological sperm sexing procedure might be developed (Blecher *et al.*, 1999), no report has apparently been published indicating that this approach has been successfully adapted to separating sperm cells. It may be noted, however, that ongoing research elsewhere into immunological possibilities for semen sexing in cattle has been reported from Slovakia (Kovacikova *et al.*, 2001) and Brazil (van Tilburg *et al.*, 2001).

In dealing with immunological studies, it might be mentioned that Beltsville workers did conduct one detailed study in collaboration with colleagues in the Netherlands in unsuccessful attempts to isolate and characterize sex-specific surface proteins in boar sperm; in this, many membrane proteins in X- and Y-bearing sperm were examined for qualitative and quantitative differences (Hendriksen *et al.*, 1996).

Separation by density gradients

The medical literature contains many papers reporting successes and failures in attempts to influence the human sex ratio at birth by the use of albumin columns and other procedures (e.g. Ericsson, 1994; Pyrzak, 1994). None the less, a recent study by Bartsich *et al.* (2001) may deserve attention; they used a static density gradient to select sex-specific human sperm. In order to avoid sperm motility as a confounding factor in their six-layer Percoll density-selection method, sperm were partially immobilized by way of a motility-inhibiting agent (lysophosphatidylcholine). Fractions recovered from the top of the gradient revealed a $62.4 \pm 2\%$ concentration of Y-bearing sperm versus $37.6 \pm 2\%$ of X-bearing cells; fractions from the bottom layer revealed a concentration of $58.5 \pm 1\%$ of X-bearing cells versus $41.5 \pm 1\%$ Y-bearing cells. Although no difference was evident in the ratio of X or Y sperm in the untreated population, treated sperm showed evidence of a significant skewing effect. According to the authors, they saw this study as representing the first step in the development of a simple, minimally invasive and effective method of gender selection of human sperm.

In cattle, one group in Brazil has reported a significant deviation towards female embryos using bull sperm centrifuged through different Percoll density gradients in an attempt to separate X- and Y-bearing sperm (Hossepian de Lima *et al.*, 2000a,b). In contrast to Bartsich *et al.* (2001), who identified separated sperm on the basis of fluorescence *in-situ* hybridization (FISH) with X- and Y-chromosome probes, the South American workers based their identification of sperm on the presence of the F body. It is believed that a more accurate estimation of sex chromosome content is by means of double-label FISH, this technique currently being the method of

choice for evaluating gender preselection methods (Flaherty and Matthews, 1996). In Sweden, Hassanane *et al*. (1999) applied double-label FISH with sex-chromosome-specific probes to detect X and Y sperm very effectively in five Friesian bulls; in Japan, Kobayashi *et al*. (1999) concluded that the FISH with a Y-chromosome-specific probe which they had developed could effectively identify Y-bearing bovine sperm.

Spermatozoal head size and volume

Several reports from Australia and elsewhere have clearly shown that human X-bearing sperm are both larger and longer than Y-bearing sperm (Cui, 1997); X-sperm heads were found to be some 6% greater in area. Concerns about possible cytoxic and/or mutagenic effects from ultraviolet (UV)-excitable DNA-specific stains used in flow-cytometric separations (Ashwood-Smith, 1994) have led some investigators to examine sperm head size (Chandler *et al*., 1999a,b) and sperm head volume (Van Munster *et al*., 1999) as possible candidates for use in of a bull semen-sexing procedure. In California, Watkins *et al*. (1995) recorded evidence of differing sex ratios according to human sperm dimensions. Although there is ample evidence, both in bulls and humans, that X- and Y-chromosome-bearing sperm can vary measurably in such features, there appears to be far too much overlap in germ cell dimensions to make these the basis of a separation technique.

10.4.4. Effect of AI timing on sex ratio

From time to time, reports appear on the skewing of the sex ratio in cattle. In Spain, for example, Martinez *et al*. (2000) reported studies on data from 423 calves born to Friesian cattle showing significant differences in the percentage of heifers born according to the time interval between the onset of oestrus and AI. Attempts to deliberately influence the gender of calves in recent years have included the use of a vaginal probe, based on evidence reported by Wehner *et al*. (1997), working in Missouri. Cows inseminated when impedance values were declining produced heifer calves, while those inseminated when values were rising gave birth to bull calves; data were derived from studies with a herd of 75 beef cows over a period of 3 years. Presumably, cattle would have to be checked twice daily to record and plot changes in the resistance of vaginal secretions, clearly a labour-intensive operation. Trials reported by Jobst *et al*. (1998), however, failed to confirm that gender may be preselected in this way. In trials on 11 farms and with 822 cows, the Virginian researchers found no evidence that gender-frequency difference was linked to the interval from oestrus onset to the time of AI. Elsewhere in the USA, Rorie *et al*. (1999a,b) also reported that the timing of insemination had no effect on the sex ratio of calves in their studies (see Table 10.11). A quarter-century earlier, readers could have found similar conclusions in a report on dairy cattle by Foote (1977).

A paper by Rorie (1999), however, has drawn attention to several conflicting reports on cattle sex ratios, the author finally concluding that any effect of time of insemination must be relatively small. None the less, it has to be noted that many mammal populations (e.g. deer) have shown significant deviations from an equal sex ratio at birth (Kruuk *et al*., 1999a,b) and occasionally there has been puzzling evidence in cattle that certain procedures (e.g. freezing of sperm/embryos; source of sperm; sperm preparation method; IVP embryos) may markedly skew

Table 10.11. Effect of time of AI after the onset of oestrus on the sex ratio in cattle (from Rorie *et al*., 1999b).

Item	Inseminated ≤ 10 h after the onset of oestrus	Inseminated ≥ 20 h after the onset of oestrus	Overall
No. inseminated	56	42	98
No. (%) confirmed pregnant and sexed	39 (69.6)	29 (69.0)	68
No. (%) bulls	21 (53.8)	15 (51.7)	36 (52.9)
No. (%) heifers	18 (46.2)	14 (48.3)	32 (47.1)

the sex ratio from the 50 : 50 value normally expected (Behboodi *et al.*, 1995, 1997; Grisart *et al.*, 1995; Lonergan *et al.*, 1995; Newcomb and Dyke, 1995; Gutierrez-Adan *et al.*, 1996; Chandler *et al.*, 1997, 1998; Pursley *et al.*, 1998; Carbonneau *et al.*, 1999; Kania *et al.*, 1999; Rheingantz *et al.*, 2000). Similar unexplained effects have been recorded in sheep; in Spain, for example, Gutierrez-Adan *et al.* (1999a,b) found evidence of significant differences in the sex ratio in sheep between those inseminated 5 h preceding ovulation and those inseminated 5 h after ovulation.

One suggestion is that the Y-chromosome-bearing spermatozoon capacitates quickly whereas female sperm are slower to capacitate and have a longer lifespan. It would be more than surprising if any genuine and hitherto undetected difference in capacitation time between the two types of bovine sperm exists. Less surprising would be effects on the sex ratio arising from fetal loss during pregnancy. In deer, there is the suggestion that male fetuses are more vulnerable than females to the mother's nutritional stress (arising from high population densities or other causes of food deprivation) because of faster male growth rates *in utero* (Kruuk *et al.*, 1999a,b); male fetuses require more nutrients and so may well be more adversely affected by food restrictions. It may be mentioned that some have suggested that the skewed sex ratio observed in IVF embryos originates from the oocytes (Carbonneau *et al.*, 1999). A paper by Larson *et al.* (2001) presented data indicating that female cattle blastocysts release more IFN-τ than male embryos during IVC; in view of the role of IFN-τ in maternal recognition, the activity of this pregnancy protein may be a factor in explaining some instances of biased sexed ratio.

10.4.5. Sperm separation in other farm animals

Pigs

As noted earlier, there are likely to be useful commercial advantages in the use of sexed semen in the pig world. The birth of piglets after IVF using sperm cytometrically sorted for gender was reported by Rath *et al.* (1996); these were the first results showing that this approach could result in healthy, normal piglets of the chosen sex. A report by Kawarasaki *et al.* (1998) showed a high purity of sorted X- and Y-chromosome-bearing pig sperm. Collaborative work between Beltsville and researchers in Missouri demonstrated that boar sperm could be sorted at a central facility and transported to a remote location to be used successfully in IVF. Studies reported by Guthrie *et al.* (2000) sought to determine the optimum laser power required for sorting; other work at Beltsville showed that pregnancies could be established with sperm doses of 100–400 million placed beyond the cervix by a modified Melrose catheter (Johnson *et al.*, 2000).

Elsewhere, other workers similarly drew attention to the need to develop a modified insemination protocol that would permit insemination with low sperm doses. AI in pigs requires 2000–3000 million sperm to achieve consistently high fertility with the current practice of inseminating into the posterior region of the cervix. A study by Krueger *et al.* (1999) demonstrated that AI with 10 million sperm, instead of the conventional 3000 million, was effective if delivered to the tip of the uterine horns. In Spain, Martinez *et al.* (2000) developed a fibre-optic endoscopic technique for deep uterine-horn insemination without requiring sedation; they reported inseminating 90% of sows within 3–7 min; insemination results obtained with this technology demonstrated that as few as 50 million sperm could be used without a decrease in fertility and litter size (Martinez *et al.*, 2000). In the same laboratory, Vazquez *et al.* (2000) reported attempts to determine critical sperm numbers required to achieve acceptable fertility using fresh, diluted and cryopreserved boar sperm; using 50 million sperm doses, results reported by Vazquez *et al.* (2002) showed that the combination of the Beltsville sorting technology and their deep intrauterine insemination could increase the application of sorted semen. In Mexico, Parrilla *et al.* (2001) monitored changes occurring in the motility characteristics of flow-cytometrically sorted porcine sperm; large changes in sperm motility patterns were recorded.

Elsewhere, Wolken *et al.* (2002) inseminated sows with low sperm doses (100–500 million in various volumes) into the distal uterine horn with a specially designed insemination

device; their results indicated a need to focus attention on repeated inseminations during oestrus In embryo production studies, Abeydeera *et al.* (1998) reported the birth of piglets after using sexed sperm in the USA. In Germany, Rath *et al.* (1999a) similarly reported the IVP of sexed embryos; six litters from sexed sperm (X-sorted) produced 33 females (97%) and one male. Other work in the same laboratory looked at the possibility of using ICSI to produce sexed pig embryos (Rath *et al.*, 1999b). A later study by Probst *et al.* (2002) reported further work on the intracytoplasmic injection of sorted sperm; although the efficiency of their ICSI technique was low, they concluded that it might be a potential method of optimizing the use of sexed semen. The German workers noted that further work is required to culture sperm-injected oocytes to the stage (morula and beyond) at which they can be non-surgically transferred.

Horses

There could be interest in some sectors of the horse-breeding industry in the prospect of being able to produce foals of the desired sex. Gender plays an important part in determining the success of horses in several areas of their sporting activities. As observed by Allen and Antczak (2000), geldings are favoured for 3-day events and mares are preferred for polo playing, to take but two examples. Using sperm sorting, foals of a predetermined sex may be produced either by the ET approach or by low-sperm-dose AI. In regard to the first possibility, it should be noted that the ICSI technique has resulted in the birth of live foals in recent years, using IVM oocytes from oestrous mares (Squires *et al.*, 1996; McKinnon *et al.*, 1998), from progestogen-treated mares (Cochran *et al.*, 1999) and from the ovaries of pregnant mares; such efforts hold out hope of using small numbers of frozen–thawed stallion sperm to produce multiple embryos from selected mares. Obviously, the high value of individual animals in the horse-breeding industry may make commercial application of this form of assisted reproduction economically feasible.

In Colorado, Buchanan *et al.* (1999, 2000) reported pregnancy rates in mares inseminated with reduced sperm numbers to the tip of the uterine horn; a dose of 25 million progressively motile sperm resulted in a pregnancy rate of 57%. Elsewhere, Morris, L.H.A. *et al.* (2000) examined the possibility of achieving fertility in oestrous mares by a single hysteroscopic deposition of a small dose of sperm (1 million) on to the uterotubal papilla, ipsilateral to the ovary bearing the preovulatory follicle. Further reports from Colorado also dealt with hysteroscopic insemination of low numbers (5 million) of flow-sorted fresh and frozen–thawed sperm; no difference was recorded in the pregnancy rates using fresh sorted and unsorted sperm but the rate was lower with frozen sperm (Lindsey *et al.*, 2002a,b).

Sheep

In Scotland, Cran *et al.* (1997) reported small-scale trials which demonstrated that pregnancy could be established and lambs of predetermined sex obtained by inseminating a low dose of sorted ovine sperm close to the uterotubal junction of the ewe. In Australia, Catt *et al.* (1997a,b) reported on attempts to improve the quality of ram sperm after sorting; they concluded that use of a presorting diluent and the identification of live sperm could be useful. A review of possible applications of sorted semen in sheep was provided by McEvoy (2000).

10.5. Embryo Sexing

Although the prospects of gender preselection by sperm sorting will certainly mean using AI as the main technique on the farm, embryo sexing must inevitably involve the use of cattle ET technology. Some of the embryo sexing methods examined in recent times are shown in Fig. 10.5. Although there have been promising developments in semen sorting, it will take time for such procedures to reach the farm in an economically viable form; for that reason, sexing of embryos is likely to remain a major route to gender preselection.

The first success with gender preselection in cattle was a calf born via the embryo sexing route on Christmas Day in 1975 in Canada. Since that day, many thousands of sexed calves have been born after various forms of embryo sexing. Not all those reporting on embryo sexing have expressed optimism about its future. In Germany, Lange

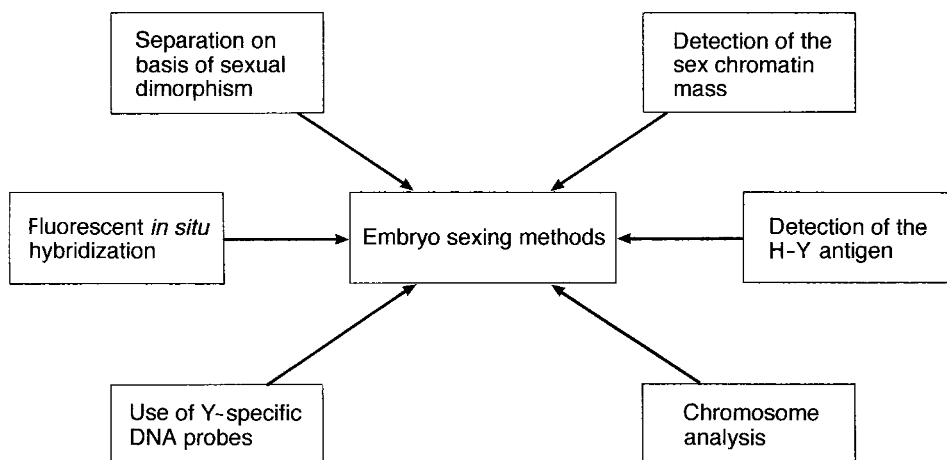

Fig. 10.5. Methods employed in the sexing of cattle embryos.

(1996) concluded that the technical problems had been underestimated, noting that frequency of sexing embryos conducted under the auspices of the Osnabrucker Herdbook Association had fallen from 26% in the early 1990s to zero at the time of writing. The reasons for such a decline included high costs and unreliable results. Reviews by Bredbacka (1998a,b) referred to the disappointing uptake of PCR sexing during the previous decade but concluded that it was difficult to estimate the general success of the technique.

10.5.1. Sexing by polymerase chain reaction technology

Studies in Finland (Bredbacka *et al.*, 1995; Jaakma *et al.*, 1998b) and reports reviewed by Bredbacka (1998a,b) showed PCR to be a highly effective and accurate method of sexing cattle embryos; its success, however, was seen to be heavily dependent on the operator and appropriate training. Bredbacka (1998a) noted that simplifications of the technique were likely to increase interest in PCR sexing and that further progress could be expected.

The stabilization of the bovine embryo by means of a protein-free medium or scratches produced on the bottom of the Petri dish has made it possible to perform a biopsy with a single microinstrument. In taking the biopsy sample from a blastocyst, this should be from the trophectoderm (TE) and not the inner cell mass (ICM). The traditional PCR sexing approach uses electrophoresis, which involves the risk of DNA contamination of subsequent assays. Such contamination, resulting in females misdiagnosed as males, is avoided by using a non-electrophoretic method in which the sex is determined based on fluorescence of unopened tubes. However, female samples cannot be distinguished from blank samples in the non-electrophoretic assay. None the less, an accuracy of about 95% is possible with both approaches. Acceptable pregnancy rates (50–70%) have been obtained with biopsied grade 1 embryos, but there is evidence that pregnancy rates with grade 2 embryos are 15–20% lower. There is evidence that pregnancies can be achieved with frozen–thawed biopsied grade 1 embryos.

Data on 4183 embryos biopsied and diagnosed for sex over a 6-year period were analysed in a report by Shea (1999) in Canada. Sex diagnosis was made using a 165 base pair (bp) Y-chromosome-specific sequence or a 210 bp autosomal sequence. Cattle embryos were biopsied by blade, aspiration or needle and were subsequently transferred non-surgically to the recipient, either fresh or after freeze–thawing. For the three biopsy methods, the percentage of embryos in which the sex could not be determined was 14%, 14% and 8%, respectively. Pregnancy rate was markedly and significantly higher for fresh than for frozen embryos (58% vs.

37%). Sex diagnosis using the PCR showed 93% agreement with results from ultrasound testing after day 60 of pregnancy. An unusual feature was the fact that significantly more embryos biopsied during the period of least daylight (October–March) were diagnosed as female than during April–September (48% vs. 44%). A report by Geldhof *et al.* (2000) noted that sexing of cattle embryos had become a routine part of the ET technology for Flemish cattle breeders. A retrospective analysis was carried out by Darrow (2002) in Canada on the pregnancy data of ETs on fresh and frozen sexed female embryos from 26 farms over a 32-month period; sexing used the protocol of AB Technology. The author concluded that embryo sexing applied routinely in ET programmes can produce acceptable pregnancy rates and grade 1 and grade 2 biopsied embryos can be frozen successfully in a protein-free medium.

Reports on PCR-based methods have come from various countries, including Finland (Bredbacka *et al.*, 1995; Jaakma *et al.*, 1998a,b; Virta *et al.*, 2002), Belgium (Geldhof *et al.*, 1999a,b, 2000), Canada (De La Fuente *et al.*, 1999), France (Thibier and Nibart, 1995; Lacaze *et al.*, 1996a,b), Germany (Neuss, 1995), Japan (Li, X. *et al.*, 1995; Kameyama *et al.*, 1996), India (Rao *et al.*, 1994; Taneja *et al.*, 1998), Ukraine (Limansky and Limanskaya, 1999), Poland (Wayda, 1999), the Netherlands (Otter *et al.*, 2000), Brazil (Hassun *et al.*, 1999; Alves *et al.*, 2000; Lopes *et al.*, 2000, 2001; Forell *et al.*, 2002), the Czech Republic (Jindra *et al.*, 2000) and Korea (Park *et al.*, 2001). The feasibility of using primer extension preamplification PCR (PEP-PCR), which is a method that enables multiple characteristics to be determined from a single biopsy, has been demonstrated by several laboratories (Hassun *et al.*, 1999; Chrenek *et al.*, 2001).

A report by Hasler *et al.* (2002) dealt with a collaborative study between P. Bredbacka and Em Tran Inc. to develop a commercially applicable sexing technique, known as Ampli-Y; the authors were able to demonstrate that their biopsy technique did not seriously compromise the survival of *in vivo*- and *in vitro*-derived cattle embryos after transfer. They were also able to show that their Ampli-Y non-electrophoretic PCR system was highly sensitive and accurate; the system provided significant advantages in speed and simplicity compared with conventional PCR sexing and was virtually free of problems with DNA contamination.

10.5.2. Fluorescence *in situ* hybridization

An accurate, reliable and rapid (< 1 h) method for determining the sex of cattle embryos was developed by Kobayashi *et al.* (1998) in Japan, using FISH with a probe designed from a cattle Y-chromosome-specific DNA (BC1.2). Lymphocyte nuclei prepared from bulls, cows and a freemartin were used in evaluating the accuracy of the procedure. A study reported by Gavio *et al.* (2002) dealt with the sensitivity and specificity of FISH in comparison with PCR in the sexing of cattle embryos; the FISH method, using the BC1.2 probe, resulted in consistently higher sensitivity. The authors concluded that the possibility of incorporating additional probes might allow the study of chromosomal abnormalities in early embryo development.

Reports with human embryos had shown FISH to be the preferred method of sexing. The use of conventional PCR methods did not enable chromosomal abnormalities to be detected and contamination by sperm or foreign DNA could pose problems. In London, Harper *et al.* (1994), using a modified spreading method and directly labelled fluorescent DNA probes, reduced the time taken in using their FISH procedure from 7 to 2 h; the reduced time ensured that embryo biopsy and transfer could be performed within the 7 h working day. Clinical experience of dual FISH for sex diagnosis in humans led Griffin *et al.* (1994) to conclude that the technique had certain advantages over the PCR method.

Sexing by male-specific antigen

In the immunological detection of a male-specific antigen on the bovine embryo, it is believed that the histocompatibility antigen (H-Y antigen) is expressed in some species as early as the eight-cell stage; in cattle, the antigen is apparently expressed at the late morula stage. There have been various attempts at sexing by identifying this male-specific antigen. In more recent times, a study reported by Gardon *et al.* (2000) used H-Y antiserum from rats to determine the sex of IVP cattle embryos; they suggested that such

embryos could be sexed by the H-Y method at the 32-cell stage. In Brazil, Moreira-Filho *et al.* (2000) reported a significant sex preselection accuracy in work with zebu embryos by indirect immunofluorescence using high-titre rat H-Y antisera; in the same laboratory, Ramalho *et al.* (2000) reported successful use of this technique in murine and bovine embryos.

10.5.3. Sexual dimorphism

In cattle, as in other farm animals, the choice between male and female development is controlled by the sex chromosomes; the presence of a Y-chromosome results in male development and the presence of testes; the testes are necessary for the development of male characteristics. The Y-chromosome encodes a dominant inducer of testis formation and the Y-linked gene(s) (known as the testis-determining factor; TDF) controls this process; TDF is activated during embryonic development and this commits the undifferentiated genital ridge to the testicular pathway; the usual consequence of testis formation is that subsequent hormone production induces male sexual differentiation. However, a number of researchers have shown that male and female bovine embryos show a dimorphic pattern of development even before the gonads develop; the possible cause of this dimorphism in the early bovine embryo has been controversial. This dimorphism is evident before the inactivation of one of the X-chromosomes of the female embryo and for that reason may be related to the expression of sex-chromosome-linked genes. On the farm, there have been a number of reports showing evidence of a disproportionate number of bull versus heifer calves born from IVP embryos (Hasler *et al.*, 1995a,b).

The results reported by various investigators have shown that certain IVC conditions support preferential development of male embryos; this difference became evident as early as the four- to eight-cell stage (Dufour *et al.*, 1994). In Italy, Lazzari *et al.* (1995) presented evidence suggesting that the culture of IVP cattle embryos in the sheep oviduct allowed more synchronous development of male and female embryos than embryos produced in culture media. In Finland, Bredbacka, K. and Bredbacka (1996) and Bredbacka, P. and Bredbacka (1996) reported that glucose controls sex-related growth differences in IVP cattle embryos. In Spain, Gutierrez-Adan *et al.* (1998) evaluated the effect of glucose on the sex ratio of IVP bovine blastocysts; the sex ratio was significantly different from the expected 1 : 1 ratio only when glucose was present in the medium. The authors suggest that a preferential loss of female embryos only takes place during IVC in the presence of glucose.

In a further report, Gutierrez-Adan *et al.* (1999c, 2000a,b) examined the possibility that developmental differences may be due to gene expression; they found that mRNA expression of glucose-6-phosphate dehydrogenase (G6PD) and HPRT (genes located on the X-chromosome) in female embryos was double that in male embryos. These are genes involved in controlling the amount of oxygen radicals and for that reason may influence embryonic development; the authors suggested that such differences are responsible for the faster development in culture of IVP male embryos. The same laboratory subsequently reported the presence of specific mRNA in female IVP cattle embryos (Gutierrez-Adan *et al.* (2000a,b); of six differential genetic markers, four were expressed in female embryos and two only in male embryos. In a study reported by Peippo *et al.* (2000a,b), the expression levels of G6PD and HPRT genes in IVP cattle blastocysts were found to be 22% and 10% higher, respectively, in females than in males. In Spain, Jimenez *et al.* (2002) evaluated the effect of glucose in their culture medium on the sex ratio of IVP embryos; they suggest that low levels of glucose (~5 mM) produce a preferential loss of female embryos whereas high levels (20 mM) produce a preferential loss of male embryos.

Further studies in Finland reported by Peippo *et al.* (2001) used time-lapse video-recording to compare the developmental kinetics of male and female cattle embryos cultured *in vitro*. In the presence of glucose in the IVC medium, males cleaved earlier than females; in the absence of glucose, females cleaved earlier than males. They found no difference in blastocyst yield in the absence of glucose, but in the presence of glucose more male than female embryos reached the blastocyst stage. The authors suggested that the difference in the number of functional X- chromosomes between the sexes in early embryonic development could

explain their findings. In female embryos, an increased capacity for oxygen-radical detoxification through the pentose phosphate pathway could result in a reduced cleavage rate; glucose might also influence the expression of enzymes located on the X-chromosome. A review of sex-influenced developmental processes in the early bovine embryo is to be found in an article by Kochhar *et al.* (2001).

In Madison, Beyhan *et al.* (1998) investigated the early development of cattle embryos produced by sexed sperm; they found evidence of a dimorphic pattern of development in male and female embryos during fertilization and first cleavage. The authors also reported a tendency for an interaction between oocyte maturational status and sex-chromosomal content of the spermatozoon. In Missouri, a study by Kimura *et al.* (2001) examined the possibility that there may be a developmental block under certain culture conditions that affects females more than males; they found that an approximate 1 : 1 sex ratio was maintained until the embryos reached the blastocyst stage but that females had difficulty negotiating the morula-to-blastocyst transition, presumably due to less than optimal culture conditions.

Fig. 10.6. Identical twins in cattle produced by embryo splitting.

10.6. Cloning in Cattle: Progress and Problems

For farmers and animal scientists alike, there has always been a certain fascination in the possibility of producing copies, or 'clones', of outstanding beef or dairy animals in some number (see Fig. 10.6). Although at one point in the early 1980s there was a view that cloning by NT was unlikely to be feasible in mammals, this proved ill-founded; numerous reports and reviews in the recent decade have shown that NT can be achieved in ruminants, using nuclei from embryos or from adult animals (Ashworth *et al.*, 1998; Campbell and Wilmut, 1998; Kato *et al.*, 1998a, 2000; Lewis *et al.*, 1998a,b; Wilmut *et al.*, 1998; Campbell, 1999; LeBourhis *et al.*, 2000), albeit at a low level of efficiency. A review by Moore (2002) has summarized some of the results achieved by various research groups performing NT in cattle and lists some of the commercial concerns carrying out work in this field. On a wider front, a book by Wolf and Zelinski-Wooten (2001) has provided a historical perspective on the development and application of assisted fertilization and NT in mammals.

It is clear from the most recent reports and reviews in the literature that NT technologies are still experimental and a considerable number of variables remain to be examined (Renard *et al.*, 1999; Moore, 2002; Spell and Robl, 2002; Tsunoda and Kato, 2002); a review by Wilmut *et al.* (2002) observes that somatic cell NT asks a great deal of molecular mechanisms designed to regulate fertilization and that it is surprising that somatic cell cloning ever produces viable young. Although evidence in a report on the production of a cloned human embryo was less than convincing (Cibelli *et al.*, 2002) and the question of human cloning is likely to be the subject of much debate and controversy (see Griffin and Wilmut, 2002), there are certainly arguments to be made for pursuing cloning in various of the non-human primates. As noted by Mitalipov *et al.* (2002) in the USA, the production of genetically identical non-human primates would reduce the number of animals required for biomedical

research and markedly influence vital studies related to human welfare, such as development of the human-immunodeficiency-virus vaccine; the Oregon workers were able to demonstrate that transfer of embryonic, although not somatic cell, NT embryos into recipients could result in a term pregnancy.

10.6.1. Introduction

Gardeners have been cloning plants for centuries. They may not always be aware of the biological implications of their activities but the fact remains that desirable plant strains are propagated by cloning technology by the millions every day. The same gardeners are likely to have carried out cloning in the animal world as well. Every time the gardener accidentally chops through his friend, the earthworm, he is likely to create two genetically identical individuals, cloning in an uncomplicated guise. Although a similar form of cloning has been carried out in cattle by deliberately splitting the early embryo, it can occur spontaneously, both in cattle and in humans. The mother looking after identical twin infants is dealing with genetic clones, not unduly uncommon in humans and cattle alike. It is, however, the cloning technology based on NT that currently occupies the attention of researchers and laymen alike. This form of cloning involves the fusion of a cell taken from an embryo or an adult to a previously enucleated oocyte; this form of NT and reprogramming dates back, in amphibians, to the work of Briggs and King with frogs in the early 1950s (see Gurdon, 1999).

The implications and potential benefits of cloning farm animals have been reviewed by many authors (e.g. Ruane *et al.*, 1997; Van Vleck, 1999; Woolliams and Wilmut, 1999; Wilmut and Paterson, 2001); they concluded that cloning was unlikely to make a significant contribution to increasing the rate of genetic gain in selection programmes and that only marginal advantages would come from the use of cloning in other areas of livestock production. In the USA, Dematawewa and Berger (1998) compared 12 models for alternative progeny-testing schemes based on genetic and economic gains that might be achieved by cattle cloning; they concluded that the total cost associated with cloning had to be < $84 for the technique to be economically viable. Other reviews suggest that there are two main ways in which NT is likely to be useful in cattle breeding (Wilmut *et al.*, 1998, 2002; Stice *et al.*, 2000): first, the possibility of producing multiple copies of élite cattle; and, secondly, NT from cultured bovine cell populations can be used to produce genetically modified progenitor cattle, to introduce genetic change into nucleus herds or for increasing the rate of genetic progress in the general cattle population. In isolated instances, cloning has already been used to preserve unique genetic material. In New Zealand, for example, Wells, D.N. *et al.* (1998c) used somatic-cell NT to clone the last surviving cow of the Enderby Island cattle breed; in the USA, Westhusin *et al.* (2001) cloned a 21-year-old Brahman steer named Chance from skin cells to produce a bull calf, which was promptly named Second Chance.

There are those who believe that one of the greatest potential benefits of the new technology may lie in therapeutic cloning – the use of somatic-cell NT to generate replacement tissues or organs (Gurdon and Colman, 1999; Wilmut, 2000). This form of cloning would avoid the risks of tissue rejection by providing a patient with new tissue of their own genetic type. The authors note that all the main steps in the therapeutic cloning procedure have already been achieved, albeit at a low level of efficiency. There is clearly a need to improve the success rate in producing NT embryos from adult somatic cells, to develop methods of generating embryonic stem-cell (ES) cultures from NT embryos and to control with accuracy the differentiation of these stem cells into the tissue or organ required. As noted by Gurdon and Colman (1999), if any new scientific technique works at all, it is probably capable of improvement in efficiency and ease of operation; this reasoning seems likely to apply to cloning technology.

Story to date

The first success in the cloning of farm livestock was that recorded by Steen Willadsen in the mid-1980s in Cambridge; a cloned sheep was produced by fusing a cell from a 16-cell embryo to an enucleated oocyte. In the studies that followed over the next several years, embryonic

cells of different stages were used as donor cells because they were young and undifferentiated (see Fig. 10.7). Clearly, the supply of donor cells for cloning was limited to those that could be obtained from the early embryo; some tried to overcome this limitation by attempting multigenerational cloning by taking cells from a previously cloned embryo on several occasions. Others looked to the possibility of using bovine ES cells, which they hoped could be established from the ICM of an early embryo, as reported by Evans and Kaufman in the early 1980s in mice.

ES cells are now widely used to genetically modify mice; ES cells can be genetically modified *in vitro* and used to target specific changes, such as gene deletions or replacements. Such ES cells would appear to be ideal for transgenesis in cattle. Unfortunately, although numerous attempts have been made to isolate ES cells from cattle, none have been successful; indeed, no ES cells have been described for any of the farm mammals. Although apparent bovine stem-cell lines have been reported, none has been able to pass the crucial test – the ability to contribute to germ cells when inserted into a developing embryo. Even in mice, genuine ES cells are only to be obtained from a limited number of inbred lines. In the mid-1990s, fortunately, came the exciting work at Roslin, in which it was shown that viable offspring could be produced, first with fetal and subsequently with adult somatic cells.

Cloned cattle, sheep, goats, pigs, cats and a variety of rodents have now been produced using adult somatic cells in NTs. Although current cloning technology is inefficient, with less than 10% of cloned embryos resulting in a live offspring, their birth has stimulated great interest and brought an increasing number of investigators into this particular research field (see Heyman *et al.*, 1998a,b; Baguisi *et al.*, 1999a; Pennisi and Vogel, 2000; Westhusin *et al.*, 2001; Behboodi *et al.*, 2002; Bondioli *et al.*, 2002; Dinnyes *et al.*, 2002; Forsberg *et al.*, 2002). The fact that many research groups are currently working on cattle cloning is due, in no small part, to the considerable amount of information that is available on IVM/IVF and embryo culture in this species. There is also the fact that the raw materials for cloning can usually be obtained at low cost from the nearby abattoir and cattle AI centre; this

Fig. 10.7. Nuclear-transfer calf derived from an embryonic cell. Several pregnancies were established using blastomeres from a 32-cell *in vitro*-produced embryo and fusing them with oocytes matured *in vitro;* this calf was allowed to go to full term and was born in 1990 at the University College Dublin farm.

means that numerous attempts at cloning can be made, even though few calves may be born. Factors influencing the quality of the IVM bovine oocyte have been studied intensively in the recent decade, mainly as a means of improving the efficiency of *in vitro* embryo production; the oocyte also plays a key role in cloning, being the only cell capable of entirely reprogramming a foreign nucleus.

Normality of clones

There are those who query the normality of any of the cloned animals that have been born thus far; it is certainly true that information is not currently available as to which genes are critical for normal embryo development and that no technology is currently available or is likely to be available in the foreseeable future to assess the ways in which such genes will be expressed (Griffin and Wilmut, 2002). It is not necessarily a question of the apparent normality of calves at birth; problems may not appear for some time after calving.

On the other hand, there have been those who regard some of the calves born as a result of their cloning technology to be as normal as any spontaneously occurring birth, even though the cloned animals represented a relatively small proportion of the NT embryos transferred to recipients (Lanza *et al.*, 2001). Attention is drawn to the growing number of physiologically normal ruminants obtained from somatic cell NT reported from different laboratories around the world (Renard *et al.*, 2002); they hold to the view that cloning can result in the birth of healthy animals but that successful application of NT to commercial practice requires greater knowledge and understanding of the basic biological processes underlying epigenetic controls and nuclear activities. Certainly, the fact that NT has been successful in producing physiologically normal calves is encouraging for those working in this field. Where such animals are bulls and are proved to be capable of normal reproduction and producing normal offspring, AI would be the obvious means of disseminating their genes widely. In the meantime, in the search for further knowledge, all would agree with the need to avoid the production of aberrant phenotypes that raise welfare problems (see Thompson, 1998).

Safety of food products

Various commercial concerns are currently developing strains of cattle produced as a result of somatic-cell NT, mainly in the USA; there is an obvious need to ensure that any food derived from such cattle (e.g. meat, milk) does not raise questions of public concern when it is marketed. The American Food and Drug Administration (FDA) is considering the various steps necessary in ensuring that the production of cloned animals is monitored and appropriately regulated. Cloning is likely to be a crucial step in the production of genetically modified animal products; any mention of genetic modification in animals is almost guaranteed to raise public concerns about food safety. The production of genetically modified organisms (GMOs) for food consumption led to a storm of protest in the media and in the field; the same reaction is to be expected at the thought of modified animal products. It is essential to have in place appropriate regulations that meet the approval of consumer groups long before any attempt is made to market products from cloned animals.

10.6.2. Embryo splitting

Work at Cambridge by Steen Willadsen in the late 1970s was the first to show that each blastomere of a two-cell sheep embryo possessed the potential to develop into a normally organized blastocyst; a technique was developed which resulted in the production of identical twins in cattle and other farm mammals. By the early 1980s, the technology had moved to splitting ruminant embryos at a later stage of development, which attracted commercial interest. For cattle, it was now possible to flush embryos from the superovulated donor animal and split them at the late morula/early blastocyst stage; this permitted the number of pregnancies obtained from a given collection of embryos to be markedly increased. A great many reports appeared on cattle embryo splitting in the late 1980s and early 1990s; occasionally some have appeared in more recent times, usually with reference to embryo sexing (Bredbacka *et al.*, 1994; Bredbacka, 1995; Wang *et al.*, 1995).

In Poland, Skrzyszowska *et al.* (1997) developed a procedure which they suggested could be

a non-invasive alternative to conventional embryo splitting in cattle. They induced a specific hatching sequence by zona drilling that led to the formation of two incomplete demi-embryos; these were finally separated with a glass needle. In Uruguay, Crispo *et al.* (2000) studied the effect of culture medium on the development of day-7 fresh and frozen–thawed cattle IVP embryos cultured either intact or after splitting; although development of whole embryos tended to be higher in TCM-199, development of fresh demi-embryos did not differ in tissue-culture medium 199 (TCM-199) or Charles Rosenkrans 1 with amino acids (CR1aa) culture media. Frozen–thawed demi-embryos, on the other hand, had a higher rate of development in CR1aa medium.

Attempts have been made to produce calves from quarter-embryos, but the general experience has been that the survival rate of such embryos is only half that found with demi-embryos, too low to be of practical interest. The advent of IVP technology, however, may revive some interest in working with early- rather than late-stage cattle embryos. In Canada, Johnson *et al.* (1995) reported the birth of four identical calves produced by separation of blastomeres from an *in vitro*-derived four-cell embryo; the quarter-embryos were co-transferred with trophoblastic vesicles and resulted in three pregnancies. Later work in the same laboratory suggested that bisection of intact IVP embryos into demi-embryos and bisection of those into quarter-embryos increased the number of transferable embryos by as much as about 180% and 280%, respectively (Rho *et al.*, 1998c,d,e).

10.6.3. Essential steps in nuclear transfer

The three main steps towards the creation of NT embryos are: (i) removal of maternal chromatin from a recipient oocyte; (ii) introduction of a donor nucleus into the recipient oocyte; and (iii) activation of the reconstructed oocyte. As observed by Renard *et al.* (2002), a growing number of physiologically normal ruminants and rodents obtained from somatic cell NT testify to the fact that cloning can be carried out without problems to the animals that are born; the same authors stress the need to limit the production of aberrant phenotypes and to work towards overcoming the reluctance of the general public to accept the potential for dramatically improving human welfare that cloning research could bring.

Quality of recipient oocytes

A study by Peura *et al.* (2002) in Australia with sheep produced evidence that the nutritional level of the ewe providing recipient oocytes had an effect on pregnancies established with somatic cell clones derived from these oocytes; they showed that a higher percentage of host sheep became pregnant and maintained pregnancies to term after transfer of clones reconstructed from oocytes recovered from ewes on a higher nutritional level. In studies reported by Bruggerhoff *et al.* (2002a,b), recipient oocytes were recovered from Simmental Fleckvieh cattle belonging to two maternal lineages; using cumulus cells from a Brown Swiss cow in somatic cell cloning, they found a significant difference in embryo yield at 7 days according to recipient cell lineage and the frequency of follicle aspiration in their OPU programme.

Enucleation

In NT studies, the bovine oocyte has to be enucleated in order to be used as a recipient cell. Oocyte chromatin is usually labelled with the UV-excitable fluorochrome Hoechst 33342 and removed by micromanipulation; the potential damaging effects of UV illumination on the ooplasm have always been a matter of some concern. Enucleation has generally involved aspirating about one-third of the ooplasm just beneath the polar body; clearly, such manipulation is accompanied by several morphological changes in the ooplasm, possibly leading to altered metabolic activity in the reconstructed embryo after NT. In work reported by Greising *et al.* (1994), it was demonstrated that recipient bovine oocytes produced by the fusion of two cytoplasts of 24 h IVM oocytes had a higher developmental capacity to the morula/blastocyst stage after integration of the donor nucleus than those used as recipient cells immediately after enucleation or after fusion of 24 h with 48 h matured cytoplasts. When the amount of ooplasm of 24 h matured and enucleated oocytes was raised to the volume of

non-manipulated oocytes by returning the amount removed during enucleation, the percentage of morulae/blastocysts increased to 38%, compared with 14% for recipient cells with reduced cytoplasm. In a subsequent ultrastructural study, Greising and Jonas (1999) reported that only cytoplasm from 24 h matured oocytes showed a development-supporting effect when fused to enucleated recipient cells before NT. It seems likely that the fusion of the two cytoplasts provided an additional source of energy for the reconstructed recipient oocyte.

A paper by Schmoll *et al.* (1997) described enucleation of bovine oocytes by laser-assisted microdissection of the zona pellucida. A study reported by Dominko *et al.* (1999b) used various fluorochromes to localize different components of the oocyte metaphase chromatin/spindle structure; they frequently observed displacement of the second metaphase plate from its expected position close to the first polar body. They suggest that the method of cumulus cell removal by manual stripping may affect the positioning of the first polar body and the metaphase II spindle; this could influence the efficiency of the enucleation process. Among other reports dealing with enucleation are those describing the centrifugation of zona-free bovine oocytes in a density gradient (Tatham *et al.*, 1995a,b,c) and in mice and goats cytoskeleton-modifying agents (e.g. demecolcine) have been used to induce enucleation of nuclear chromatin at acceptable rates without some of the adverse effects associated with mechanical enucleation (Karnikova *et al.*, 1998; Baguisi and Overstrom, 2000; Fischer *et al.*, 2002; Ibanez *et al.*, 2002). Treatment of the IVM bovine oocyte with demecoline results in a membrane protrusion that contains a condensed chromosome mass, which can be readily removed by aspiration (see Fig. 10.8).

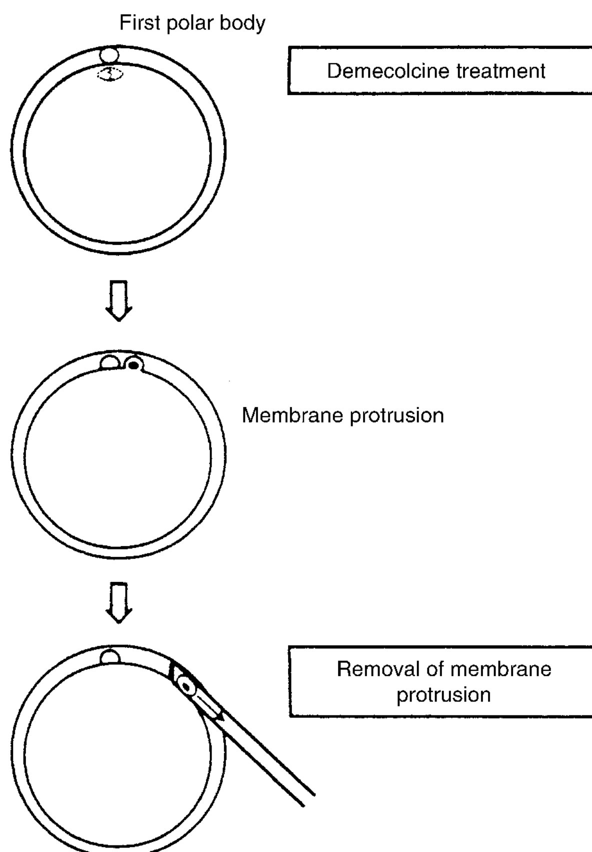

Fig. 10.8. Chemically assisted removal of maternal chromosomes (from Yin *et al.*, 2002).

This simple, chemically assisted method for removing maternal chromosomes has been successfully used with pig oocytes as well as with cattle and rabbit oocytes (Yin *et al.*, 2002).

In Connecticut, a study of interest was reported by Liu *et al.* (2002) who demonstrated that a 0.3 M sucrose treatment of cattle oocytes facilitated the location of the metaphase chromosomes under normal light microscopy and enabled enucleation to be accomplished with great efficiency without compromising the *in vitro* development of the reconstructed embryos. It appears that the sucrose treatment induces the formation of a projection around the chromosome and spindle area in most metaphase II bovine oocytes; the authors at Storrs suggest that this enucleation procedure has the potential to significantly improve overall NT efficiency in cattle.

Telophase enucleation

Telophase enucleation has proved to be an efficient method for removing chromatin with minimal loss of cytoplasm and without exposure to UV illumination or DNA stain (Bordignon and Smith, 1998). A study by Liu *et al.* (2000) demonstrated that cattle oocytes enucleated at telophase II and subzonally injected with donor somatic cells showed a higher rate of embryonic development *in vitro* than did those enucleated at metaphase II.

Introduction of donor nucleus

The introduction of the diploid nucleus into the recipient cytoplast usually involves fusion between the plasma membranes of the bovine cytoplast and the donor cell. Membrane fusion has been achieved by a variety of means, including the use of Sendai virus or polyethylene glycol or by means of direct current (DC) electrical fusion. The latter method has been almost universally adopted in view of the consistency of the response achieved. Electrofusion is a powerful method not only for inducing fusion between nucleus and enucleated oocyte but for triggering activation of the oocyte; fusion is usually carried out in a chamber attached to the electrofusion apparatus and in a non-electrolyte fusion solution (e.g. Zimmerman cell-fusion medium; see Table 10.12).

Table 10.12. Zimmerman mammalian cell-fusion medium formulation.

Compound	Mol. wt	g/l	Molarity
Sucrose	342.3	95.84	0.28 M
$Mg(C_2H_3O_2)_2 \cdot 4H_2O$	214.5	0.107	0.5 mM
$Ca(C_2H_3O_2)_2$	158.2	0.016	0.1 mM
K_2HPO_4 (anh)	174.2	0.174	1.0 mM
Glutathione	307.3	0.031	0.1 mM
Bovine serum albumin pH 7.0	25,000	0.01	0.01 mg/ml

anh, anhydrous.

Some bovine cloning protocols involve simultaneous fusion of the donor nucleus and activation of the recipient cytoplast; others involve separate fusion and activation steps. In France, LeBourhis *et al.* (2001), using simultaneous fusion and activation, examined the kinetics of cytoplasmic activation by analysing nuclear modifications in recipient oocytes; they found evidence that premature chromatin condensation (PCC) may not be essential in achieving a high rate of development of NT embryos to the blastocyst stage. It has been generally assumed that the competence of artificially matured metaphase II oocytes in reprogramming somatic cell nuclei is related to a high maturation-promoting factor (MPF) activity, which leads to PCC, but the French study did not support that view.

Although the majority of NT reports show that incorporation of nuclei into the enucleated bovine oocyte has been by electrofusion, some have taken the route commonly used in mice. A technical feature of mouse NT that distinguishes it from that used in farm animals is the fact that cloning is usually by direct injection of the donor cell into the oocyte (Wakayama *et al.*, 1998); there are, however, reports in which NT by electrofusion has been shown to be effective in this species (Ogura *et al.*, 2000). In cattle, the direct intracytoplasmic injection of an isolated nucleus has been used to a limited extent to produce embryos and live calves (Goto *et al.*, 1997; Lacham-Kaplan *et al.*, 1999; Rho *et al.*, 1999). In horses, results presented by Choi *et al.* (2002b) demonstrated that direct injection of somatic cells using the Piezo drill and activation by injection of stallion sperm cytosolic extract was effective as a means of NT.

Choice of donor cell and cell-cycle stage

Early cloning studies in farm animals used cells (blastomeres) taken from cleavage-stage embryos; further work showed that donor nuclei could be taken from progressively later stages of embryonic development. In Wisconsin, Keefer *et al.* (1994b) demonstrated that the nuclei of ICM cells from expanded bovine blastocysts were pluripotent and that such nuclei, after NT to enucleated oocytes, could direct embryo and fetal development and result in live births. In New Zealand, Vivanco *et al.* (2000) reported the production of live normal calves from embryos reconstructed by the transfer of blastomeres from IVP embryos. The choice of donor cell used in cattle NT studies changed from embryonic to somatic cells after the birth of Dolly; among donor cells currently used are granulosa and cumulus cells, mammary epithelial cells, blood cells, fetal and adult fibroblasts, leucocytes, chondrocytes and fetal genital-ridge cells (Chesne *et al.*, 1997; Vignon *et al.*, 1998a,b, 2000; Zakhartchenko *et al.*, 1998, 1999a,b,c,d; Galli *et al.*, 1999b; Goto *et al.*, 1999; Shin *et al.*, 1999; Wells *et al.*, 1999b; Akagi *et al.*, 2000; Beyhan *et al.*, 2000; Bordignon *et al.*, 2000; Katska *et al.*, 2000a,b; Kishi *et al.*, 2000; Knott *et al.*, 2000; Kubota *et al.*, 2000; Lee, C.K. *et al.*, 2000; Mohamed Nour and Takahashi, 2000; Mohamed Nour *et al.*, 2000; Oikawa *et al.*, 2000; Saikhun *et al.*, 2000; Arat *et al.*, 2001; Im, G.S. *et al.*, 2001; Wilmut, 2001; Forsberg *et al.*, 2002; Lucas-Hahn *et al.*, 2002; Palma *et al.*, 2002; Taverne *et al.*, 2002).

They have included cultured and non-cultured cumulus cells (Akagi *et al.*, 2002), cells from sexed parent embryos (LeBourhis *et al.* (1998) and cells from many individual cell lines (Mitalipova *et al.*, 2002). In Australia, several papers have dealt with recycling bovine embryos for NT (Peura and Trounson, 1998; Peura *et al.*, 1998); even the particular genotype within the one breed of cattle may be a factor influencing development of NT embryos (Kasinathan *et al.*, 2002). In Korea, Hwang, W.S. *et al.* (2000) and Cho *et al.* (2002a,b) compared the efficacy of different cattle somatic-cell types for oocyte reconstruction; cumulus and ear fibroblast cells were more efficient than uterine or oviductal cells. In Wisconsin, Forsberg *et al.* (2002) sought to determine which cell types produced healthy cloned calves that would allow for transgenic manipulation. In general, cultured fetal cells performed better than adult cells, with the exception of cumulus cells, which produced the highest overall pregnancy and calving rates. The cell type that combined relatively high pregnancy and calving rates with growth characteristics that allowed for extended proliferation in culture were fetal genital ridge cells; such cells initiated pregnancies in 40% of recipient heifers and resulted in a 9% calving rate.

In view of evidence suggesting that the cell-cycle stage of donor cells is important in determining the developmental ability of NT embryos (Samake and Smith, 1995, 1996, 1997; Fulka *et al.*, 1996b), attempts are being made to ensure that cells are at the appropriate stage (Kasinathan *et al.*, 2000; Yang *et al.*, 2000; Beyhan *et al.*, 2002; Hayes *et al.*, 2002; Wells *et al.*, 2002). Cell-cycle checkpoint controls (see Fig. 10.9) ensure the fidelity of cell division and are divided into two groups: (i) there are the metaphase checkpoints, which monitor the completion of the preceding phase before the entry into the next phase; and (ii) there are M-phase checkpoints controlling the precise separation of chromosomes during meiosis and mitosis.

The fact that there is considerable variation in embryo development between individual donor cell populations used for constructing NT cattle embryos has led some to check the stability of their chromosomal complement (Slimane *et al.*, 2002); this revealed an incidence of X-chromosomal aneusomy in donor cells of 23%, a figure reflected in the reconstructed NT embryos. On the question of storing donor cells, a study by Li, G.P. *et al.* (2002) in Colorado showed that somatic cells cold-stored (4°C) for 5–7 days supported the development of NT cattle embryos to the blastocyst stage, whereas dead cells did not. On the other hand, a study in sheep reported by Loi *et al.* (2002) showed evidence of successful reprogramming of nuclei from somatic cells rendered non-viable by heat treatment (55°C or 75°C); success took the form of reconstructed embryos developing to the blastocyst stage *in vitro* and into fetuses and viable offspring after transfer to foster-mothers.

Although there are those who believe that the combination of G0/G1-arrested donor nuclei and metaphase-arrested cytoplast is likely to be the most appropriate, alternative approaches

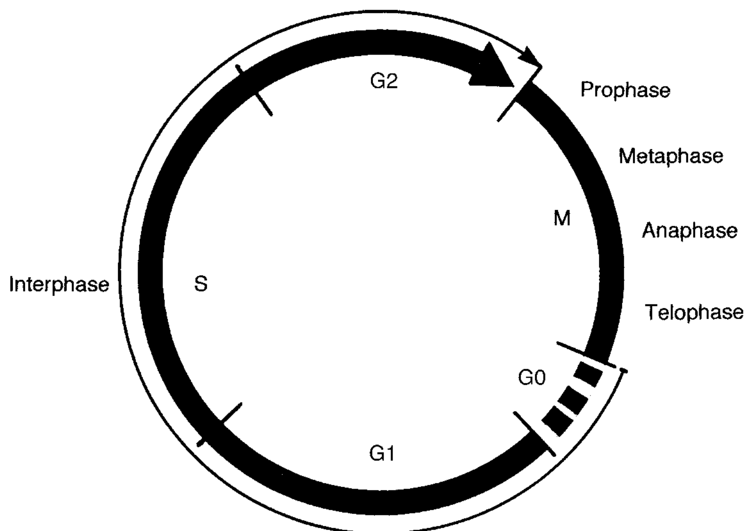

Fig. 10.9. Phases of the mammalian cell cycle. The mammalian cell cycle, made up of phases of mitotic division (M) separated by interphase. During interphase, synthesis of DNA during the S phase is preceded and followed by gaps (G0, G1 and G2) state of non-division (G0) and the duration of the other phases differs as development progresses.

have been followed in some laboratories. In Canada, Bordignon and Smith (2002) found that cattle NT embryos reconstructed with cyclic somatic cells and telophase-enucleated oocytes showed similar developmental rates to those reconstructed using G0/G1 stage nuclei and metaphase II oocytes; it appeared that complete chromatin remodelling of somatic cells can be achieved in different cell cycle environments. In Japan, Kurosaka *et al.* (2002) sought further information on the effect of donor and recipient cell cycle on DNA synthesis and the development of the embryo; they found that embryos reconstructed from G0 cells started DNA synthesis according to the time course of the recipient cell cycle and those reconstructed from G1/S cells started according to the time course of the donor cell cycle. In Georgia, Gibbons *et al.* (2002b) used the cyclin-dependent kinase 2 inhibitor roscovitine to maximize cell cycle synchrony and to produce donor cells (granulosa) that responded more reliably to nuclear reprogramming; they found that roscovitine-treated cells were more efficiently synchronized in the quiescent G0/G1 phase of the cell cycle than were serum-starved cells. The authors recorded enhanced fetal and calf survival in the roscovitine treated group (seven surviving calves from six pregnancies) compared with serum-starved controls.

Fetal or adult somatic cells

A study reported by Heyman *et al.* (1999) dealt with the transfer of NT embryos derived from fetal or adult bovine fibroblasts; it was found that fetal losses were much higher after cloning of adult cells as compared with fetal cells (78.5 vs. 43%), with most losses occurring in the second month of pregnancy. In the same laboratory, a report by Vignon *et al.* (1999) showed evidence of a significant difference in blastocyst yield in favour of fetal cells. In Cornell, on the other hand, Hill *et al.* (2000a,b,c) concluded that somatic NT embryo development rates were similar whether adult or fetal cells, from the same genotype, were used as donor cells; they also found that serum starvation of adult donor cells did not improve development rates of NT embryos but similar treatment of fetal cells led to a significant increase in blastocyst yield. In France, Heyman *et al.* (2002) used intensive ultrasonographic and biochemical monitoring in recipient cattle during pregnancies established by transfer of cloned (somatic adult, somatic fetal or embryonic) and IVF co-cultured embryos. They recorded a dramatic decrease in fetal survival, especially in recipients that received an embryo from adult somatic cells (see Table 10.13).

Table 10.13. Pregnancies in recipients that received three different clone types and IVP embryos (from Heyman *et al.*, 2002b).

	Embryos cloned from somatic adult cells (n = 133)	Embryos cloned from somatic fetal cells (n = 40)	Embryos cloned from embryonic cells (n = 67)	Control IVF co-cultured embryos (n = 51)
% presumed pregnant day 21	55.6	57.5	62.6	62.7
% found pregnant day 35	33.8	27.5	49.2	52.9
% found pregnant day 50	27.1	22.5	41.8	50.9
% found pregnant day 70	14.3	22.5	37.3	49.0
% found pregnant day 90	12.0	22.5	34.3	47.0
% calves at term	6.8	15.0	34.3	49.0

Quiescent or proliferating cells

Several studies have shown that viable somatic-cell cloned offspring can be produced by NT using either quiescent (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Lacham-Kaplan *et al.*, 1999) or actively proliferating donor cells (Cibelli *et al.*, 1998a,b,c). In the first instance, donor cells are cultured in medium containing a low concentration of serum for several days to induce a quiescent state and then they are detached for NT. The second method involves culturing cells in medium with a normal concentration of serum and detaching cells for NT when they reach 70–80% confluency. In Texas, Hill *et al.* (1999) compared the two cell categories, which they produced using either 10% or 0.05% fetal calf serum in their culture medium; they found that serum-starved fetal fibroblasts gave improved morula/blastocyst yields but were uncertain whether this was due to improved synchronization of cells in G0/G1 or to an alteration in unknown nuclear and cytoplasmic factors. In Japan, Ueda *et al.* (2000) recorded no significant difference in their NT results between treatments with or without serum deprivation when culturing bovine cumulus donor cells for more than 10 days. In Korea, Cho *et al.* (2002a,b) concluded from their studies that serum starvation was effective in improving the development of NT embryos constructed with cumulus or ear fibroblast cells.

Non-viable cells as donors

In studies reported by Loi *et al.* (2002) in sheep, it was shown that physically denatured nuclei taken from adult sheep donors could be reactivated after NT and develop into blastocysts *in vitro* and to viable offspring *in vivo*. There are those who suggest that for somatic cell cloning all that may be necessary is nuclear DNA without its chromosomal proteins and a functional centriole. It is known that freeze-dried sperm retain their genetic integrity and are able to give rise to normal healthy young (Wakayama and Yanagimachi, 1998); it may well prove possible to store somatic cells in the freeze-dried state at refrigerator or even ambient temperature.

Activation

Artificial activation of NT cattle oocytes is designed to imitate sperm fusion at fertilization and so induce the series of events that include the initiation of cleavage. Activation treatments have been the subject of many reports (Ushijima and Eto, 1994; Du *et al.*, 1995; Jung *et al.*, 2000; Kubelka *et al.*, 2000a,b, 2002). The two commonly used methods to artificially activate oocytes after NT involve the use of either cycloheximide or 6-dimethylaminopurine (DMAP), both treatments being preceded by brief exposure of the oocyte to factors that induce calcium oscillations (e.g. calcium ionophore, ionomycin, ethanol or electrical pulse). The first treatment is designed to elevate intracellular calcium and the second to inhibit protein synthesis. In Georgia, Respess *et al.* (2002) showed that exposure to calcium ionophore following a single direct electrical pulse (40 V) was not necessary to produce NT embryos; cleavage and blastocyst development was similar for NT embryos treated with or without calcium ionophore. In Brazil, Yamazaki *et al.* (2002) evaluated the effect of strontium in activating reconstructed bovine oocytes in comparison with the standard protocol of activation

using ionomycin and DMAP; strontium was found to activate NT embryos with the same efficiency as the standard method. Other work with cattle oocytes in the same laboratory led to the conclusion that strontium ($SrCl_2$, 20 mM) could be efficiently combined with the standard activation treatments (ionomycin + DMAP) for activating young (matured for 22 h) oocytes (Meo *et al.*, 2002).

Activation may be timed to occur simultaneously with fusion or several hours later. In the USA, Edwards, J.L. *et al.* (1999) concluded that embryonic development to the blastocyst stage was similar for reconstructed embryos activated immediately or 4 h after fusion using DMAP. In France, LeBourhis *et al.* (2002) investigated the kinetics of cytoplasmic activation and its consequence for the chromatin and the nuclear envelope of the donor nucleus; results showed that NT into a metaphase II enucleated oocyte accompanied by an immediate activation procedure allowed a remodelling of the somatic nucleus without inducing PCC. Although it is generally assumed that the competence of the bovine oocyte in reprogramming a somatic nucleus is related to high MPF activity in the cytoplasm, which induces PCC, the evidence of the French group indicated that PCC may not be an essential step in somatic reprogramming in cattle. In the USA, Sawyer *et al.* (2002) demonstrated that activation immediately prior to fusion yielded significantly greater developmental rates than that of fusion into preactivated ooplasts. In Massachusetts, Knott *et al.* (2002b) found that porcine sperm factor supported activation and development of bovine NT embryos; the efficacy of this approach may be limited because of the premature cessation of the induced oscillations.

10.6.4. Nuclear reprogramming

The successful development of cattle clones depends on the reprogramming of transferred nuclei in the enucleated oocyte; there have been several reviews dealing with problems associated with nuclear reprogramming and the many questions that require an answer (see Fulka *et al.*, 1996a, 2001a,b; Kono, 1997; Kanka, 1999a,b; De Sousa *et al.*, 1999; Jones, 2001; Korfiatis *et al.*, 2001; Tani *et al.*, 2001; Wilmut, 2001; Gao *et al.*, 2002; Miles *et al.*, 2002). It seems that oocytes are unique in being able to convert nuclei, already differentiated, into the undifferentiated stages that are normally found in the fused pronuclei of the freshly fertilized zygote. It was the birth of Dolly, the sheep produced at Roslin from a mammary gland cell taken from an adult ewe, that established for the first time that a differentiated adult cell could be fully reprogrammed. The pattern of gene expression in the adult cells of cattle is very different from that in the cells of the early bovine embryo; genes expressed in the early days of embryonic life are not expressed in adult cells and some genes expressed in embryonic cells are no longer expressed in the adult cow. When examined after the transfer of a somatic cell nucleus, the reconstructed bovine embryo shows evidence of gene expression indistinguishable from that found in the normal embryo; the exchange of cytoplasm around the nucleus from that of an adult cell to that of an oocyte has resulted in a dramatic switch in gene expression in a matter of hours. The nucleus that was once part of a COC or a fibroblast has been transformed into that of an embryonic cell.

A study by Gao *et al.* (2002) has demonstrated that germinal vesicle (GV) material is essential for nucleus remodelling after NT; these workers observe that information about what factors are released from the GV during GV breakdown (GVBD) may permit a better understanding of the molecular basis of nuclear reprogramming.

There is, however, an exception to the general rule that a somatic-cell nucleus can be transformed into an embryonic cell; this is in the matter of the imprinted genes of cattle. Imprinting, an event crucial to normal embryonic and fetal development, is believed to involve about 50 genes that are apparently marked during oocyte and sperm formation in such a way that they are switched off in the bovine embryo (Surani, 2002). One of the first genes known to be imprinted was murine IGF-II which was later shown to be imprinted in sheep; changes in IGF-II receptor have been found in sheep tissues taken from abnormal NT offspring and this has been associated with a loss in DNA methylation. In Korea, Kang *et al.* (2001) have reported evidence indicating that developmental anomalies of cloned bovine embryos could be due to incomplete epigenetic reprogramming of donor

genomic DNA. In Scotland, a paper by Young *et al.* (2001) concluded that the loss of methylation provided a plausible epigenetic mechanism for the LOS. As noted in a review by Jones (2001), it seems possible that the unpredictable phenotypes observed in clone development may be caused by inadvertent manipulations of the imprinting mechanism; the same author notes that gene expression may also be influenced by the confluency of donor-cell cultures as well as the presence or absence of serum prior to NT or during embryo culture.

There is also the matter of the inactive X-chromosome. During early development of the female bovine embryo, one of its two X-chromosomes is randomly inactivated in those tissues that make up the fetus; in the tissues that contribute to the placenta, however, the paternal X-chromosome is always the one that is inactivated.

10.6.5. Simplifying nuclear-transfer protocols

Current NT techniques employed in cattle call for skilled personnel and lengthy periods of micromanipulation. For such reasons, a laboratory at the Danish Institute of Agricultural Sciences in Tjele, in conjunction with workers from the Cooperative Research Centre in Australia, has attempted to simplify the procedure. The group reported a simplified technique that involved zona-free oocyte enucleation by free-hand bisection, electrofusion with embryonic cells from early cattle embryos and the IVC of the cloned embryo for 6–7 days (Lewis *et al.*, 2000). After two or more embryos produced by such means were transferred to 94 recipient cattle, 17 calves were born alive and apparently healthy. Subsequently, and this time using somatic rather than embryonic donor cells, the group presented further details (Booth *et al.*, 2001a,b,c). Their method involved the bisection of zona-free bovine oocytes and the reconstruction of embryos comprising two half cytoplasts and a somatic cell (granulosa cell) using phytohaemagglutin (PHA), followed by culture in microwells (termed wells of wells WOWs; see Section 7.5.9) to prevent embryonic disaggregation (see Fig. 10.10). The donor cell was stuck to one half cytoplast with PHA and fused to a second half cytoplast by electropulse. Successfully reconstructed NT embryos were activated with Ca ionophore for 5 min, followed by DMAP after 4 h before culture in WOWs for 7 days in modified SOF (mSOF) medium. The authors suggested that their novel zona-free NT technique was capable of generating cattle blastocysts in similar numbers and of similar quality to those produced by conventional procedures. In a paper by Booth *et al.* (2002), the workers in Denmark reported successful reconstruction of pig NT embryos as well as cattle.

In Australia, Lewis *et al.* (2002) compared pregnancy rates achieved by embryos produced by their simplified NT procedure with those produced by conventional methods; their results indicated that the simplified procedure may offer the means of producing large numbers of genetically identical animals, which may help in increasing the rate of genetic gain in both beef and dairy cattle. Reports by Vajta *et al.* (2002a,b) in the same laboratory dealt with the optimization of certain steps in the simplified procedure; modifications included extension of the time of

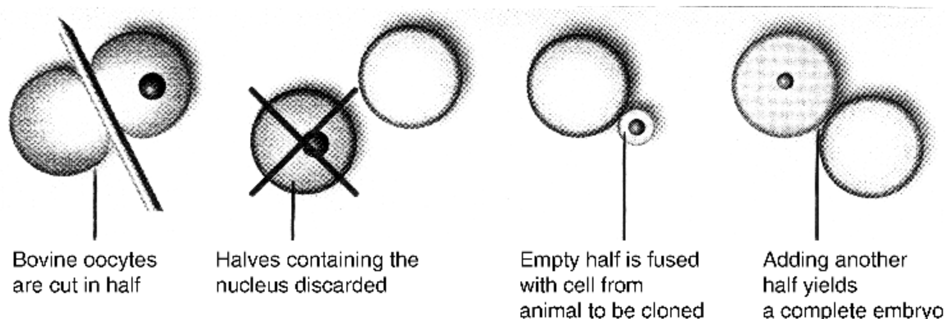

Fig. 10.10. Simplification of cloning technology in cattle (from Vajta *et al.*, 2002b).

DMAP incubation at activation and improved culture conditions. The workers note that, in 14 consecutive experiments with either week-old granulosa cells or fetal fibroblasts as nuclear donors, an average blastocyst rate of 51% per reconstructed embryo was achieved. As with the studies of Greising *et al.* (1994), the fusion of the two cytoplasts raised embryonic volume to that of a non-manipulated embryo; it is possible, as suggested by Greising and Jonas (1999), that such fusion made much needed energy available to the reconstructed embryo.

In Japan, Varisanga *et al.* (2002) compared two incubation systems – a simple portable system and a standard carbon dioxide incubator – for production of cattle embryos by electrofusion of quiescent fetal fibroblast nuclei to enucleated oocytes matured *in vitro*; they found the portable system to be effective in the production of NT embryos. They also recorded that the portable system was inexpensive and reduced the costs involved in setting up an embryology laboratory; due to its simplicity, the system avoided problems with CO_2 incubators and was easy to disinfect.

10.6.6. Preserving donor cells, cytoplasts and cloned embryos

Refrigeration

Attempts to simplify procedures in the preparation of donor cells for use in NT include storage at refrigeration temperatures. In China, the studies of Liu *et al.* (2001) indicated that refrigeration for 1–2 weeks was a feasible procedure for preparing cumulus donor cells for bovine somatic NT; they presented evidence that this could be done without compromising development *in vitro* and early development *in vivo*.

Freezing

It is generally believed that NT embryos are more sensitive to freeze–thawing damage than IVP or *in vivo*-produced embryos; it is thought that this may be due to quality factors such as low cell number or the loss of an intact zona pellucida in such embryos. Studies reported by workers in Japan have shown the developmental potential of frozen–thawed bovine oocytes following NT to be poor (Ito *et al.*, 1999a,b); the same laboratory also found that treatment of IVP cattle embryos with linoleic acid–albumin (LAA) improved their post-thaw viability (Hochi *et al.*, 1999). Further studies by the group investigated the effect of LAA in the medium used to mature oocytes that were subsequently enucleated before freezing (Hochi *et al.*, 2000b); results indicated that LAA treatment of the oocytes during IVM, enucleation and activation improved the ability of such cytoplasts after freeze–thawing to develop to blastocysts after NT.

Vitrification

The post-thaw *in vitro* survival of vitrified cloned cattle embryos was reported by Vajta *et al.* (1997b) in Denmark; they recorded that the mean survival rate of embryos was only 10% below that found with controls. A study by Peura *et al.* (1999a,b) assessed the vitrification of cytoplasts by Vajta's open pulled straw (OPS)-method for potential use in NT procedures; their results suggested that cytoplasts are resistant to cellular damage but the physiological and biochemical properties of the cytoplasm were adversely affected. None the less, the 4–9% morula/blastocyst yields achieved by NT were regarded as encouraging. The developmental potential of NT cattle embryos constructed from vitrified cytoplasts was studied by Booth *et al.* (1997, 1998b,c, 1999a,b,c) in Denmark; in comparison with controls, fusion rates were not affected by vitrification but cleavage rate (55.7 vs. 92.8%) and blastocyst yield (7.2 vs. 32.6%) were markedly reduced.

In the USA, Dinnyes *et al.* (2000a,b) used a novel, solid-surface vitrification method to cryopreserve recipient oocytes; they recorded a high survival and cleavage rate and the development of good-quality NT blastocysts. In Japan, Nguyen *et al.* (2000b) reported on the vitrification of cattle blastocysts derived from NT with cumulus cells in a comparison with vitrification of IVP-embryos; they concluded that NT embryos could be preserved without loss of viability by a simple and efficient method using a combination of partial dehydration and vitrification. In Australia, French *et al.* (2002) found no significant difference in the early pregnancy rate between fresh and vitrified cloned embryos when the OPS vitrification method was employed (see Table 10.14). Notwithstanding several encouraging

Table 10.14. Pregnancy rates after transfer of fresh or vitrified cloned embryos. Pregnancy rates following the transfer of fresh and vitrified somatic-cell cloned embryos produced using standard nuclear-transfer techniques. (From French *et al.*, 2002.)

NT embryo treatment	No. of embryos	No. of recipients	No. pregnant at 35–45 days post-transfer (%)	No. of live births[a]
Fresh	484	169	28[a] (16.5)	2[b]
Vitrified	237	78	15[a] (19.2)	2

[a]Five fresh and seven vitrified ongoing pregnancies.
[b]One transgenic calf died shortly after birth.

reports, the majority of cloning laboratories currently transfer NT embryos fresh because of a lack of proven cryopreservation systems for such embryos.

10.6.6. *In vitro* culture and evaluation of nuclear-transfer embryos

A small-scale study reported by Aoyagi *et al.* (1999) in Japan dealt with the *in vitro* development and IVP of calves from NT embryos cultured in serum-free medium. Elsewhere in that country, Shiga *et al.* (2001) presented results indicating that SOF medium containing BSA supported full-term development of somatic cloned embryos under high (20%) and low (5%) oxygen tension, whereas protein-free SOF only supported embryonic development under 5% oxygen but not under 20% oxygen. Such findings suggested that proteins, including serum and albumin, protected embryos from damage, such as free-radical damage, induced by high oxygen tension. In Korea, Im *et al.* (2000) found evidence suggesting that NT embryos cultured in modified CR1aa in an atmosphere of 5% carbon dioxide, 5% oxygen and 90% nitrogen supported a high level of blastocyst development without the need for somatic cell co-culture.

Also in Korea, Jang *et al.* (2002) found that supplementation of their mSOF culture media with 0.5 mg/ml hyaluronic acid, heparin or chondroitin sulphate improved the development of NT bovine embryos to the blastocyst stage; such supplementation also increased the TE cell numbers in the reconstructed embryos. In the same country, Choi *et al.* (2002d) sought to establish an effective culture system to support *in vitro* development of bovine embryos, cloned from ear fibroblasts, and to determine how far the IVC system could enhance pregnancy and delivery outcomes after transfer; they found mSOF to be more effective than modified CR2aa (mCR2aa) and that the successive addition of BSA (early culture) and fetal blood serum (FBS) (late culture) to this medium provided the most effective regime. The authors concluded that a much larger study would be necessary to evaluate fetal development.

Ploidy analysis

NT embryos exhibit a higher rate of embryonic loss after ET compared with those fertilized *in vitro* (see Heyman and Renard, 1996). In Denmark, the ploidy level was accurately estimated in NT blastocysts (Booth *et al.*, 1999a,b,c, 2000, 2001a,b); it was concluded that the high rate of embryonic and fetal loss in NT embryos was independent of ploidy abnormalities, at least to the blastocyst stage of development. It was also evident that NT embryos possessed no greater ploidy errors than IVP embryos.

Ribosomal RNA gene activation

Nucleolar ultrastructure in cattle NT embryos has been studied by several groups (Kanka *et al.*, 1997, 1999; Hyttel *et al.*, 2000a,b,c; Laurincik *et al.*, 2000a,b,c). It has been suggested that ribosomal RNA (rRNA) gene activation and the associated nucleolus formation may be used as a marker for embryos originating from different culture systems, including NT embryos; NT cattle embryos displayed a lack of localization of nucleolar proteins to the nucleolar anlage as compared with IVP embryos. It is possible that this may be a factor contributing to the abnormalities seen in a proportion of cloned offspring.

Apoptosis

A study by Gjorret *et al.* (2002) examined the chronology of apoptosis in bovine embryos reconstructed by NT; not surprisingly, the incidence of apoptosis was found to be significantly higher in the NT embryos than in embryos produced *in vivo*.

ICM and TE cells

Some workers have suggested that early developmental failures of NT cattle embryos may be the result of aberrant allocations of the ICM and the TE. In Korea, Lee, K.K. *et al.* (2002) recorded that NT blastocysts showed a significantly higher ICM : total cells ratio than either IVP or *in vivo*-derived cattle embryos; they suggest that placental abnormalities or early fetal losses may be the result of such differences. A further study by Koo *et al.* (2002) in the same laboratory assigned bovine blastocysts to four groups (I: < 20%; II: 20–40%; III: 40–60%; IV: > 60%) according to the ratio of ICM : total cells; they found that most NT blastocyts were placed in groups III and IV, whereas most IVF and *in vivo*-derived blastocysts were distributed in group II.

Gene expression patterns

Results presented by Wrenzycki *et al.* (2001a) demonstrated that modifications of the NT protocol could alter the expression pattern of developmentally important genes in NT-derived cattle embryos compared to their IVP and *in vivo*-derived counterparts; it was concluded that recent developments in complementary DNA (cDNA) array technology permitting the simultaneous determination of several thousand gene transcripts are likely to be valuable in investigating the effects of nuclear transfer at the molecular level.

Mitochondrial heteroplasmy

When a normal cattle embryo is produced by the union of a spermatozoon and an oocyte, the male gamete contributes few, if any, mitochondria to the embryo. The general belief is that mitochondria are derived solely from the maternal oocyte. In the normal course of events, mitochondrial DNA (mtDNA) is maternally inherited; while the mammalian spermatozoon contains a limited number of copies of such DNA (< 100), the oocyte itself is likely to contain 100,000–200,000 copies of maternal mtDNA. It has been speculated that the paternal mtDNA may be highly mutated due to the spermatozoon's high energy requirements and accompanying generation of free radicals of oxygen causing extensive damage. According to a report by Sutovsky *et al.* (1999), sperm mitochondria inside the fertilized bovine oocyte are tagged by the recycling marker protein, ubiquitin, an imprint that occurs during spermatogenesis and which results in the degradation of the sperm mitochondria by the oocyte's cytoplasmic proteasomes and lysosomes. The same authors noted that learning more about the destruction of paternal mtDNA may be important in evaluating the safety and efficacy of cloning; they speculated that there may be problems in cloned offspring due to heteroplasmy, the condition of mismatched mitochondria.

The reconstruction of oocytes by NT can result in mixed populations of mtDNA being transmitted to the offspring, in contrast to the unimaternal inheritance of mtDNA in IVP and *in vivo*-produced cattle embryos. The fate of donor-cell mitochondrial DNA in cloned cattle embryos was examined in Korea by Do *et al.* (2002); they presented results indicating that the foreign cytoplasmic genome from donor cells was not destroyed by the cytoplasmic events during early embryonic development that followed NT. A paper by Hiendleder *et al.* (2002) showed that zebu cattle mtDNA was fully compatible with the nuclear-encoded mtDNA replication machinery of European cattle; they concluded that cross-subspecies transmitochondrial cloned cattle with zebu mtDNA was feasible. A review by St John (2002) notes that there is much still to be investigated in this field.

Telomerase activity

The telomere is the end structure of the DNA molecule. Telomerase is the ribonuclear enzyme that enables the telomere to elongate; otherwise, the telomere would shorten with each cell division that occurs in DNA replication. Concern was expressed when an analysis of telomere lengths in cloned sheep showed these to be significantly smaller than in control animals

(Shiels *et al.*, 1999); the fear was that this might be indicative of premature ageing in cloned animals. With such concerns in mind, a study by Xu and Yang (2000) involved assaying telomerase activity in cattle embryos; they demonstrated that telomerase activity was present in early embryos but that its level varied according to the developmental stage. The activity was relatively low in matured oocytes, increased after fertilization and then decreased gradually until the eight-cell stage. After the eight-cell stage, telomerase activity increased again and reached its highest level in the blastocyst stage. The authors suggested that the length of the telomere may be reprogrammed during early embryo development. A subsequent paper by the same authors examined telomerase activity in NT cattle embryos; similar levels of activity were found in NT and IVP embryos (Xu and Yang, 2001).

A paper by Miyashita *et al.* (2002) in Japan focused attention on differences in telomere lengths depending on the source of donor cells employed in NT. Using nuclei of donor cells derived from muscle, oviduct and mammary and ear skin, they produced 14 cloned cattle, which exhibited remarkable differences in telomere lengths. In clones derived from muscle cells from an aged bull, telomere lengths were longer than those of a control donor animal but were within the variation shown by normal calves. In clones derived from oviductal and mammary epithelial cells from an equally aged cow, telomere lengths were shorter than those found in control cattle. The findings were taken as indicating that cloning does not necessarily restore the telomere clock, but that NT itself may often trigger an elongation of telomeres, probably influenced by the type of donor cell employed.

10.6.7. Gestational and perinatal losses

Papers by Chavatte-Palmer *et al.* (2000a,b) in France have reviewed the incidence of LOS in somatic and embryonic cloning in their laboratory. It is often noted that gestation length is prolonged and live calves occasionally exhibit a respiratory distress syndrome and several types of abnormalities that may hinder their survival. A striking feature of NT studies is the high incidence of gestational losses in early pregnancy and in the late fetal and perinatal periods. Although the majority of losses are usually observed in the first third of pregnancy, there is a distinct syndrome, the LOS, which may occur in late pregnancy and result in perinatal death. Such defects may be apparent for some time in the developing fetuses; a paper by Garry *et al.* (1998) demonstrated marked differences in energy regulation between bovine fetuses from NT clones and naturally derived embryos.

It is clear from many reports that losses of NT bovine embryos/fetuses during the first trimester of pregnancy may sometimes exceed 50%. In a study of such attrition, De Lille *et al.* (2001) in Colorado examined tissues from NT and control conceptuses at 75 days of gestation; they found large and medium placentomes to be significantly heavier and the umbilical cord to be enlarged (see Table 10.15).

In Texas, Hill *et al.* (2000a,b) traced the viability of 40 cattle clone fetuses by ultrasonics and examination of fetal/maternal tissues; by day 40 40% of cloned fetuses had died and by day 60 only 28% of the fetuses remained viable. It was concluded that failure of placentome development was the likely cause of early fetal loss. In France, Chavatte-Palmer *et al.* (2000a,b) found evidence that concentrations of IGF-I and IGF-binding proteins (IGFBPs) vary between neonatal cloned and control calves, regardless of their

Table 10.15. Tissue characteristics of cloned and control fetuses (from De Lille *et al.*, 2001).

	Clones (n = 10) (mean ± SEM)	Controls (n = 5) (mean ± SEM)
Mean placentome weight (g):		
Large	3.64 ± 0.51	2.16 ± 0.18[a]
Medium	1.13 ± 0.11	0.74 ± 0.08[a]
Small	0.21 ± 0.02	0.18 ± 0.03
Fetal weight (g)	59.8 ± 3.04	59.5 ± 2.23
Fetal length (mm)	135.9 ± 2.65	129 ± 5.63
Umbilical cord (g/cm)	0.46 ± 0.03	0.35 ± 0.03[b]
Brain (g)	2.33 ± 0.07	2.30 ± 0.07
Heart (g)	0.50 ± 0.04	0.48 ± 0.07
Kidneys (g)	0.45 ± 0.04	0.34 ± 0.06
Liver (g)	2.64 ± 0.15	2.48 ± 0.09

[a] $P \leq 0.01$; [b] $P \leq 0.05$, Student's t test.
SEM, standard error of the mean.

clinical status; such findings may reflect abnormalities in growth and placental development in fetal life. In Japan, Yamada *et al.* (2001) examined the development of NT cattle embryos and found evidence suggesting that early fetal loss, especially in the first half of pregnancy, may be caused by placental malfunction; the most striking feature recorded was the incomplete formation of chorionic villi. A study reported by Hill *et al.* (2002) examined the possibility that altered immunological status was a contributory factor to early embryonic losses; they concluded that major histocompatibility complex class I (MHC-I) expression in the trophoblast of cloned cattle fetuses was abnormal and likely to have caused increased numbers of maternal lymphocytes, which could be detrimental to the maintenance of pregnancy.

In Japan, Hirako *et al.* (2002) examined the developmental ability of somatic cell cloned fetuses; of eight NT blastocysts at time of transfer, only one remained alive at day 132; the authors found evidence of growth retardation, placental hypoplasia and disruption of chorionic membranes. Studies reported by Chavatte-Palmer *et al.* (2002a,b) analysed IGF receptor 2 (IGFR2) and cyclo-oxygenase 2 (COX-2) expression in the placenta of cattle clones derived from somatic cells; they found evidence that IGFR2 may play a role in the origin of pathological clones and that COX-2 may be a factor in the associated late onset of contractions.

Neonatal care

As part of a campaign to generate cloned cattle for the production of pharmaceuticals, a specialized in-house neonatal unit was set up in Wisconsin to provide intensive follow-up of both late pregnant heifers and newborn calves (Salaheddine *et al.*, 2002); 58 cloned calves were delivered by Caesarean section within 2 weeks of their due delivery date. Despite a number of life-threatening problems observed in the postnatal period of such calves, the mortality rate did not exceed 12%. In France, Heyman *et al.* (2002a,b) reported experiments suggesting that maternal Pregnancy Specific Protein (PSP)60 assays could be a good predictor of abnormal fetal development after somatic cloning in cattle and that cloning from adult cells induced higher levels of abnormalities and losses than cloning from embryos. In the same country, Constant *et al.* (2002) reported that the most likely cause of increased PSP60 concentrations in abnormal clone pregnancies was hypertrophy of placental tissue rather than deregulated proliferation of binucleate cells. In the USA, Crosier *et al.* (2002) studied the effect of *in vitro* embryo production on the development and regulation of skeletal muscle in cattle fetuses in late pregnancy; they recorded evidence of altered development of muscle fibres and identified myostatin as the candidate gene whose expression may contribute towards such changes.

Preventing LOS?

According to a report by Hwang *et al.* (2001), a combination of AI and ET proved effective in preventing LOS in cloned cattle production. They reported work in which 51 NT embryos were transferred to inseminated recipient cattle and noted the occurrence of freemartins in some of the mixed-sexed twin pregnancies. Presumably such problems could be solved by the use of sexed semen and using NT embryos of the appropriate gender. The *modus operandi* of this approach to avoiding problem clones is, if not due to chance, not immediately obvious.

10.6.8. Development of clones after birth

Much research attention has been focused on the fate of NT offspring during pregnancy and at calving. A paper by Gartner *et al.* (1998) drew attention to the high variability of body sizes that occurred with cattle clones born in their studies. There are fewer reports dealing with the development of clones after birth. Studies by Harris *et al.* (1994) dealt with growth and carcass quality characteristics in NT calves. In France, Chavatte-Palmer *et al.* (2002b), in the light of their experience, concluded that apparently healthy cloned calves cannot be considered as physiologically normal until at least 50 days of age. In the USA, Tian *et al.* (2002) examined the growth patterns of clones obtained from a high-yielding cow whose genetics were 13 years older than those of controls; they found no significant difference between clones and normally reproduced calves in their intake and growth

patterns. It was evident that clones produced from aged adult somatic cells were similar to their naturally produced peers. The authors took such evidence as supporting the view that the biological clock of the aged animal had been reset in the cloning process. A study reported by Enright *et al.* (2002) compared reproductive characteristics of cloned heifers, including puberty, follicular dynamics and hormone profiles during the oestrous cycle,with controls produced by AI; they found that the cloned animals, derived from the cells of an aged cow, exhibited normal reproduction. Other studies in the same laboratory, reported by Govoni *et al.* (2002), dealt with systematic studies on the normalcy of somatic clones in comparison with age- and weight-matched calves produced by AI; they found that the developmental patterns of cloned calves, in terms of the hormones involved in growth (growth hormone, IGF-I, IGFBP-3), were similar to those in controls.

In Wisconsin, Pace *et al.* (2002) reported on the survival, growth and development of 117 calves produced over a 2-year period using NT and non-embryonic cells derived from various tissues; they demonstrated that 85% of clones alive 2 days after birth were still alive and healthy, ranging in age from 5 to 29 months. The authors concluded that, even allowing for current technical inefficiencies, the cattle produced using NT technology offered many possibilities for use in human health care and agriculture.

10.6.9. Embryonic stem cells

ES cells are pluripotent cells derived from the ICM of the blastocyst that can be propagated indefinitely in an undifferentiated state. Such cells are capable of differentiating to all cell lineages *in vivo* and to many cell types *in vitro*. In the early 1980s, ES cells were isolated from the ICM of the developing mouse embryo by Evans and Kaufman; such cells proved capable of being maintained in an undifferentiated state in culture indefinitely. Although ES cells have also been isolated from human embryos, their use in research as well as in therapeutics has been the subject of much debate and controversy. The discovery of ES cells in mice and humans was of great interest to those who later became involved in cattle NT research, who believed that young, embryonic cells were what was required for success. However, despite many attempts to isolate bovine embryonic cells over the years, none has proved successful (see Ito *et al.*, 1996; Stice *et al.*, 1996; Wolf *et al.*, 2000); however, there have been a number of reports claiming to have established pluripotent cell lines in cattle (Hamano *et al.*, 1998). In human medicine, a commentary by Edwards (2001) has drawn attention to studies indicating that the time is fast approaching when close control over directed differentiation of human stem cells into each germ layer or into specific tissues will become possible; such developments will be immensely valuable in alleviating currently incurable human conditions. A recent commentary by Orkin and Morrison (2002) discussed the relative merits of using ES and adult stem cells for research in human medicine.

10.7. Transgenic Cattle

Modern livestock farming, with the various breeds of cattle that graze the fields, is largely the result of centuries of patient selection by farmers on the basis of animal appearance and performance. However, change is in the air, change that would have been greeted with incredulity by farmers and their advisers a half-century ago. The most dramatic of these changes is likely to be genetic engineering (transgenesis). Transgenesis can be defined as the alteration of the animal genome with the intent of modifying a specific physical trait. Great progress has been made in developing genetic linkage maps, which will enable researchers to identify chromosomal regions that influence traits of economic importance in cattle (see Kappes, 1999). The first genetically modifed farm livestock were described in 1985 by workers at the USDA Beltsville centre in Maryland; transgenic sheep and pigs on that occasion were produced by pronuclear DNA injection, a method described several years earlier in mice (see Fig. 10.11). In farm animals, however, the pronuclear injection technique was laborious and costly and could not be easily applied on the farm. The first decade of transgenic research saw most attempts aimed at producing recombinant pharmaceutical proteins in

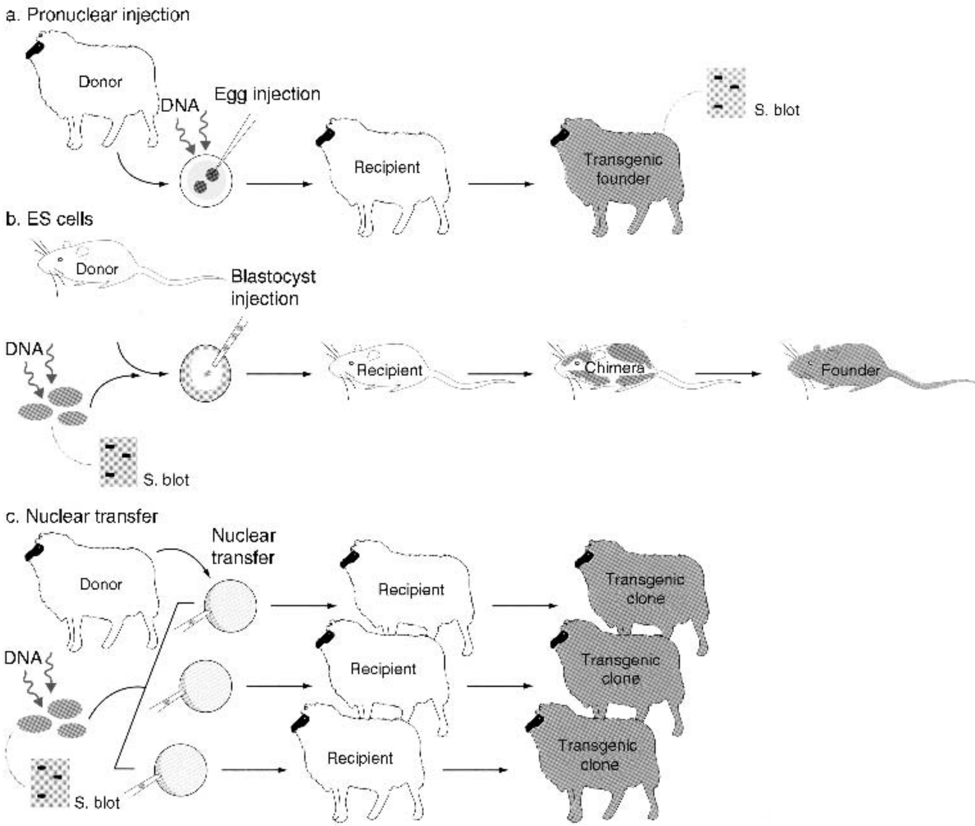

Fig. 10.11. Approaches to the production of transgenic sheep (from Clark, 1998).

milk. A clear advance in cattle work was evident in the 1990s, when it became possible to generate one-cell embryos by IVM and IVF of oocytes and to culture the microinjected embryos until the blastocyst stage. This led to a considerable reduction in the number of cattle required to produce transgenics. However, with microinjection of DNA into the pronuclei of fertilized oocytes, only a small proportion of animals integrated the transgene DNA into their genome, making transgenesis expensive and relatively ineffective. A few years later, in 1997, somatic cloning opened the way to combining NT with transgenesis in cattle.

Cloning techniques, including gene transfer into cultured fetal or adult cells used as the source of nuclei, has greatly simplified gene addition in cattle. Using current technology, during IVC of somatic cells over several passages, it is possible to transfect stably the cells with a construct containing the gene in question, linked to a promoter known to be expressed in the mammary gland or other appropriate target organs. After that step, co-transfection of cells in culture with a selectable marker enables the transgenic cell clone to be isolated; such selected cells, which are all transgenic, can then be used as a source of nuclei for somatic NT to create transgenic embryos and young more efficiently. The relative ease by which cultured somatic cells may be rendered transgenic, coupled with the use of such cells for NT, holds out the possibility of generating transgenic animals, initially for biomedical purposes, but eventually for agriculture.

Future developments in transgenic technology are likely to see precise genetic modifications being achieved by site-specific recombination in the somatic cells before NT; gene targeting would greatly facilitate the introduction and propagation of genes, widening the scope of applications in transgenic cattle and other farm livestock, as well as providing additional opportunities for

understanding gene products and the mechanisms regulating gene expression (see Espanion and Niemann, 1996; Jafar and Flint, 1996; Campbell and Wilmut, 1997; Heap, 1997; Eyestone, 1998; Heyman *et al.*, 1998a,b; Lubon, 1998; Robl *et al.*, 1998; Seidel, 1998a; Stice *et al.*, 1998; Ward and Brown, 1998; Wilmut, 1998a,b; Chan, 1999; Colman, 1999; First *et al.*, 1999; Mepham *et al.*, 1999; Robl, 1999; Bulfield, 2000; Houdebine, 2000; Piedrahita, 2000; Polejaeva and Campbell, 2000; Lavoir, 2001; Wheeler and Walters, 2001).

10.7.1. Development of transgenic technology in cattle

In vitro embryo production techniques have facilitated the development of current methods employed in generating, evaluating and breeding transgenic cattle. Studies in bovine oocyte maturation, fertilization and embryo culture in laboratories around the world have provided much of the basic information currently used in cattle transgenic technology; in the initial stages of genetic engineering in cattle, most transgenic calves were derived from IVP zygotes. In Virginia, for example, Eyestone (1999) reported the microinjection of 36,530 such zygotes with a gene construct designed to cause the production of human α-lactalbumin in mammary glands; the eventual outcome was the birth of 18 transgenic calves.

Although pronuclear injection was rapidly adopted as the method of choice for generating transgenic mice in the early 1980s, the application of this technique in the mid-1980s presented serious difficulties with cattle. One problem was the opacity of the zygotic cytoplasm, which made the pronuclei impossible to view for microinjection of DNA. This was solved without undue difficulty by centrifuging the bovine zygote, a procedure that fortunately did not have a serious effect on subsequent embryonic development. None the less, pronuclear injection soon showed itself to be a very inefficient way of generating transgenic cattle; it was not unusual for fewer than 1% of microinjected cattle zygotes to end up as live births. Further drawbacks to this approach were related to the random integration process, which could result in mosaicism, insertional mutations and varying expression due to position effects.

The ability to clone sheep and cattle from cultured somatic cells was to have far-reaching implications for the generation and propagation of transgenic ruminants. Experiments conducted at Roslin by Schnieke *et al.* (1997) resulted in the birth of the first transgenic sheep by way of NT; in this work, sheep fetal fibroblasts were co-transfected with a neomycin-selectable marker gene and a gene encoding human factor IX, linked to regulatory elements from the sheep β-lactoglobulin gene. Within a year, the cell-based route for transgenesis had been repeated in cattle (Cibelli *et al.*, 1998a,b). Although the Roslin workers considered that their success with somatic-cell NT might have been due to the use of donor cells in the G0 phase of the cell cycle, the US group achieved their success using non-quiescent donor cells.

The discovery that cattle can be cloned by NT from cultured somatic cells now means that it is possible to achieve gene targeting in this species. Several reviews discussing prospects and technical challenges for introducing targeted changes into the germ line by this route may be consulted (see Clark, 1998; Clark *et al.*, 2000).

10.7.2. Potential advantages of transgenic cattle

Various authors have reviewed how transgenic technology can be applied to the modification of milk composition in cattle (Houdebine, 1995, 2000, 2001; Rudolph, 1997, 1999; Wall *et al.*, 1997; Eyestone *et al.*, 1998; Garner and Colman, 1998; Ziomek, 1998; Zuelke, 1998, 1999; Pintado and Gutierrez-Adan, 1999a,b); the simplification of the methodology and the consistent reduction of the time taken to carry out transgenic studies enables many possible ways of modifying milk components to be tested. For the production of large amounts of foreign protein, transgenic milking cows would seem to be the most appropriate. There are those who have suggested that purification costs could be reduced if the protein in question is produced in a less complicated fluid than milk, such as urine (Meade and Ziomek, 1998; Wall *et al.*, 1998), although the aesthetic consequences of this

route to pharmaceuticals may not have strong appeal. A review by Yang *et al.* (2000) noted estimates that, in the near future, the world's demands for human pharmaceutical proteins may largely be met by transgenic farm animals; the same authors suggest that recent developments on cloning, ES cells and alternative transgenic methods are likely to further expand transgenic applications in farming and biomedicine.

Among the interesting possibilities that the future holds, increased resistance to disease, by introducing specific genes into cattle, is one that has obvious attractions (Muller and Brem, 1994). The identification in the early 1970s of single genes in the MHC which influence the immune response was a major factor in recognizing the genetic basis of disease resistance or susceptibility. Manipulation of the MHC in cattle through NT transgenesis could have a major beneficial effect on disease resistance in this species. Moves aimed at reducing or eliminating the need for prophylactic use of drugs or at improving human safety (e.g. producing prion-free cattle) are likely to warrant higher priority in the public at large than increasing growth rates and feed efficiency. A review by Murray and Anderson (2000) dealt with factors affecting the acceptance of genetically engineered animals by industry, including its economic benefits and whether consumers are prepared to buy the resulting products.

10.7.3. Methods of genetic modification in cattle

Although cloning by NT and recombinant DNA technology had quite separate origins, in practice the two technologies have developed side by side in the past two decades. Both technologies involve manipulation of the early bovine embryo and its culture *in vitro*. Gene-transfer methods have been reviewed by several authors (Wall, 1996, 2002; Niemann and Kues, 2000). Many of the methods developed in the mouse are likely to be employed in achieving precise modification of the bovine genome. These may include the application of artificial chromosomes from yeast (YAC) or bacteria (BAC) for position-independent and copy-number-dependent expression of a transgene.

Pronuclear injection

Only a few years ago, the standard procedure for producing genetically modified cattle involved the injection of one of the pronuclei into the recently fertilized oocyte. Pronuclear microinjection was the first gene-transfer method designed specifically to produce transgenic animals. The microinjection method took advantage of the unique DNA-processing events that occur in the pronuclei, especially the larger male structure; the pronucleus provides the specialized nuclear environment for the incorporation of DNA sequences and for their inclusion in a functional chromosomal region. Although pronuclear injection of DNA proved to be an acceptable approach in mice, in cattle the lower efficiency of the technique and the costs involved with the recipient herds required to carry clones to term severely limited the uptake of the technology; it might often be necessary to inject more than 1000 zygotes to produce a single transgenic calf. The low efficiency of the technique in cattle was attributable to poor embryo survival to term, low transgene integration rates and unpredicatable transgene behaviour. In common with several other reports, Eyestone *et al.* (1995) found that pronuclear injection had an adverse effect on embryo development to day 7. In New Zealand, Hagemann (1995) showed that the use of a physiologically appropriate buffer in pronuclear injection of DNA may be a factor influencing the development of manipulated embryos. Not unexpectedly, there have been those who have reported that initial oocyte quality is an important factor in selecting zygotes for pronuclear injection (Chauhan *et al.*, 1999a,b). In Japan, Saeki *et al.* (2000) investigated the fate of DNA injected into bovine zygotes by detecting the fluorescence of luciferase cDNA, which had been fluorescence-labelled prior to microinjection; their results suggested that injected DNA degraded markedly.

Although microinjection of pronuclear-stage bovine embryos became the conventional route to the creation of transgenic cattle for several years, workers in Poland found that development to the morula/blastocyst stage was about 20% greater for bovine embryos obtained from microinjected two-cell embryos compared with zygotes (Jura *et al.*, 1994). Other work, this time in the USA, examined the production of

transgenics by injecting one- to four-cell *in vivo*-derived embryos (Echelard *et al.*, 2000); their results indicated that transgenic cattle could be created by injecting two-cell embryos.

Transfected cells for nuclear transfer

Although true ES cells are currently only available in the mouse and human, some researchers have been able to isolate, culture and genetically transform primordial germ cells (PGC) derived from cattle embryos (Lee, C.K. *et al.*, 2000); it is possible they might be used to achieve genetic modification in this species. Earlier studies by Lavoir *et al.* (1994), Cherny and Merei (1994) and Cherny *et al.* (1994) had shown that PGC-derived cells could be isolated from suspensions of gonadal cells in the early months of gestation; a paper by Strelchenko and Stice (1994) noted that such cells might be an alternative to ES cells. A report by Brink *et al.* (2000) described the use of PGC cells from the genital ridge of bovine fetuses, aged 40–60 days; the introduction of transgenes into these cells is achieved by standard transfection methods. Selection of cells that have correctly integrated the desired transgene into the genome is achieved by using a selectable marker encoding antibiotic resistance; by employing female PGC-derived cells for NT, all the calves born will be heifers, transgenic and non-mosaic. No longer is it necessary to wait for some time before a production herd is established, as was the case when pronuclear injection of DNA and conventional breeding methods were employed (see Fig. 10.12); now it is possible to establish a herd of genetically identical cattle instantly. As noted by Brink *et al.* (2000), given the advantages of preselection of transgenic embryos, genetic consistency and shorter time required for product development, it is easy to understand why NT is rapidly becoming the technology of choice to generate transgenic cattle.

Although fetal fibroblasts have been the cells of choice for the production of transgenic NT animals because of their potential for many cell

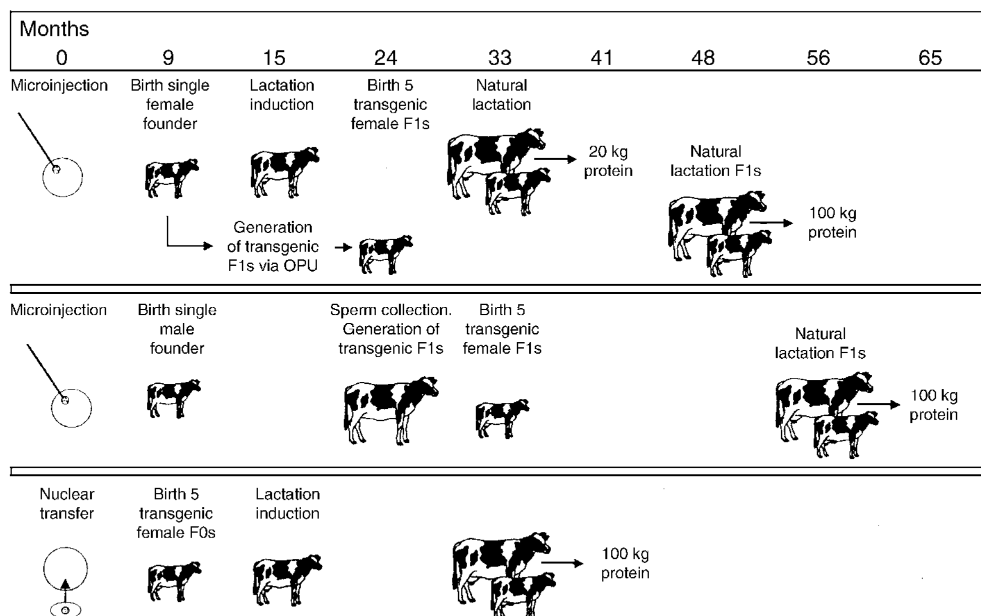

Fig. 10.12. Transgenic cattle to produce recombinant protein in milk. Three scenarios for the generation of transgenic cattle producing recombinant protein in milk. Time lines (in months) for the production of 100 kg of recombinant protein are outlined when the production herd is generated via microinjection and conventional breeding, or nuclear transfer. Calculations are based on the assumption that a single cow produces 10,000 l of milk over a lactation period of ~8 months. Concentration of recombinant protein in milk is 2 g/l. (From Brink *et al.*, 2000.)

divisions, it has been found that adult cells can be similarly employed. A study in the USA by Arat *et al.* (2002a,b), using tissue taken from the ear of an aged cow, showed that adult fibroblasts could be cloned as stable transfected cell lines and used in the production of transgenic bovine embryos.

Gene targeting

For more than a decade, researchers have been able to modify endogenous genes in the mouse by manipulating ES cells; they have been able to generate specific mutations and alter specific gene sequences in cultured ES cells. Such modified cells retained their developmental potential and when inserted into a developing embryo could contribute to all its tissues, including sperm and oocytes. The ability to use cells to transfer a predetermined genetic modification to the whole animal would have several benefits in farm animals, including cattle. McCreath *et al.* (2000) in Roslin described efficient and reproducible gene targeting in fetal fibroblasts and showed that viable sheep could be produced by NT; a similar technology can be used in cattle. The Roslin work showed that NT in transgenesis does not require ES or PGC-derived cells and avoids the need to generate chimeric animals, which is costly and time-consuming. In Georgia, Arat *et al.* (2002a,b) examined various cloning strategies that could be used for gene targeting in cattle; transfection was via a plasmid (pEGFP-N1) containing the enhanced green fluorescent protein (GFP) and neomycin-resistant genes. They reported that adult and fetal cells could complete clonal propagation, including transfection and selection, and be used to produce transgenic NT embryos.

Sperm-mediated DNA transfer

The report of Lavitrano and colleagues in Italy in the late 1980s describing the production of transgenic mice using sperm as a vector of exogenous DNA into the oocyte attracted much attention and some amount of scepticism. According to several reviews since that report, there is insufficient evidence available to understand what happens to sperm-associated DNA on entry into the oocyte at fertilization (Niemann, 1996; Sperandio *et al.*, 1996, 2000; Gandolfi, 1998a,b, 2000; Wall, 2002). Although the binding of DNA molecules to sperm is now firmly based on experimental evidence, whether this gives rise to real transgenic animals has been a matter of some controversy. Currently, it has yet to be resolved whether sperm-mediated gene transfer is a possible way of manipulating the genome or if evolution has imposed insoluble difficulties on its use in producing transgenic cattle. A study by Chan *et al.* (2000) was able to demonstrate the uptake of rhodamine-tagged DNA on the sperm of cattle and several other mammalian species; they were able to use this labelling system to monitor the dynamics of DNA binding on sperm and in the selection of DNA-bound sperm for sperm injection. Compelling evidence of successful sperm-mediated gene transfer has recently been provided by Lavitrano *et al.* (2002), who have reported the production of transgenic pigs for use in xenotransplantation; they found a 25-fold improvement in their success rate in comparison with pronuclear injection. According to the Italian team, it is necessary to free the sperm of seminal plasma because of its IFN content, which usually prevents sperm from accepting the new DNA. The majority of piglets born (up to 80%) had the gene incorporated into the genome and most transcribed the gene (human decay-accelerating factor (hDAF)) in a stable manner; the gene was transmitted to progeny.

An article by Kim and Shim (2000) noted that transgenesis using ICSI may be particularly useful in pigs. According to a report by Lee, H.T. *et al.* (2002) in Korea, sperm-mediated DNA transfer has the potential to simplify the generation of transgenic pigs; they investigated the expression of a transgene after co-injection of a spermatozoon or sperm head with green fluorescent protein (GFP) into IVM pig oocytes. They found that membrane-disrupted sperm could attach exogenous DNA and that ICSI might prove to be a useful tool for producing transgenic pigs. According to Wall (2002), it is likely that sperm-mediated gene transfer protocols will continue to be refined; if reliable gene expression can be achieved, then the method may become the method of choice in species in which manipulation of oocytes and zygotes presents particular difficulties.

Workers in Israel have combined restriction enzyme-mediated integration (REMI) with sperm-mediated gene transfer to produce

transgenic cattle (Shemesh *et al.*, 2000); they found that REMI-lipofected sperm could be used in both IVF and AI. A later review by Shemesh *et al.* (2002) concluded that the use of REMI and lipofection of sperm for gene transfer could be a highly effective method of producing transgenic animals. In Canada, Rieth *et al.* (2000) showed that transgenesis by homologous recombination was possible, using electroporation of bull sperm to carry DNA containing highly repetitive sequences into oocytes.

Retroviral infection of early embryos

Haskell and Bowen (1995) in Colorado attempted to produce transgenic cattle by retroviral infection of one- to four-cell embryos; these embryos were exposed to a replication-defective retrovirus by microinjection of retrovirus-producing cells into the perivitelline space. In Madison, a study by Chan *et al.* (1998) involved the injection of pseudotyped replication-defective retroviral vector into the perivitelline space of metaphase II cattle oocytes; they were able to demonstrate the production of transgenic cattle by reverse-transcribed gene transfer. The authors discuss the implications of this mechanism as a means of producing animals. In Korea, Ko *et al.* (2000) demonstrated the successful integration of enhanced green fluorescent protein (EGFP), erythyropoietin (EPO) and neomycin-resistant genes in IVP cattle embryos following NT with fetal fibroblasts that had been transfected with these genes by retrovirus-mediated infection; the authors saw the procedure as an attractive method of producing transgenic cattle. According to Wall (2002), the advantages of retrovirus-mediated gene transfer include a high frequency of gene transfer across embryonic membranes, high integration into the oocyte/zygotic genome and minimal embryo manipulation. Disadvantages mentioned by the same author include the fact that the technique can only deal with relatively small amounts of genetic information (< 10 kb) in a field where increasing emphasis is on the use of increasingly longer sequences; there is also the complexity of introducing the transgenes into the retrovirus, a process involving many steps. A final difficulty may be that of the public perception of this approach, bearing in mind the inevitable connection between viruses and certain disease conditions.

10.7.4. Transgenic embryos in the laboratory

Predicting transgene integration

The ability to identify those embryos carrying gene inserts before they are transferred to recipient animals is of great practical value; it avoids the need to maintain a large number of recipients for ETs. Various methods have been reported. An early attempt to identify transgenic embryos was a system designed by Bowen *et al.* (1994) in Colorado; a biopsy sample consisting of ten to 30 trophoblast cells was analysed by PCR for the presence of the transgene. In Finland, Hyttinen *et al.* (1994) reported the successful generation of transgenic dairy cattle developed from IVP embryos that had been screened for transgene and sex prior to ET; even with a successful screening technique, the authors noted that thousands of microinjections of DNA had to be carried out to achieve a single pregnancy with a transgene-positive embryo. In France, Menck *et al.* (1998a,b) described a luminescent screening test to select transgenic cattle embryos; on the basis of their results they suggested that selecting luminescent blastocyts on the basis of signal intensity and distribution could markedly reduce the number of recipients required. In Japan, Nakamura *et al.* (1998) reported the rapid detection of firefly luciferase gene expression in live developing cattle embryos by photon-counting.

In Wisconsin, Chan *et al.* (2000) suggested the possibility of using specially designed GFP as a marker for transgenic cattle embryos. The screening of bovine embryos by either biopsy or GFP was employed by Chen *et al.* (2002) in attempts to ensure that calves born would be transgenic; the day-40 pregnancy rate of the biopsy group (40%) was lower than that of the GFP group (57%) but the calf birth rate of the biopsy group was higher (40% vs. 21%). The authors note that this was the first report of biopsy-screened cloned transgenic animals and that both the screening methods were useful in detecting transgenic NT embryos without negatively affecting their development into viable offspring.

Preserving embryos

It is only to be expected that the sensitivity to freezing of cattle embryos would not be

improved by gene injection; pronuclear injection involves high-magnitude *g* forces and making a small opening in the zona pellucida and oolemma. However, the cryopreservation of transgenic embryos prior to transfer would avoid the need to maintain an excessivly large recipient herd and make the production of transgenic animals more cost-effective. Cryopreservation of *in vitro*-derived cattle blastocysts microinjected with foreign DNA at the pronuclear stage was reported by Ito *et al.* (1998a,b) in Japan; their results showed that microinjected day-7 blastocysts could be successfully frozen by conventional two-step freezing or vitrification. A study by Han *et al.* (2000) in Korea showed that the viability of microinjected IVP embryos was markedly affected by freezing in comparison with fresh controls (13.6 vs. 26.5%); embryo quality and stage of development were factors influencing the response of embryos to their conventional freezing technique (slow freezing in 10% glycerol solution).

10.7.5. Losses in transgenic embryos, fetuses and calves

The factors involved in the low pregnancy rates associated with the transfer of microinjected IVP embryos have been the subject of numerous reports. In the Netherlands, Garcia and Salaheddine (1999) found that IVP microinjected embryos harvested on day 7 resulted in higher pregnancy rates than those transferred on day 8. Other authors have reported on the fetal and neonatal abnormalities found in cloned transgenic calves; it is possible that many such defects are a follow-on to NT procedures rather than to the incorporation of a transgene. In Texas, Hill *et al.* (1999a) reported clinical and pathological features of cloned transgenic fetuses and calves derived from the same male fetal-fibroblast cell line transfected with a β-galactosidase marker gene; the authors concluded that the cloning technique and/or embryo culture conditions probably contributed to these abnormalaties. Elsewhere in the USA, Behboodi *et al.* (2000) presented results for bovine microinjected embryos showing that embryo development to the morula/blastocyst stage was significantly higher using *in vivo*- rather than *in vitro*-derived oocytes; calf weights were also found to be significantly higher with the IVM oocytes.

70.7.6. Transgenic cattle on the farm

Germ-line mosaic bulls

Germ-line mosaic founder bulls have transgene copies in only some of their cells and they transmit the transgene locus at low frequency (< 50%).One way of reducing the high cost of unwanted pregnancies in dealing with such bulls by AI or conventional ET methods is by transferring only transgenic female embryos to recipients. A study reported by Hendolin *et al.* (2000) in Finland sought to develop an efficient herd-generation procedure for a mosaic human lactoferrin founder bull by a combination of MOET, embryo biopsy and multiplex PCR techniques. By transferring only transgenic female embryos, they produced 29 transgenic heifer calves; if they had used AI, they would have required about 450 recipients and would have had more than 250 unwanted pregnancies to achieve the same result.

Transgenic cows

A report by Echelard *et al.* (2002) described a herd of transgenic cows expressing high levels of human serum albumin (hSA) in their milk; the herd was produced using transfected bovine fetal cells carrying an hSA expression gene in a somatic-cell NT programme. Twenty calves showing high-level expression of hSA survived from the 27 born alive. In Korea, Cho *et al.* (2002a) reported attempts to produce transgenic cows for the production of recombinant α-1-antitrypsin by somatic-cell NT using transfected adult fibroblast cells.

10.7.7. Welfare of transgenic cattle

The genetic modification of animals is a controversial subject and there is active opposition to work in this area of biotechnology. In Cambridge, a review by Broom (1998) dealt

with the effects of biotechnology on animal welfare, in particular the welfare of transgenic animals. A major concern of those directing transgenic technology must be to ensure that it does not compromise farm-animal welfare; there have been several unfortunate examples that are widely quoted to the detriment of researchers working in this area. On the other hand, it may well be that the modification of disease resistance or disease susceptibility by gene transfer will eventually come to be seen as a major asset to animal welfare. As noted by Piedrahita (2000), studies in mice have already shown that targeted transgenesis can be used to produce animals resistant to spongiform encephalopathy; a similar approach in cattle could be of great value, both for the welfare of the animals themselves and for the peace of mind of the human subjects consuming their products.

A study of the welfare aspects of a project generating transgenic cattle for the production of human lactoferrin and lysozyme in milk was carried out by Van Reenen and Blokhuis (1997) in the Netherlands; in the 23 progeny of a transgenic bull, the incidence of anomalies was no greater than that found in 23 progeny sired by a non-transgenic bull. In a later report, Van Reenen *et al*. (2001) identified critical components of a scheme for evaluating the welfare of transgenic farm animals; they anticipated that systematic research into the welfare of farm animals involved in transgenesis will facilitate the use of the safest experimental protocols, which will enable progress in this field to be ethically justified.

Appendix A: Embryo Production Protocols

Numerous protocols have been described for the laboratory production of cattle embryos. The details provided in this appendix are based on the production systems currently followed in the Dublin *in vitro* fertilization (IVF) laboratory and the Guangxi University laboratory. The Dublin laboratory is arranged in three areas: (i) an ovary reception area, regarded as non-sterile; (ii) an area for the handling of gametes and embryos: and (iii) an area for ancillary activities, such as media preparation and embryo freezing. Both (ii) and (iii) are regarded as tissue-culture areas and fully aseptic precautions are taken when using them. Chemicals used in the various media described below have been obtained from the Sigma Chemical Company unless otherwise stated.

(I) University College Dublin Protocol

(Protocol by courtesy of Dr. Pat Lonergan)

1. Oocyte collection

Cumulus–oocyte complexes (COCs) are obtained by aspirating follicles from the ovaries of heifers/cows, either at slaughter or using ovum pick-up (OPU). Where ovaries are collected from slaughtered animals, they should be separated from the reproductive tract immediately after removal of the internal organs and returned to the laboratory within 3 h. The ovaries are transported in vacuum flasks containing presterilized phosphate-buffered saline (PBS) with antibiotic cover (penicillin/streptomycin; kanamycin) held at 30°C. An alternative is to use 0.9% sterile saline (9 g/l NaCl) as the ovary storage medium (with antibiotic cover).

Recovery of oocytes

Ovaries from slaughtered animals are washed twice in fresh, sterile PBS and dried lightly on sterile filter-paper. COCs are recovered from 2–8 mm antral follicles by aspiration using a sterile 18-gauge needle attached to a sterile 5 ml syringe. After aspiration, the contents of the syringe are slowly dispelled into a sterile test-tube (Vacutainer, Becton Dickinson) with minimum disruption of the COC. Once the last ovary of a particular batch is processed, the COCs are allowed a few minutes to settle to the bottom of the test-tube. The supernatant is then decanted and the remaining medium, containing the COCs and other follicular debris, is poured into a series of 60 mm sterile Petri dishes (Sterilin Ltd) for subsequent search. The recovery work is undertaken in a sterile environment in a Laminair flow cabinet with the room temperature held at 25°C.

Once located in the Petri dish, COCs are lifted in a sterile glass pipette (bore diameter 400 μm) and transferred into a dish of fresh medium. Oocyte quality is assessed on the basis of morphological appearance and the following criteria: (i) oocyte surrounded by at least four to five layers of cumulus cells; (ii) oocyte showing bright, ungranulated, even cytoplasm; and (iii) cumulus cells unexpanded, compact and even.

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2. *In vitro* maturation (IVM) procedure

COCs are washed four times in PBS supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamycin and 0.5 mg/ml bovine serum albumin (BSA) (Sigma, catalogue number A-9647). The maturation medium is tissue-culture medium 199 (TCM-199) supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma, F-2442), 10 ng/ml epidermal growth factor (Sigma, E-4127) and trace amounts of gentamycin (50 μg/ml). Groups of up to 50 COCs are transferred to four-well dishes (Nunc, Roskilde, Denmark) containing 500 μl of medium and are cultured for 24 h at 39°C under an atmosphere of 5% carbon dioxide in air at maximum humidity.

3. Sperm preparation

Frozen bull-semen straws are obtained from an appropriate source (cattle artificial insemination centre). Where possible, straws from several bulls are used; each male should have a proven field record of high fertility. Straws are thawed in a water-bath at 35–37°C for 30–40 s; each straw is wiped with 70% alcohol before being opened. Motile sperm are obtained by centrifugation of the semen on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (2.5 ml 45% (v/v) Percoll) over 2.5 ml 90% (v/v) Percoll) for 8 min at 700 *g* at room temperature. Viable sperm, collected at the bottom of the 90% fraction, are washed in HEPES-buffered Tyrode's and pelleted by centrifugation at 100 × *g* for 5 min. Sperm are counted in a haemocytometer and diluted in the appropriate volume to give a concentration of 2 × 10 million sperm/ml.

4. IVF procedure

The fertilization medium is Tyrode's medium with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate and 6 mg/ml fatty acid-free BSA; for capacitation and fertilization, 10 μl/ml heparin-sodium salt (184 units/mg heparin; Calbiochem, San Diego, California) are added. Matured COCs are washed in PBS and then in the fertilization medium before being transferred in groups of up to 50 into four-well Nunc dishes containing 250 μl of fertilization medium per well; a 250 μl aliquot of the sperm suspension is added to each well to give a final concentration of 1 million sperm/ml. The Nunc dishes are incubated for 24 h at 39°C under an atmosphere of 5% carbon dioxide in air at maximum humidity.

5. *In vitro* culture (IVC) of the early embryo

At 24 h after insemination (hpi), presumptive zygotes are denuded of cumulus cells by vortexing for 2 min in 2 ml of PBS. The zygotes are washed four times in PBS and once in the IVF medium before being transferred to 25 μl culture droplets. Culture of the embryo is carried out in a modified synthetic oviduct fluid (SOF) medium under mineral oil in a humidified atmosphere of 5% carbon dioxide, 5% oxygen and 90% nitrogen; after 24 h of culture (48 hpi), 10% (v/v) FCS is added to the droplets.

6. Assessing outcome of IVM/IVF/IVC procedures

Embryo cleavage is assessed at 48 hpi and blastocyst development recorded on days 6–8 (day 0 = day of insemination).

(II) Protocol Used in Guangxi University

(Protocol by courtesy of Professor Kehuan Lu)

1. Oocyte collection

Ovaries are collected on a daily basis from sexually mature heifers or cows slaughtered in a local abattoir and returned to the laboratory within 4 h of slaughter. Ovaries are removed within 30 min of animal slaughter without reference to stage of oestrous cycle or breed type. Appropriate records are kept of the source of the ovaries (breed type/age). Ovaries are transported to the laboratory in vacuum flasks containing presterilized PBS held at 25–30°C. An alternative is to use sterile NaCl (0.9%) or Ringer's solution as the ovary storage medium.

2. Oocyte recovery

In commencing processing, ovaries are washed twice in fresh, sterile PBS and dried lightly on sterile degreased gauze. Primary oocytes are recovered from 2–8 mm antral follicles by aspiration of the follicle containing the oocyte with an 18-gauge needle attached to a 10 ml syringe.

The medium used for oocyte recovery and washing is TCM-199 with Earle's salts, L-glutamine, 10 mM HEPES and 5 mM $NaHCO_3$, supplemented with trace amounts of antibiotic (60 μg/ml penicillin G and streptomycin sulphate) and 3% (v/v) steer serum (SS) or newborn calf serum (NBCS) heat-treated at 56°C for 30 min. The pH is adjusted to 7.3–7.4, using either HCl or NaOH, and the medium is sterilized by filtration through a 22 μm Millipore filter and warmed in an incubator without carbon dioxide with maximum humidity at a temperature of 38.5–39.0°C for at least 2 h prior to use.

Before commencing aspiration, the needle and syringe are first primed with approximately 0.5–1.0 ml of the aspiration medium; after aspiration, the contents of the syringe are slowly discharged into a sterile test-tube with minimum disruption of the COC. Once the last ovary of a particular batch is processed, the oocytes are allowed a few minutes to settle to the bottom of the test-tube. The supernatant is then decanted and the remaining medium, containing the COCs and other follicular debris, is poured into a series of 60 mm sterile glass dishes for subsequent searching. The recovery work is undertaken in a sterile environment in a laminar-flow cabinet with room temperature held at 25°C.

Once located in the glass dish, oocytes are lifted in a sterile glass pipette (bore diameter 400 μm) and transferred into a dish of fresh medium. Oocyte quality is assessed on the basis of morphological appearance and only oocytes exhibiting a compact cumulus investment and uniform ooplasm are selected for culture.

3. Harvesting granulosa cells

After removal of all oocytes from the glass dishes, the remaining aspiration medium containing granulosa cells and follicular debris is washed twice by centrifugation for 5 min at $500 \times g$. The final pellet of granulosa cells is suspended in the IVM medium and the resultant suspension is passed three times through an 18-gauge needle attached to a 1 ml syringe, to disperse the cells.

4. *In vitro* maturation of the primary oocyte

Selected oocytes (20–100 in 1 ml volume) are matured for 24 h in TCM-199 (with Earle's salts, L-glutamine, 26 mM $NaHCO_3$ and 5 mM HEPES) supplemented with 5% heat-inactivated SS or oestrous cow serum (OCS), 2% bovine follicular fluid (bFF), 0.1 μg/ml follicle-stimulating hormone (FSH) (Sigma) and trace amounts of antibiotic (60 μg penicillin G and 100 μg streptomycin sulphate. The pH is adjusted to 7.3–7.4 and the medium is sterilized by filtration through a 22 μm Millipore filter and equilibrated with 5% carbon dioxide in air for 2 h before use. The medium is contained in 25 mm glass dishes, the temperature held at 38.5°C and the medium exposed to 5% carbon dioxide in air atmosphere at maximum humidity.

5. *In vitro* capacitation of bovine sperm

Frozen bull semen is obtained from an appropriate source (cattle AI centre); to avoid variation due to individual bulls, three straws from each of three males are used for each 'swim-up'. Semen straws are thawed in a water-bath at 35–37°C for 30–40 s; approximately 0.25 ml of thawed pooled semen is layered under 1.5 ml of fertilization medium in each of two conical test-tubes. Each straw is wiped with 70% ethanol before being opened. The fertilization medium consists of modified Tyrode's medium (without lactate) supplemented with 50 μg/ml heparin (Sigma, H-3125 sodium salt), 2.5 mM caffeine and 0.6% BSA; the pH is adjusted to 7.4–7.6 prior to sterilization by filtration through a 22 μm Millipore filter. The capacitation medium is equilibrated in a humidified atmosphere of 5% carbon dioxide in air at a temperature of 38.5°C for at least 2 h before use.

After incubation for 30 min (tubes angled at 45°), the uppermost 0.5–0.8 ml of medium

containing the more motile sperm is removed from each tube and pooled in a sterile conical centrifuge tube; this sample is washed twice by centrifugation at $500 \times g$ for 5–10 min. The resultant pellet is measured using an adjustable micropipette, and the suspension diluted with fresh fertilization medium to give a final concentration of 50 million sperm per ml; the sperm suspension is passed three times through a micropipette to separate the sperm cells before insemination of the fertilization microdroplets is carried out.

6. *In vitro* fertilization procedures

Fertilization is carried out in 20 μl microdroplets of fertilization medium under sterile mineral oil (Sigma M 8410). After preparation of the fertilization microdroplets, the Petri dishes are placed in an incubator and allowed to equilibrate for at least 1 h at 38.5°C in a carbon dioxide in air gas atmosphere at maximum humidity. After maturation, oocytes are partially denuded of surrounding expanded cumulus cells (mechanical stripping in a micropipette). Before transfer to the fertilization microdroplets, oocytes are washed by passing through two successive dishes of fresh fertilization medium. Groups of ten to 15 oocytes are then arranged in each of the microdroplets before a volume of 2–3 μl of sperm suspension is added so as to achieve a final concentration of sperm in the microdroplet of 1–1.5 million per ml.

The Petri dish containing the microdroplets is then placed in an incubator at 38.5–39.0°C for 20–24 h, maintaining a gas phase of 5% carbon dioxide in air at high relative humidity. In performing IVF, strict aseptic precautions are observed throughout to avoid contamination of the medium by conducting all operations in a sterile atmosphere at 25°C.

7. *In vitro* culture of the early embryo

At 20–24 hpi, oocytes are removed from the fertilization microdroplets, treated by vortexing to remove their surrounding cumulus cells and washed by passing successively through two to three dishes of modified TCM-199, supplemented with 3% SS or OCS, before being placed on a granulosa-cell monolayer for further IVC. At 44–48 hpi, the cleavage of oocytes is recorded by examination under a stereoscope and blastocyst yield recorded after 6–8 days of culture.

8. Preparing the granulosa cell monolayer

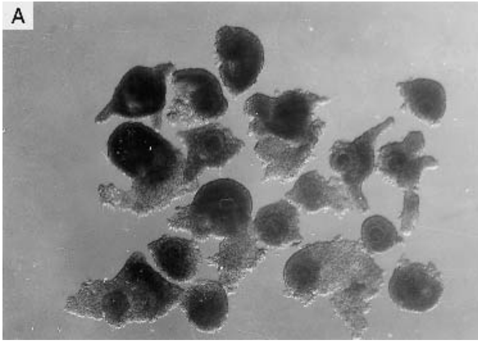
After removal of oocytes from the searching glass dishes (see step 3 above), the remaining aspiration medium, containing granulosa cells and cellular debris, is washed twice by centrifugation for 5 min at $500 \times g$. The final pellet of granulosa cells is then suspended in modified TCM-199 supplemented with 3% SS or OCS and trace amounts of antibiotics (penicillin G and streptomycin sulphate) until the appropriate cell concentration (1.5 million per ml) is achieved; the resultant suspension is passed several times through an 18-gauge needle to disperse the granulosa cells.

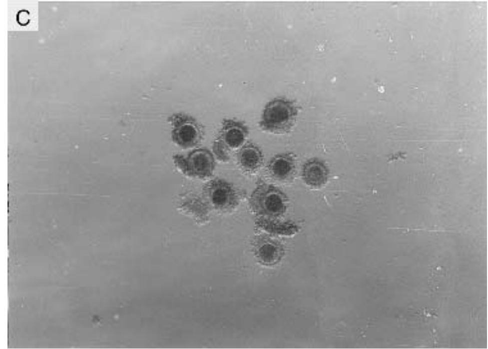
Droplets of 30 μl volume of this dilute suspension are then placed in a 60 mm tissue culture dish and covered with sterile mineral oil. After 72 h of culture, a confluent monolayer of cells should have formed at the base of the droplet. Before transferring embryos to the monolayer at 20–24 hpi, the medium is replenished by removing 20 μl and replacing with 20 μl of fresh culture medium. This ‘cleaning’ process is subsequently carried out every 48 h until the end of the culture period; the zygotes are placed in the droplets of IVC medium in groups of 20–50 and cultured at 38.5°C in a gas phase of 5% carbon dioxide in air at maximum humidity.

(III) Bovine Oocyte and Embryo Features

Acceptable and non-acceptable primary bovine oocytes for IVM are featured in Fig. A1 and Fig. A2, respectively. Photographs illustrating bovine oocytes before and after IVM are shown in Fig. A3.

Photographs illustrating stages in the development of the bovine embryo from the late





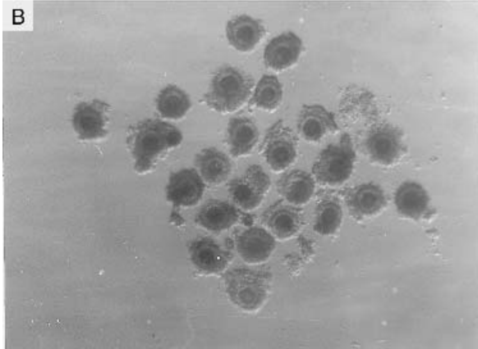
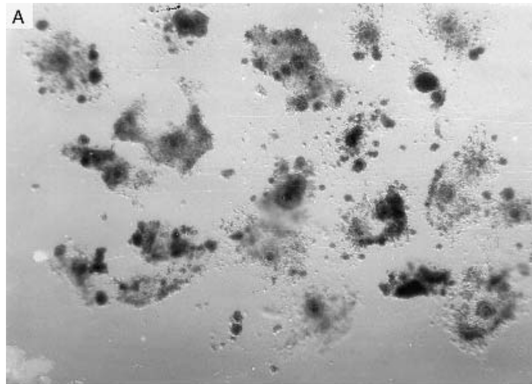


Fig. A1. Acceptable-quality bovine primary oocytes: (A) oocytes showing many tight layers of cumulus cells; (B) oocytes showing three to four cumulus cell layers; (C) oocytes showing two or three cell layers.



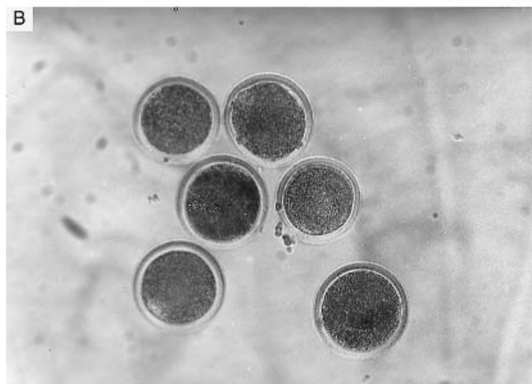


Fig. A2. Unacceptable-quality bovine primary oocytes: (A) bovine oocytes with expanded cumulus cells scattered in dark clumps in a jelly-like matrix; (B) denuded oocytes.

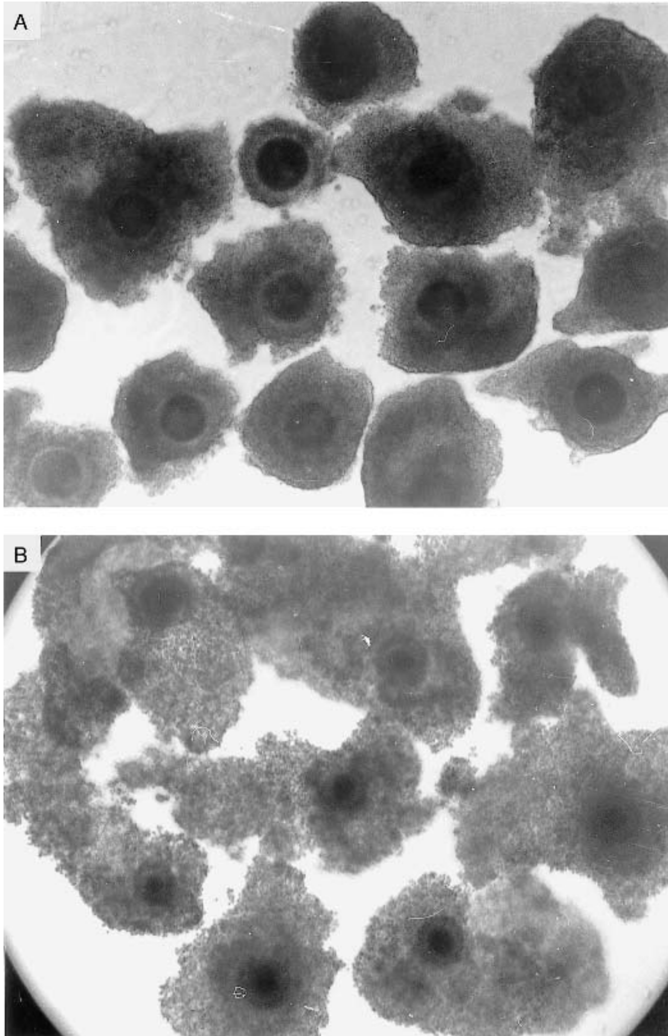


Fig. A3. Before and after *in vitro* maturation of the bovine oocyte: (A) bovine primary oocytes showing many layers of tightly packed cumulus cells; (B) bovine secondary oocytes showing expanded cumulus cells.

morula to the expanded blastocyst stage are shown in Fig. A4. The changes in the dimensions of embryos as they progress from morula to expanded blastocyst are illustrated in Fig. A5; by the time the blastocyst is fully expanded, the diameter has increased by 50% or so.

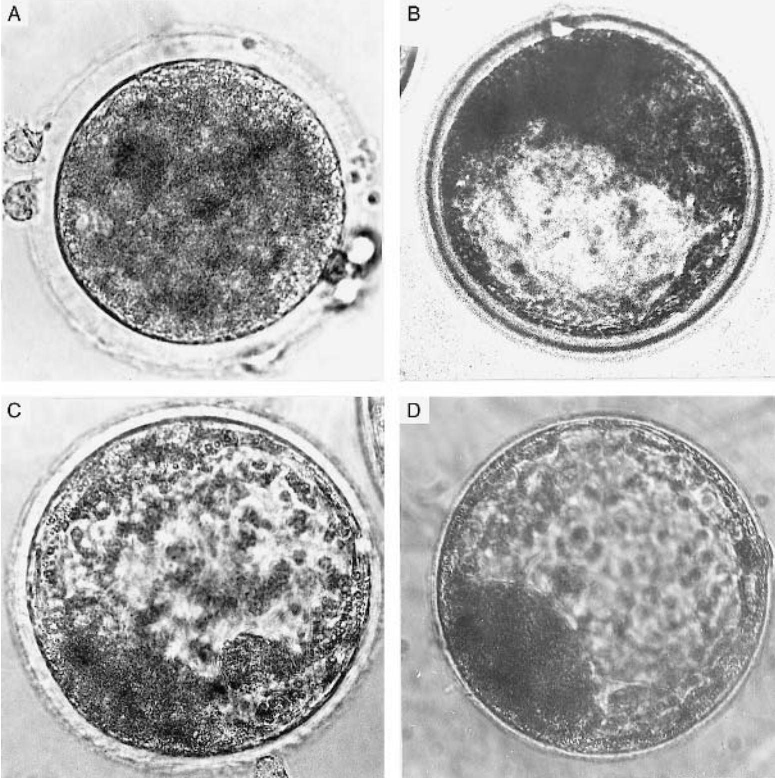


Fig. A4. Stages in the progress of the bovine blastocyst. A late morula/early blastocyst is shown in (A) with a blastocoel just starting to form; in (B) this day-7 blastocyst is showing a well-defined blastocoel; in (C) the blastocyst has reached the expanding stage, with noticeable thinning of the zona pellucida; in (D) the blastocyst continues to expand, with the zona becoming still thinner.

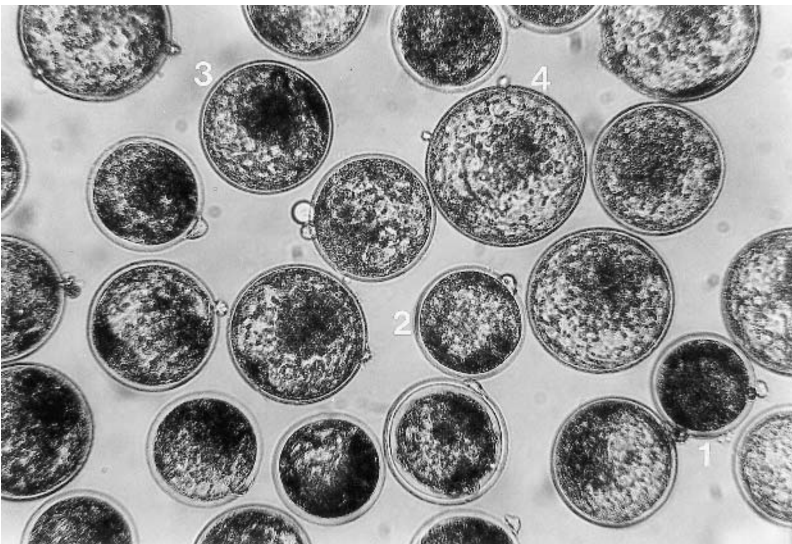


Fig. A5. From bovine morula to expanded blastocyst: (1) morula; (2) non-expanded blastocyst; (3) expanding blastocyst; (4) expanded blastocyst.

Appendix B: Preparation of Culture Media

Table B1. Preparation of phosphate-buffered saline (PBS).

Solution A		
Ingredient	mM	g/l (10 ×)
NaCl	136.89	80.0
KCl	2.68	2.0
$Na_2HPO_4{\cdot}2H_2O$	8.09	29.0
or		
Anhydrous Na_2HPO_4	8.09	11.5
KH_2PO_4	1.47	2.0
Solution B		
Ingredient	mM	g per 100 ml (10 ×)
$CaCl_2{\cdot}2H_2O$	0.457	0.67
or		
$CaCl_2{\cdot}6H_2O$	0.457	1.0
$MgCl_2{\cdot}6H_2O$	0.492	1.0

Sterilize solutions A and B separately and then take 100 ml of A and 10 ml of B and 890 ml of H_2O to make 1000 ml of PBS.

Table B2. Preparation of oocyte washing medium (modified HEPES-buffered Tyrode's medium).

Ingredient	mM	mg per 100 ml
NaCl	114.0	666.0
KCl	3.2	23.8
$NaHCO_3$	2.0	16.8
$NaH_2PO_4{\cdot}2H_2O$	0.4	6.2
Na lactate	10.0	112.1
$MgCl_2{\cdot}6H_2O$	0.5	10.0
$CaCl_2{\cdot}2H_2O$	2.0	29.4
HEPES	10.0	240.0
Phenol red		1.0
Sodium pyruvate	0.5	5.5
Bovine serum albumin		300.0
Glucose	5.6	100.0
pH		7.4
mosmol		270–290

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Table B3. Preparation of sperm capacitation medium (modified Ca^{2+}-free Tyrode's medium).

Ingredient	mM	mg per 100 ml
NaCl	112.0	654.5
KCl	2.7	20.0
$NaHCO_3$	25.1	210.0
$NaH_2PO_4 \cdot 2H_2O$	0.4	6.2
Na lactate	10.0	112.1
$MgCl_2 \cdot 6H_2O$	0.5	10.0
HEPES	5.0	120.0
Sodium pyruvate	1.0	11.0
Bovine serum albumin		600.0
Glucose	13.9	250.0
pH		7.4
mosmol		290–310

Table B4. Preparation of fertilization medium (modified Tyrode's medium).

Ingredient	mM	mg per 100 ml
NaCl	114.0	666.0
KCl	3.2	73.8
$NaCO_3$	25.0	209.0
$NaH_2PO_4 \cdot 2H_2O$	0.4	6.2
Na lactate	10.0	112.1
$MgCl \cdot 6H_2O$	0.5	10.0
$CaCl_2 \cdot 2H_2O$	2.0	29.4
HEPES	10.0	240.0
Phenol red		1.0
Sodium pyruvate	0.5	5.5
Bovine serum albumin		600.0
Glucose	5.6	100.0
pH		7.8
mosmol		290–310

Table B5. Preparation of motility-stimulating mixture: penicillamine–hypotaurine–epinephrine (adrenalin) (PHE).

MUST be prepared in low light

Solution A

- Step 1: Measure 200 ml of saline in beaker
- Step 2: (a) 0.2003 g sodium metabisulphite ($Na_2S_2O_5$)
  (b) 0.6562 g Na lactate (CH_3.CHOH.COONa, 70% w/w)
- Step 3: Add them into 200 ml of saline and measure pH 4.004 at room temperature 21.3 °C

Solution B

Separately prepared before mixing with solution A

- Step 4: Add 0.011 g hypotaurine (Sigma, H-1384, lot 69F7800) in 100 ml saline
- Step 5: Add 0.0301 g penicillamine (Sigma, N-55F-0634) in 100 ml saline
- Step 6: Add 0.0185 g epinephrine (adrenalin) (Sigma, E-4125, lot 105F7702) into 10 ml of solution A (low density of light)
- Step 7: Put 1 ml of step 6 solution into 39 ml of solution A
- Step 8: Put in one container and add:
  32 ml saline
  16 ml of epinephrine (adrenalin) stock (step 7 solution)
  20 ml of penicillamine stock
  20 ml of hypotaurine stock
- Step 9: Make aliquots in small vials and freeze at –20 °

Table B6. Procedures for fixation and staining of oocytes and embryos.

Fixative: acetoethanol

1. Mix one part of acetic acid into three parts of ethanol (1 : 3 v/v mixture)
2. Store mixture in sealed container at 5 °C

Stain

0.5% Acetolacmoid stain

Acetic acid	45.00 ml
Lacmoid	0.50 g
Distilled water	55.00 ml

Aceto-orcein stain

Purchased from BDH company

Filter both stains before use (0.22 μm)

Acetoglycerol decolorizer

Acetic acid	20.00 ml (1 part)
Glycerol	20.00 ml (1 part)
Distilled water	60.00 ml (3 parts)

Preparation of vaseline : paraffin-wax mixture

1. Weigh out 2 g paraffin wax and 40 g vaseline (1 : 10, w/w) into a 100 ml beaker
2. Heat the beaker to melt contents and stir gently with a glass rod to mix the mixture
3. When the mixture has melted and mixed completely, put into a 5 or 10 ml syringe
4. Cool the syringe to 4 °C
5. Attach a blunt 18-gauge needle before use

Table B7. Salt concentrations in CR1 and CR2 media.

	Medium	
Salt (mM)	CR-1	CR-2
Sodium chloride	114.7	109.5
Potassium chloride	3.1	3.1
Calcium chloride	–	2.5
Magnesium sulphate	–	0.5
Sodium bicarbonate	26.2	26.2
EDTA	–	0.01

CR, Charles Rosenkrans; EDTA, ethylenediamine tetra-acetic acid.

Appendix C: Cryopreservation Procedures

Embryo Freezing

Glycerol and conventional slow freezing

For acceptance, blastocysts should be day-7 and of the highest grade (grade 1). The embryos are equilibrated in a freezing solution consisting of 10% (v/v) (1.4 M) glycerol in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS). The blastocysts are loaded into 0.25 ml straws (IMV, L'Aigle, France) and placed in a programmable freezer precooled to −6°C; after 3 min, seeding is induced. After a further 12 min at −6°C, straws are cooled at −0.6°C/min to −32.5°C, after which they are plunged into liquid nitrogen and stored.

For thawing, embryos are held in air for 10 s and then in water at 35°C until the ice in the straw has melted. Embryos are recovered from the straws and moved sequentially through three dishes containing: (i) 0.94 M glycerol + 0.3 M sucrose; (ii) 0.47 M glycerol + 0.3 M sucrose; and (iii) 0.3 M sucrose, spending 5 min in each solution. The embryos are then washed three times in PBS + 10% FCS. They are then loaded individually into straws for non-surgical transfer.

Ethylene glycol and direct transfer

The blastocysts are equilibrated for 10 min in a freezing solution consisting of 1.8 M ethylene glycol (EG) in PBS supplemented with 10% FCS at 25°C. They are then loaded into the mid-portion of a 0.25 ml straw in a small volume of the freezing medium, separated by air bubbles on each side from larger volumes of the same medium and cooled to −7°C at 1°C per min. At −7°C, the freezing straw is seeded, held at 10 min at that temperature and cooled at 0.3°C per min to −30°C; the straw is then plunged directly into liquid nitrogen for storage.

For thawing, the plastic straw is held for 6 s before being plunged into a water-bath held at 35°C; it is then loaded into an appropriate cattle embryo transfer instrument (IMV, L' Aigle, France) and transferred immediately. Prior to transfer, the recipient animal receives epidural anaesthesia (lignocaine) and an appropriate tranquillizer (e.g. Buscopan, Boehringer, Ingelheim) for its comfort during the transfer procedure. The ovary containing the corpus luteum is identified and the embryo deposited in the mid-horn of the associated (ipsilateral) uterine horn. In the transfer procedure, care is taken at all times to maintain sterility in the handling and manipulation of the straw. In cold weather, precautions are necessary to ensure that transfer instruments and straws are not exposed to temperatures below 25°C up to the time when the transfer is made.

Vitrification Using the Open Pulled Straw (OPS) Method

Blastocysts for vitrification are first equilibrated in tissue-culture medium 199

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(TCM-199) + 10% FCS (holding medium (HM)) with 10% EG and 10% dimethyl sulphoxide (DMSO) for 2 min at 25°C and then secondly 20% EG, 20% DMSO and 0.6 M sucrose in HM for 30 s at 4°C. Embryos are vitrified in a volume of less than 1 μl in an open pulled straw.

Monitoring Pregnancy in Recipients

In the absence of information about a 'repeat' oestrus, the recipient's uterus is checked by suitably experienced operatives using appropriate real-time ultrasonics at day 35.

Appendix D: Journals, Books and On-line Sources of Information Relevant to the *in Vitro* Production and Transfer of Cattle Embryos

Journals

AgBiotech News and Information. CAB International, Wallingford, UK.

American Journal of Veterinary Research. American Veterinary Medical Association, Schaumburg, USA.

Animal Breeding Abstracts. CAB International, Wallingford, UK.

Animal Reproduction Science. Elsevier Science BV, Amsterdam, the Netherlands.

Animal Science. British Society of Animal Science, Penicuik, UK.

Biology of Reproduction. Society for the Study of Reproduction, 309 West Clark Street, Champaign, Illinois 61820, USA.

British Veterinary Journal. Baillière Tindall, 33 Redmond Square, London WC1R 4SG, UK.

Canadian Journal of Animal Science. Agricultural Institute of Canada, Ottawa, Canada.

Cloning and Stem Cells. Mary Ann Liebert Inc., Larchmont, USA.

Embryo Transfer Newsletter. International Embryo Transfer Society, 309 West Clark Street, Champaign, Illinois 61820, USA.

Fertility and Sterility. Elsevier Science Inc., New York, USA.

Human Fertility. Journal of Reproduction and Fertility, 22 Newmarket Road, Cambridge CB5 8DT, UK.

Human Reproduction. European Society of Human Reproduction and Embryology, Oxford University Press, Oxford, UK.

Human Reproduction Update. European Society of Human Reproduction and Embryology, Oxford University Press, Oxford, UK.

International Journal of Fertility. Ben Franklin Press, Pittsfield, Massachusetts, USA.

Irish Veterinary Journal. Irish Veterinary Association, Dublin, Irish Republic.

Journal of Agricultural Science (Cambridge). Cambridge University Press, Cambridge, UK.

Journal of the American Veterinary Medical Association. 600 S. Michigan Avenue, Chicago, Illinois, USA.

Journal of Animal Science. American Society of Animal Science, 309 West Clark Street, Champaign, Illinois 61820, USA.

Journal of Assisted Reproduction and Genetics. Kluwer Academic/Plenum Publishers, New York, USA.

Journal of Dairy Science. American Dairy Science Association, 309 West Clark Street, Champaign, Illinois 61820, USA.

Journal of Reproduction and Development. Japanese Society of Animal Reproduction, Tokyo, Japan.

Livestock Production Science. Elsevier Science BV, Amsterdam, the Netherlands.

Molecular Human Reproduction. European Society of Human Reproduction and Embryology, Oxford University Press, Oxford, UK.

Molecular Reproduction and Development. John Wiley & Sons, Inc., 1 Wiley Drive, Somerset, New Jersey 08875, USA.

Nature. Nature Publishing Group, London, UK.

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Nature Biotechnology. Nature Publishing Group, London, UK.

Reproduction. Journal of Reproduction and Fertility Ltd, 22 Newmarket Road, Cambridge CD5 8DT, UK.

Reproduction in Domestic Animals. Blackwell Wissenschafts-Verlag GmbH, Berlin, Germany.

Reproduction, Nutrition, Development. Institut National de la Recherche Agronomique, Paris, France.

Science. American Association for the Advancement of Science, Washington, USA.

Theriogenology. Elsevier Science, The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK.

Veterinary Record. British Veterinary Association, 7 Mansfield Street, London, UK.

Zygote. Cambridge University Press, Cambridge, UK.

On-line Journals

Reproductive Biology and Endocrinology. rbe@utk.edu

Books

Clark, A.J. (ed.) (1998) *Animal Breeding: Technology for the 21st Century*. Harwood Academic Publishers, Amsterdam, Netherlands.

Elder, K. and Dale, B. (2000) *In Vitro Fertilization*. Cambridge University Press, Cambridge, UK.

Fields, M.J., Sand, R.S. and Yelich, J.V. (eds) (2002) *Factors affecting Calf Crop: Biotechnology of Reproduction*. CRC Press, Boca Raton, Florida, USA.

Foote, R.H. (1999) *Artificial Insemination to Cloning: Tracing 50 Years of Research*. Cornell University, Ithaca, New York State, USA.

Hafez, E.S.E. and Hafez, B. (eds) (2000) *Reproduction in Farm Animals*, 7th edn. Lippincott Williams & Wilkins, Baltimore, Maryland, USA.

Holland, A. and Johnson, A.J. (eds) (1998) *Animal Biotechnology and Ethics*. Chapman & Hall, London, UK.

Knobil, E. and Neill, J. (eds) (1994) *The Physiology of Reproduction*. Raven Press, New York, USA.

Lauria, A., Gandolfi, F., Enne, G. and Gianaroli, L. (eds) (1998) *Gametes: Development and Function*. Portland Press, London, UK.

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Renaville, R. and Burny, A. (eds) (2001) *Biotechnology in Animal Husbandry*. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Stringfellow, D.A. and Seidel, S.M. (eds) (1998) *Manual of the International Embryo Transfer Society*, 3rd edn. International Embryo Transfer Society, Savoy, Illinois, USA.

Trounson, A. and Gardner, D.K. (eds) (2000) *Handbook of In Vitro Fertilization* 2nd edn. CRC Press, Boca Raton, Florida, USA.

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Abdoon, A.S.S. and Kandil, O.M. (2001a) Factors affecting number of surface ovarian follicles and oocytes yield and quality in Egyptian buffaloes. *Reproduction, Nutrition, Development* 41, 71–77.

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