A review of in vitro gamete maturation and embryo culture and potential impact on future animal biotechnology

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Abstract

This review considers the relationship of in vitro gamete maturation and embryo culture to the future development of animal biotechnology. The areas reviewed are oocyte maturation in vitro and embryo culture and their importance for successful in vitro embryo production. The rapidly developing area of spermatogonial cell transplantation and culture is also reviewed. The scientific milestones leading to the development of each area, the problems and prospects for future development and the possible significance of major advances in each area are discussed.

Keywords: Oocyte maturation; In vitro fertilization; Embryo culture; Spermatogonial culture; Spermatogonial transplantation; Biotechnology

1. Introduction

The 21st century seems set to see a revolution in the application of biotechnological procedures to farm animals. This revolution has been heralded by the production of transgenic mice by Brinster et al. (1982), followed rapidly by the production of transgenic cattle, sheep and pigs (Wall et al., 1992; Ebert and Schindler, 1993), the development of practicable sperm sexing in cattle (Seidel et al., 1997, 1999; Seidel and Garner, 2002) and the dramatic production of the sheep, Dolly, cloned from a somatic cell by Campbell et al. (1996) and Wilmut et al. (1997) with all the possibilities that offers for the production of genetically modified animals (Cibelli et al., 1998; Park et al., 2002). However, the successful development and application of this and related technologies is critically dependent on a whole
range of basic reproductive technologies such as in vitro maturation of oocytes, in vitro fertilization, in vitro culture of embryos and in vitro culture of sperm precursors. Without significant improvements in these reproductive technologies, application of developments in cloning and the production of transgenic farm animals will remain limited and extremely costly. This chapter examines the problems and limitations of these technologies and how substantial progress in overcoming these limitations might change the face of reproductive biotechnology. In order to provide a context for the discussion, this chapter also reviews very briefly the significant discoveries or milestones along the way to the current state of our knowledge in these areas.

2. In vitro production of embryos

In the provision of in vitro produced (IVP) embryos, the processes of in vitro maturation of oocytes (IVM) and of in vitro fertilization (IVF) are almost inextricably linked and in this review the two processes will be considered together.

2.1. Milestones in IVM and IVF

Investigators of mammalian oogenesis usually refer to oocyte maturation as the set of processes occurring from the germinal vesicle stage to completion of the second meiotic division with the formation of the first polar body (McGaughey, 1983). Milestones in the study of in vitro maturation of oocytes include the discovery by Pincus and Enzmann in 1935 that release of immature rabbit oocytes from the inhibitory influence of the follicle allowed them to resume maturation in culture (Pincus and Enzmann, 1935), and the demonstration by Minato and Toyoda in Japan in 1983 and by Schroeder and Eppig in 1984 in the USA that in vitro matured mouse oocytes fertilized in vitro could result in live offspring (Schroeder and Eppig, 1984). Other milestones include the finding that 39°C was the optimal temperature for maturation of cattle oocytes (Lenz et al., 1983), the birth of live calves from in vitro matured and fertilized cattle oocytes (Lu et al., 1988) and the birth of a live mouse pup after complete development from primordial oocyte to in vitro fertilized embryo and transfer to a host mother (Eppig and O’Brien, 1996). This last work, which was carried out in Eppig’s laboratory, involved a two step procedure for maturation of the oocytes in which the ovaries of new born mice were first cultured for a number of days, the ovaries then dissociated with enzyme, the oocyte–granulosa cell complexes matured in vitro, fertilized and cultured in vitro to the 2-cell and blastocyst stages. The live pup was born after transfer of a 2-cell embryo to the oviduct.

Milestones in IVF include the discovery of the need for sperm capacitation in rabbits by Austin (1951) and Chang (1951), the definitive proof of in vitro fertilization in rabbits by Chang (1959), the birth of the first human in vitro fertilized baby in 1978 as a result of the work of Edwards, Steptoe and co-workers (Steptoe, 1980), the birth of the first in vitro fertilized calf in Brackett’s laboratory (Brackett et al., 1982), the discovery that 39°C was the optimal temperature for bovine IVF (Lenz et al., 1983) and the development of improved methods for in vitro capacitation of bull sperm such as the use of heparin (Parrish et al., 1986).
2.2. Problems and prospects in the production of IVP embryos

Production of IVP embryos usually involves the collection of immature oocytes by puncture of follicles followed by oocyte aspiration, IVM and IVF and some period of embryo culture. Originally much of this work was done with slaughterhouse ovaries and this is still the case for much research work. Currently, however, one of the major commercial methods of IVP embryo production involves the use of ovum pick up procedures guided by ultrasound to collect oocytes from both fertile and infertile genetically valuable cows. Such collections can be carried out on a weekly basis for periods of, in some cases, over a year with the number of usable oocytes per collection being about four to five (Hasler, 1962; Hasler et al., 1995; Galli et al., 2001). One problem that shows up with in vitro matured oocytes, whether they are collected using ovum pick up procedures or from slaughterhouse material, is that, while a high percentage may cleave after IVF, a much lower percentage usually develop to blastocysts. This is less of a problem in the case of oocytes that are surrounded by four or more cumulus cell layers, suggesting that the problem is mainly one of follicle maturity. For a recent review on the influence of follicle development on the in vitro competence of oocytes see Hendriksen et al. (2000).

However, a central problem with the use of non-ovulated immature oocytes collected from the ovary is not only their degree of maturity but also the fact that many oocytes in the ovary are undergoing a process of programmed cell death or apoptosis of the oocyte or follicle leading to atresia (Hsueh et al., 1994). Knowledge of apoptosis in oocytes and follicles and particularly on how early follicle apoptosis affects oocyte viability is seriously lacking. Presumably apoptosis begins in the follicle and then affects the oocyte. There is some evidence that even good quality cumulus oocyte complexes isolated from bovine follicles with a high degree of apoptosis in the follicle wall lose their developmental capacity (Jewgenow et al., 1999). On the other hand, it has been suggested recently that a limited degree of atresia or apoptosis in the cumulus may be associated with improved oocyte competence (Hendriksen et al., 2000). Basic information on the control of apoptosis in the ovary and in cells and tissues generally is beginning to emerge (for reviews see Hsueh and Hsu, 1997; Kaipia and Hsueh, 1997; Martinmeau and Tilly, 1997; Driancourt and Thuel, 1998; Morita and Tilly, 1999; Hsu and Hsueh, 2000; Markström et al., 2002). The application of this knowledge may eventually allow us to detect apoptotic oocytes by using a simple vital stain and, in cases of incipient apoptosis, allow us to reverse the process. For instance there is evidence in some cells that activation of protein kinase C inhibits apoptosis (Zhuang et al., 1998) and it is possible that activation of protein kinase C may be used to inhibit incipient apoptosis in oocytes and thus improve rates of oocyte maturation in vitro.

Studies on apoptosis do not constitute the only approach to improving oocyte maturation and therefore IVP embryo production. There is a considerable amount of information now available on the role of various signal transduction systems in oocyte maturation and activation—see reviews as follows: cAMP (Tornell et al., 1991; Conti et al., 1998), calcium (Homa, 1995), inositol trisphosphate (Carroll et al., 1996; Stricker, 1999) and PtdIns(3,4,5)P3 (Anas et al., 1998; Carnero and Lacal, 1998). There is also a vast body of information on the molecular control of the cell cycle particularly on the role of cyclin-dependent kinases, much of it based on work with oocytes (for reviews see Parrish et al., 1992; Dekel, 1996; Whitaker, 1996; Sagata, 1997; Taieb et al., 1997; Winston, 2001). The
application of this basic information on apoptosis, signal transduction and cell cycle regulation has the potential to lead to the development of better systems for in vitro oocyte maturation in farm animals.

Another developing research area with the potential for improving in vitro maturation of oocytes is that of follicular fluid meiosis-activating sterols (Byskov et al., 1995). While as yet there has been little or no investigation of the role of these compounds in the oocytes of farm animals, the meiosis-inducing properties of these compounds have been studied extensively in mouse, rat and human; there is evidence that they may have a role in mediating the action of the gonadotrophic surge on oocyte maturation (reviewed by Byskov et al., 2002; Tsafiri et al., 2002).

The work on in vitro grown or IVG oocytes by Eppig and O’Brien (1996) in which complete development takes place in vitro in oocytes from primordial follicles of newborn mice has enormous potential if it could be applied to domestic animals. However, as yet, even in mice, the success rate is extremely limited; out of 190 2-cell-stage embryos developed from IVG oocytes transferred to host mothers, two females produced one pup each, one of which lived. Thus there is a considerable amount of work to be done on this system in order to achieve an acceptable success rate (reviewed by Smitza and Cortvrindt, 2002). It has been reported (Wu et al., 2001) that oocytes from in vitro-grown pig preantral follicles can acquire meiotic competence and undergo fertilization and in vitro culture to blastocysts.

In looking at the problems of IVP embryo production in farm animals, it is well nigh impossible to disentangle the role that poor conditions of oocyte maturation, in vitro fertilization or embryo culture may play in the failure of embryos to develop to viable blastocysts capable of implantation and development to live offspring. For most species of farm animals the most important problems probably relate to conditions of oocyte maturation and embryo culture rather than fertilization. A problem associated with IVP embryos which manifests itself much later is that of abnormal fetal and neonatal development and growth, known as large calf or large offspring syndrome; this is discussed later in Section 3.2.

2.3. Biotechnological implications of the development of successful reliable systems for IVP embryo production

The immediate effect of improvements in IVP cattle embryo production is likely to be an increased supply of embryos from high genetic merit cows using oocytes obtained by ovum pick up procedures. Improved and more efficient procedures for the supply of these embryos would widen the use of such techniques in genetic improvement.

A second effect might be to facilitate the production of twins in beef cattle using IVP embryos. Either two embryos could be transferred non-surgically to the uterus of a synchronized recipient or, as suggested by Gordon, the recipient could be bred to AI in the usual way and later receive an IVP embryo by embryo transfer (Gordon and Boland, 1978; Gordon and Lu, 1990). At one time, there was considerable research interest in such an approach to twinning using IVP embryos from slaughterhouse material. However, interest in this approach has declined and this decline has probably been accelerated by fears about disease transmission from slaughterhouse material. However, if the techniques for IVG oocytes developed by Eppig’s group in the mouse could be adapted and improved for
cattle oocytes to allow the production of thousands of fertilizable oocytes from fetal calf ovaries, this would enormously improve the IVP embryo supply situation and could change the attitude to twinning in beef cattle.

A spin off from improvements to the in vitro maturation part of the IVP embryo production procedure would probably be improvement in the production of suitable oocytes for use in embryo cloning procedures.

Progeny testing of female farm animals as compared with male farm animals has always been drastically hindered by the small number of offspring produced by one female. The repeated use of ovum pick up procedures in the same animal has to a limited extent overcome this problem. However, the development of successful procedures for the production of thousands of viable IVG oocytes from a single fetal calf ovary would dramatically change the whole area of female progeny testing in cattle. IVP embryos produced from IVG oocytes could be stored frozen while embryos from the same cohort were being transferred to recipients for progeny testing. Because the semen from one bull can fertilize a huge number of oocytes, it should be possible to improve the efficiency of the progeny testing procedure by using semen from a limited number of carefully selected bulls to fertilize the IVG oocytes.

Extension of successful IVP embryo production techniques could have major importance in the preservation of endangered species. A caveat here is that the usage of animals in the research and development of these techniques should not of itself increase the danger of species extinction before the techniques could be put to beneficial effect.

The availability of large numbers of normal IVP embryos would be a very useful research tool for researchers wishing to study early development. The morula and blastocyst stages of development are characterized by the first development of cell junctions, the initiation of tissue differentiation with the formation of a functioning transporting epithelium, the trophectoderm, and by the start of true embryonic growth. All of these processes are of fundamental biological interest and, while they can readily be studied in mouse embryos, it is important to have at least one other animal model such as the IVP cattle embryo in which these processes can be studied.

3. In vitro culture of embryos

3.1. Milestones in embryo culture

Milestones in the development of in vitro culture of embryos include the first attempt at mammalian embryo culture by Brachet (1912) using coagulated blood plasma to culture 5–7 day rabbit blastocysts in glass dishes, the cinematographic studies of Lewis and Gregory (1929) on cleaving rabbit embryos, and the discovery by Hammond in 1949 that 8-cell mouse embryos could be cultured to blastocysts in a simple salt solution supplemented with hen egg white and yolk (Hammond, 1949). This was followed by the absolutely crucial discovery of Whitten in 1957 that the addition of lactate as an energy source allowed culture of 2-cell mouse embryos to blastocysts in a simple Krebs Ringer bicarbonate solution supplemented with serum albumin (Whitten, 1957), and the demonstration by McLaren and Biggers (1958) that embryos cultured to the blastocyst stage in this simple medium developed to live young after transfer to host mothers. This discovery of Whitten’s, which was subsequently
extended by the work of Brinster, Biggers, Whitten and their collaborators, laid the basis for the subsequent huge expansion of research into preimplantation development in the mouse and other species and without this discovery embryo biotechnology would not exist.

Later milestones in embryo culture include the finding in Foote’s laboratory that, unlike the mouse, amino acids were essential for blastocyst formation in the rabbit (Kane and Foote, 1970), and the cleavage (Edwards et al., 1970) and development to blastocysts of in vitro fertilized human embryos (Steptoe et al., 1971). A major milestone for farm animal embryo culture was the discovery in First’s laboratory in 1983 of the importance of using a temperature equal to the animal’s core body temperature (about 39 °C for cattle) for in vitro maturation and fertilization of cattle oocytes (Lenz et al., 1983); this led to the later realization that the same temperature should be used for sheep (Gandolfi and Moor, 1987; Fukui et al., 1988) and cattle embryo culture (Fukui and Ono, 1988; Fukuda et al., 1990). A further very significant factor in the development of embryo culture in farm animals was the finding that embryos tended to develop better in gas phases with oxygen concentrations lower than 20%, especially when cultured in the absence of somatic cell support (Tervit et al., 1972; Fukui et al., 1991).

3.2. Problems and prospects in embryo culture

The preimplantation embryos of cattle, sheep and pigs differ radically from those of the mouse and most other laboratory species with respect to the degree of growth that takes place before implantation. The 1-cell mouse embryo contains about 20 ng of protein and the blastocyst just before implantation also has only about 20 ng (Brinster, 1967; Schiffner and Spielmann, 1976); thus, true growth before implantation is minimal or non-existent. In contrast, while the 2-cell cattle embryo has about 132 ng of protein and the day 8 early blastocyst has a protein content of only about 185 ng, the day 16 elongated blastocyst (depending on size) can have a protein content of more than 900 μg (Grealy et al., 1996)

There is a similar picture in the pig (Wright et al., 1981, 1983). This difference in the degree of preimplantation growth has major consequences for embryo culture; it explains why it is possible to culture mouse embryos over a period of about 3–4 days from 1-cell to blastocyst in a simple medium devoid of amino acids, vitamins or trace elements; it also partly explains why it is difficult to obtain good blastocyst development of the embryos of farm animals in vitro.

The contrast between the limited growth and small increase in protein content up to the early blastocyst stage of cattle, pig and sheep embryos and the huge increase in growth and protein content after blastocyst hatching, results in markedly different types of culture problems for early cleavage and blastocyst stages. In general, culture from 1-cell to early blastocyst stages is possible even if success rates are much less than 100%. Nowadays, almost all culture of cattle embryos is carried out using IVP embryos; good success rates with these embryos seem to lie in the range of 30–45% of matured oocytes reaching the blastocyst stage following IVF and embryo culture with pregnancy rates of 40–60% following embryo transfer (Hasler, 1962; Hasler et al., 1995). Results depend to some extent on the degree of oocyte selection (Hawk and Wall, 1994). In contrast to the situation for embryo cleavage and early blastocyst formation, there appear to be no reports of in vitro culture of cattle early blastocysts to elongated blastocysts. However, in vivo culture of IVP early cattle blastocysts
to expanded and hatching blastocysts in the sheep oviduct followed by culture to elongated blastocysts in the cow uterus is possible (Galli et al., 1997).

Predicting how improvements in the in vitro culture of embryos of cattle and other farm animals might be made is difficult. Current techniques of farm animal embryo culture from 1-cell to early blastocysts seem to fall into one or other of two categories. The first category involves a co-culture system using a complex tissue culture medium with various cell types such as oviducal epithelial cells, granulosa cells and even cells of a tissue culture cell line such as BRL cells, e.g. Reed et al. (1996). Much effort at improving these media has been concentrated on the type of co-culture cells. The second category uses a cell-free system based very often on a simple medium such as synthetic oviduct fluid (SOF) supplemented to varying degrees with amino acids, vitamins, serum and other components (Tervit et al., 1972; Holm et al., 1999). In recent years co-culture systems appear to have been largely abandoned by commercial operators and replaced by culture in SOF-BSA-AA under a low oxygen concentration (Galli et al., 2001); this emphasizes the importance of amino acid supplementation of the medium. Efforts to improve such media consist mainly of varying concentrations of the standard medium constituents and trying out new constituents, so far with only limited success. For instance, two nutrients, inositol and citrate, first shown by our laboratory (Kane, 1988, 1989; Gray et al., 1992) to be essential for rabbit blastocyst growth, appear to improve cattle blastocyst development (Keskintepe et al., 1995; Keskintepe and Brackett, 1996; Holm et al., 1999; Vajta et al., 2000) but the effect does not appear to be very substantial except in the case of inositol in protein-free medium (Holm et al., 1999). There is also no clear evidence that peptide growth factors added to the medium improve culture of embryos to any marked degree (Kane et al., 1997); most effects seem to be minor and do not give a marked enough improvement to be used commercially on a routine basis. However, it is possible that in the absence of serum or bovine serum albumin in the medium, there may be beneficial effects of growth factors. Also some unusual growth factors may be beneficial, e.g. parathyroid hormone-related protein (Nowak et al., 1999), vasoactive intestinal peptide (Servoss et al., 2001) and lysophosphatidic acid.

The use of oviductins in embryo culture is an area which might be worth exploring. The oviductins are a family of glycoproteins synthesized and secreted by oviduct cells, which bind to the zona pellucida of the oocyte after ovulation (Malette and Bleau, 1993). It is possible that their only function is merely to change the properties of the zona so as to provide increased protection to the embryo but one cannot exclude the possibility that they may affect the embryo directly.

While most embryo culture research is concentrated on studies of the effects of medium composition, it is possible that improvements in farm animal embryo culture will come from changes in factors other than medium composition. One such advance in the past seems to have been the move to 39 °C for cattle embryo culture; interestingly this important change in embryo culture technique was adopted with no fanfare and little or no formal documentation of its advantages in the literature.

One factor that needs examination is the volume of medium in which embryos are cultured. Currently embryos are cultured in relatively large volumes of medium in spite of the fact that a mammalian embryo of just over 150 μm diameter has a volume of only 1.5–2.0 nl and in the oviduct it is probably surrounded by mere picoliters of fluid at any one time. Thus as I have previously suggested (Kane et al., 1992) “the requirements for nutrients etc.
that we do see for cleavage in vitro could well be an artefact caused by the fact that the embryo is swimming in an ocean of fluid into which it is leaking its own internal nutrients’. Thus it is possible that culture of cleavage stage embryos in volumes of medium 1 µl or less might markedly improve development in culture. One possible problem with this approach is exhaustion of nutrients in the medium due to usage by the embryos. Another more immediate obvious problem is the physical one of managing such small culture volumes and the danger that these small culture droplets with a high surface area to volume ratio might facilitate uptake of toxic substances from the environment. The possibility that culture conditions commonly used are seriously abnormal is highlighted by the discovery (Schini and Bavister, 1988a) that the 2-cell block in hamster embryos in culture is due to the presence in the medium of phosphate and glucose, two substances which are ubiquitous in biological fluids; this effect is almost certainly to some degree an artefact of the culture conditions. This is at least partially supported by the finding of the same group (Schini and Bavister, 1988b) that culture of hamster embryos at high embryo density in 0.75 µl microdrops also overcame the incidence of the 2-cell block to some degree. Unfortunately this observation has not been followed up to any great extent. Galli et al. (2001) found that increasing cattle embryo density in 20 µl drops did not improve embryo development. However, a new method of culturing cattle embryos in miniature wells (0.04 and 0.15 µl) within larger wells (the Well of the Well or WOW system) has been reported to give improved development of cattle embryos in culture (Vajta et al., 2000).

Other possible changes to culture conditions might usefully be examined, e.g., very mild agitation could be used to mimic the movement of the female reproductive tract although there is evidence that in some circumstance at least agitation is harmful (Hickman et al., 2002). Another curious finding which has seemingly never been explored further was the report (Elliott et al., 1974) that increased atmospheric pressure in culture improved rabbit embryo development.

Little if any current work has been reported on the in vitro culture of cattle, sheep or pig early blastocysts to elongated blastocysts, almost certainly because of the difficulty of making progress in this area and because its commercial usefulness seems to be limited. One approach, at least to improve blastocyst expansion, might be to use co-culture on polarized uterine epithelial monolayers; such monolayers are produced by growing the cells on collagen-impregnated filters with culture medium above and below the filter (Dickens et al., 1993). It is possible that such monolayers might constitute an environment analogous to the uterine environment. For later development, a perfusion system such as that used by Pincus and Werthessen (1938) in early work on growth of the rabbit blastocyst might be useful in promoting growth of cattle and sheep blastocysts. Growth factors may well have a role to play here.

Bavister has emphasized the importance of the possible toxic effects of relatively ordinary constituents of complex culture media at the concentrations normally used in these media (Bavister, 1995). A very early indication of this problem was the finding that the nucleic acid precursor, thymidine, at the concentration used in Ham’s F10 medium, was inhibitory to rabbit blastocyst formation (Kane and Foote, 1971). Thus, when adding new constituents to a medium, consideration should always be given to possible negative as well as beneficial effects. Toxic contaminants in culture medium ingredients, including the water, are always a major concern. Adding agents to remove toxic contaminants or metabolites is one
area of possible improvement. EDTA has been added both to remove toxic heavy metals (a role possibly also carried out by albumin) and to help prevent free radical production (Nasr-Esfahani et al., 1992). Another problem has been the production of toxic metabolites from medium constituents, e.g. the production of toxic ammonia from amino acids in the medium; an enzyme, glutamate dehydrogenase which removes the ammonia by converting it to glutamic acid has been added to culture media to deal with this problem (Lane and Gardner, 1995). It has been suggested that free oxygen radicals produced in culture media block embryo development (Legge and Sellens, 1991). Various methods of remedying this problem have been used. Reducing the oxygen concentration in the gas phase probably helps (Umaoka et al., 1992). Other approaches have included the use of antioxidants, e.g. thioredoxin and superoxide dismutase (Nonogaki et al., 1991), EDTA (Nasr-Esfahani et al., 1992), catalase (Nasr-Esfahani and Johnson, 1992) and vitamins C and E (Vermeiden and Bast, 1995). Recently, Seidel’s laboratory examined the effects of EDTA and vitamins E and C on culture of IVP cattle embryos; vitamin E markedly improved blastocyst development but EDTA and vitamin C had no obvious beneficial effect (Olson and Seidel, 2000).

If one is considering the effects of factors that are toxic to embryo culture, one should also consider possible toxic factors other than medium constituents. For example, the number of times and the conditions under which embryos are taken out of the incubator for examination may be important. Taking embryos out of the incubator exposes them to conditions of temperature, light and gas phase CO2 to which they would not be exposed in vivo. There is evidence that exposure to light and room temperature negatively influences embryo development (Hegele-Hartung et al., 1991; Noda et al., 1994), and exposure to a CO2-free gas phase is rapidly toxic to hamster embryos. Although short-term cooling of cattle oocytes to 25 °C does not appear to be inhibitory to later embryo viability (Hasler et al., 1995), it is probable that removal of cultured embryos from the incubator should be as limited as possible and the nature of the environment in which they are handled and examined kept as non-toxic as possible. Species differences here may be of major importance.

In considering the whole area of embryo culture, the one thing that does seem clear is that some radically new thinking on the subject is needed if the field is to make significant advances in the future.

One issue that has to be resolved in relation to IVP embryo production is the problem of abnormal fetal and neonatal development and growth, known as large calf or large offspring syndrome, sometimes seen with such embryos (for reviews see Kruip and den Daas, 1997; Young et al., 1998; McEvoy et al., 2001). The most obvious feature of this syndrome is large size at birth often resulting in dystocia (Behboodi et al., 1995); other associated features may include breathing difficulties and an increased incidence of perinatal death (Young et al., 1998). The growth and development of body organs is often seriously disturbed. The syndrome is caused by the exposure of pre-elongation embryos to unusual environmental conditions. It is not clear exactly what environmental changes are important but a major cause may be embryo culture conditions, and in particular, in some cases at least, the use of serum and co-culture (Sinclair et al., 1999). The cause of the embryo culture effects may be due to some factor(s) in the culture medium such as ammonia, oxygen radicals, growth factors, etc. There is evidence that embryos cultured in a defined medium such as SOF with polyvinylalcohol (PVA) without serum or co-culture are much less susceptible to develop the large offspring syndrome (Jacobsen et al., 2000). However, such a medium
tends to produce a smaller proportion of blastocysts. Thus the approach to solving the large offspring syndrome may be to concentrate on modifying simple media such as SOF with the object of increasing the yield of viable blastocysts while at the same time checking that the modifications do not give rise to later developmental problems.

A more productive and less costly approach to the large offspring syndrome may be to investigate abnormal DNA methylation in IVP embryos in relation to culture conditions. There is evidence that in vitro embryo culture and manipulation causes abnormal DNA methylation which in turn causes abnormal gene expression both in preimplantation and fetal stages resulting in fetal overgrowth and large offspring syndrome (Niemann and Wrenzycki, 2000; Young et al., 2001). Changing the culture conditions so that normal gene methylation is present in IVP embryos might reduce or abolish the incidence of large offspring syndrome.

3.3. Biotechnological implications of the development of successful reliable embryo culture systems

Culture systems which would allow the development of >90% of normal 1-cell cattle, sheep or pig embryos to early blastocysts would have a huge general impact on the development of related areas of reproductive biotechnology; reported results from large commercial groups using ovum pick up and optimal sources of oocytes (pregnant heifers and first-parity cows) indicate figures of at best about 60% of cleaved IVP embryos reaching the stage of transferable morulae or blastocysts (reviewed by Galli et al., 2001). Such a culture system would be a very powerful research tool; the study of oocyte maturation and the development of optimal oocyte maturation systems, the study of fertilization and the development of optimal IVF systems, and the study of cloning and development of optimal cloning systems would benefit enormously from the availability of such culture systems for testing the normality and viability of oocytes or embryos developed from the system under study. Any experimental manipulation which affected the oocyte or embryo so as to reduce development to the blastocyst stage would readily be detected with minimal numbers of oocytes or embryos. It must be emphasized, however, that such a culture system would not detect the type of deleterious effect which affected development only after the blastocyst stage was reached.

Perhaps the most important and certainly the most obvious biotechnological implication at this time is that in addition to facilitating the production of better embryo cloning systems, the availability of such an optimal culture system would greatly increase the availability of cloned embryos simply because very few embryos would be lost due to damage by the culture process. With current systems, the embryo undergoes the double insult of the cloning procedure and a less than optimal culture procedure. These two insults are probably not just additive but rather negatively synergistic in their effects. One can expect an embryo which has been injured by one procedure to be much less capable of surviving injury from a second procedure.

Currently almost all cattle embryo transfer is carried out non-surgically using blastocysts and this necessitates the use of a reliable culture system. In combination with optimal methods of superovulation, ovum pick up, in vitro maturation (IVM) of oocytes and IVF, optimal methods of embryo culture would facilitate greatly the use of IVP embryos. In dairy cattle this would lead to a better use of the genetic merit of high yielding dairy cows.
4. In vivo and in vitro culture of spermatogonial stem cells

The area of in vivo and in vitro culture of spermatogonial stem cells may not seem appropriate in a review dealing mainly with in vitro oocyte maturation, in vitro fertilization and in vitro embryo culture because as yet work on spermatogonial stem cells mainly involves the transplantation of stem cells from one testis to another and there is as yet a very limited amount of work on the in vitro culture of stem cells. However, the transplantation procedure offers a real opportunity to develop and test out systems of in vitro spermatogonial stem cell culture and the development of such culture systems in farm animals has great biotechnological potential.

4.1. Milestones in the in vivo and in vitro culture of spermatogonial stem cells

After the development of the procedures for artificial insemination and frozen semen storage in farm animals about the middle of the last century, the major emphasis and excitement of biotechnological development in reproduction shifted to the preimplantation embryo and the oocyte. Brinster, Biggers and their co-workers were a major influence in this shift with their pioneering work in embryo culture and embryo studies generally. Thus, it is all the more extraordinary that, in the last few years, Brinster’s laboratory has switched its attention to the male and suddenly opened up the whole area of sperm research with consequent enormous implications for the biotechnology of reproduction in the male. In 1994 Brinster and Zimmermann showed that spermatogonial stem cells from the testes of donor male mice were able to repopulate the testes of sterile mice after transplantation (Brinster and Zimmermann, 1994), and Brinster and Avarbock (1994) also showed that sperm developed from these transplanted cells were capable of fertilization and production of viable offspring.

A much more surprising discovery by the same laboratory was the demonstration that rat spermatogonial stem cells could, on transplantation to the testes of immunocompromized mice, complete spermatogenesis and produce rat sperm with normal morphology (Clouthier et al., 1996). Thus, xenogeneic transplantation and xenogeneic in vivo culture is possible in some species at least. However, transfer of hamster cells to mouse testes was not as successful in producing hamster sperm with normal morphology (Ogawa et al., 1999) indicating that there may be strong barriers to xenogeneic transplantation of testis cells in other species. This conclusion has recently been born out by further work from Brinster’s group showing that while cattle, pig, horse and baboon spermatogonial stem cells can colonize the mouse testis after transplantation, they do not undergo spermatogenesis (Dobrinski et al., 2000; Nagano et al., 2001). Colonization of pig testis by transplanted spermatogonial stem cells has been shown (Honaramooz et al., 2002) and this is a first step in the extension of these techniques to farm animals.

The finding that mixtures of testis cells could be stored frozen for extended periods and then undergo spermatogenesis after transplantation (Avarbock et al., 1996) has opened up the prospect of prolonged storage of spermatogonial stem cells with the prospect of the later production of large numbers of sperm after transplantation. Brinster’s group has also found that mouse testicular stem cells can be maintained in culture in vitro for 4 months and then complete spermatogenesis with the production of mature sperm after transplantation.
In these experiments, the degree to which the stem cells divided during culture was not demonstrated and it is possible, if perhaps unlikely, that they remained in a quiescent non-dividing state during culture. However, a recent report (Izadyar et al., 2003) demonstrating proliferation and some degree of differentiation of bovine spermatogonial stem cells in vitro, is an exciting and very promising development. These findings are of enormous interest with huge potential implications for biotechnology because of the possibilities they open up for genetic manipulation of sperm. In preliminary experiments, it has been shown that it is possible to transfer genes into mouse spermatogonial stem cells using retrovirus-mediated gene delivery (Nagano et al., 2000). There are a number of recent reviews of this work (Johnston et al., 2000; Russell and Griswold, 2000; de Rooij, 2001; Jiang, 2001; Brinster, 2002).

The method of genetic manipulation of sperm via exposure to gene constructs in vitro has considerable biotechnological potential but does not fall within the scope of this review; interested readers should consult the review by Wall (2002).

4.2. Problems of spermatogonial stem cell culture

While the technology of spermatogonial stem cell culture has very great potential, it is still at a very early stage in its development and there are still huge obstacles to its biotechnological exploitation. The first major problem is that as yet the transplantation technology is almost entirely confined to the mouse; to date, with the exception of the work of Honaramooz et al. (2002) there appear to be no reports of successful spermatogonial transplant experiments in farm animals—although this is likely to change rapidly. Also it has not been possible to use xenotransplantation to get the spermatogonial stem cells of farm animal to undergo spermatogenesis in the mouse testis (Dobrinski et al., 2000). A second major problem is the inability as yet to obtain pure preparations of stem cells. Some progress has been made in this regard by Brinster’s laboratory (Shinohara et al., 2000); by selecting cryptorchid testis cells using fluorescence-activated cell sorting, based on light scattering properties and expression of the cell surface molecule α6-integrin and negative or low expression of α2-integrin, it has been possible to enrich spermatogonial stem cells by about 166-fold. The third problem is that information on spermatogonial stem cell proliferation in vitro is very limited. However, the demonstration (Nagano et al., 1998) that mouse spermatogonial stem cells can at least survive in vitro for up to 4 months is encouraging and even more encouraging is the work of Izadyar et al. (2003) with cattle spermatogonia.

4.3. Biotechnological implications of the development of successful reliable spermatogonial stem cell culture systems

The potential biotechnological implications of in vivo and in vitro spermatogonial stem cell culture are, as I have stated already, enormous. The ability to culture pure preparations of bovine spermatogonial stem cells in vitro, to modify the genetic makeup of these cells in culture, to transplant these genetically modified stem cells and achieve normal spermatogenesis in host testes previously denuded of their own stem cells, and to collect and use these sperm for cattle breeding using normal commercial artificial insemination (AI) procedures...
would transform the cattle breeding industry. However, it must be remembered that the process of spermatogenesis in producing sperm from spermatogonial stem cells leads to a rearrangement of the sperm DNA and therefore one is not going to get cloned identical sperm by this process, even if one is starting from clones of identical spermatogonial stem cells.

The major advantage of this system for progress in cattle breeding is that one would be grafting on a new technique to the standard commercial system of cattle breeding with all the advantages resulting from using a tried and tested technology; one would not be trying to set up a totally new system from the beginning. A second major advantage would be that one can deal readily with large numbers of sperm in bulk; this contrasts with systems of IVP embryo production and transfer where each embryo has to be handled individually by highly skilled personnel.

The development of such a spermatogonial stem cell culture system capable of producing genetically modified sperm in farm animals and its linkage to commercial AI procedures could broaden the scope for genetic improvement in farm animals. As well as improving production efficiency, one could possibly genetically engineer in traits for disease resistance at the same time, provided that traits for disease resistance and production efficiency are not mutually exclusive. Even if this were true, it might be possible to engineer resistance to specific diseases into the animal’s immune system.

Progress in the area of spermatogonial stem cell culture would be a very useful research tool in the study of spermatogenesis. It could shed light on both the systemic and local control of spermatogenesis and on the influence of growth factors on the process. For instance, teasing out the reasons why xenogeneic transplantation works between some species and not others could be very informative in terms of understanding normal spermatogenesis and transplantation physiology.

5. Conclusions

The aim of reproductive biotechnology in farm animals can be very simply summed up as the use of advances in reproductive techniques to increase both reproductive efficiency and overall production efficiency. This should be coupled with respect for the welfare of the animals.

Much of the interest of reproductive biotechnology is presently and will probably continue for some time to be centered on the use of these techniques to improve productivity and quality by gene modification, e.g. improvement of milk yield and quality in dairy cows, more rapid and more efficient rates of live weight gain in cattle and pigs, better meat quality and improved fertility in all farm animals. Another area of major interest is biopharming where the genetic makeup of the animal is modified to secrete medically useful products in large quantities in the milk. While this process of improvement of animal productivity can be regarded as the acceleration of a process in which human beings have been engaged for many centuries, there are potential dangers involved in gene modification and embryo cloning and I can understand the concerns of many reasonable individuals about these procedures. These concerns include concerns over animal welfare, over the possible narrowing of the gene base of domestic animals (cryopreservation of embryos in a gene bank could alleviate this
problem), over the safety of food from genetically modified animals and over the possibility that genetically modified animals might give rise to novel and possibly untreatable diseases. While some but not all of these fears may be of dubious validity, they do concern many people particularly in Europe.

There are, however, a number of uses of gene modification combined with either spermatogonial cell culture or embryo cloning which it should be possible for everyone to welcome. One such area is the use of the technology to reduce problems caused by intensive farming, one example of which is the phosphorous pollution produced by intensive pig farming. Pigs are normally not able to digest plant phytate, an inositol hexakisphosphate compound present in abundance in cereal grains and their by-products. The phosphorous in the phytate is thus not available for the nutritional needs of the animals and is consequently excreted to contribute to pollution of rivers and lakes; the nutritional needs of the animals for phosphorous may then be met by dietary phosphorous supplements. One possible solution to this problem is the development of the “phytase pig” which has been genetically engineered to produce salivary phytase, thus digesting phytate, making the phosphorous available to the animal and greatly reducing fecal phosphorous excretion (Golovan et al., 2001).

Another such use of the technology would be to produce resistance in farm animals to various diseases caused by organisms ranging from insect and worm parasites to viruses and bacteria. Use of such technology against bacterial disease in animals might reduce the use of antibiotics in farm animals and thereby decrease the rate at which the efficiency of antibiotics in animals is being curtailed by overuse. There are also concerns, set out by the Australian Joint Expert Advisory Committee on Antibiotic Resistance (Anonymous, 1999), that overuse of antibiotics in animals may affect bacterial resistance in humans even though it is generally admitted that most bacterial resistance in humans is due to use/overuse of antibiotics in humans. It is also possible that the technology could be used to produce cattle which are resistant to prion-dependent diseases such as bovine spongiform encephalopathy (BSE). We should not forget that human beings and their domestic animals are part of a constant war with disease-producing organisms. Our present situation where most infectious diseases are under control in the Western World may be analogous to a traveler coming to an oasis while travelling across the Sahara desert. Just because we have come to a friendly environment does not mean that this type of environment will continue. The human race cannot afford to throw away any potential weapons in the battle against disease in either human beings or farm animals.

The development of disease resistant strains of animals should not be confined to the Western World. There is even more scope for improvement of disease resistance in animals in the Third World. The use of genetically modified sperm could be of enormous value here. I believe that the technique with by far and away the greatest potential importance for farm animal reproductive biotechnology is the use of spermatogonial cell culture coupled with in vitro genetic modification by homologous recombination and selection of the modified spermatogonia. The major precautionary notes that must be repeated here are firstly the need to show clearly that spermatogonial stem cells proliferate reliably in vitro and secondly the fact that the transplantation technology is still almost entirely confined to rodents and has not yet been extended to farm animals. The reason for saying that it has the greatest potential importance is that as already stated one would be marrying it to the existing
reliable commercially tested technique of AI. Thus if efficient procedures for spermatogonial stem cell culture and gene modification can be developed for use in farm animals, the rapid dissemination of the modified sperm via the AI system offers huge scope for genetic modification of farm animals.

Spermatogonial stem cell technology is followed in potential importance for reproductive biotechnology by embryo cloning. It has the immediate advantage that the technology is already here, even if in very imperfect and expensive form for farm animals. Improvements in embryo culture would greatly facilitate the use of embryo cloning. However, if efficient procedures can be developed for the exploitation of spermatogonial stem cell culture in farm animals, it is possible that use of this technology could render the use of embryo cloning (no matter how much embryo cloning technology is perfected) of somewhat limited direct use in the production of widespread genetic modification. However, even if this does happen, embryo cloning may have still have a use in the production of genetically modified bulls whose modified genes would then be spread by artificial insemination.

Gazing into a crystal ball and predicting future developments in science and its applications is not a very fruitful occupation. However, one can perhaps safely predict that procedures for controlled genetic modification in farm animals and for spreading these modifications in the farm animal population will continue to be developed and will have a major impact in the 21st and later centuries. The importance of such developments will perhaps lie not so much in increasing production in farm animals but in adapting that production to future consumer needs and in coping with change in a world which is evolving industrially, biologically and socially.

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References


Galli, C., Duchì, R., Crotti, G., Lazzari, G., 1997. Elongated bovine blastocysts can be obtained from IVM-IVF embryos cultured in vivo from day 2 to day 7 in the sheep oviduct and from day 7 to day 14–16 in the synchronised cow uterus. J. Reprod. Fertil. Abstr. Ser. 19, 11.


