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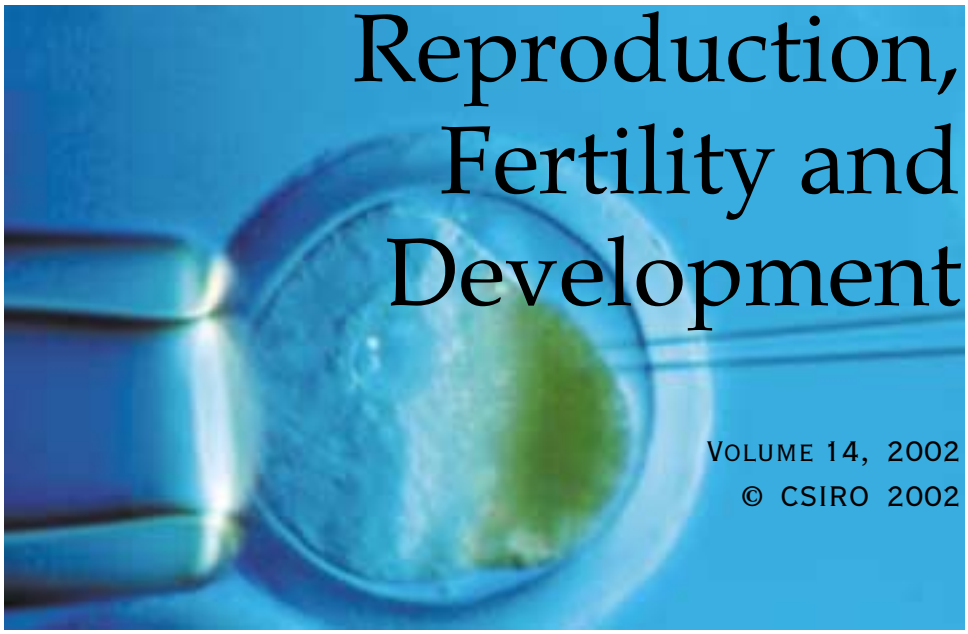

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In vitro production of pig embryos: a point of view

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Abstract. Porcine embryos have become raw materials for different programmes of reproductive biotechnology and the *in vitro* production of embryos has some advantages over *in vivo* production in gene transfer programmes and for xenotransplantation. Despite this promising future, several problems limit the success of the *in vitro* production (IVP) of viable porcine embryos. Porcine IVP has not been fully developed because of several problems associated with different techniques, such as incomplete final maturation status after *in vitro* maturation, a high incidence of polyspermy after *in vitro* fertilization and a low development rate and poor quality of blastocysts at the end of culture. The results could be improved with studies comparing *in vivo* and *in vitro* conditions, standardization of techniques for sperm processing, testing new additives in the culture media and developing intracytoplasmic sperm injection procedures. The first objective of the present article is to summarize the main studies published on the subject. Second, we provide a guide for researchers starting work on the IVP of pig embryos, making special mention of first papers and the most recent achievements for each of the different techniques. Third, we provide suggestions for future experiments designed to improve the results of each technique.

Introduction

In vitro production of pig embryos (p-IVP) is a useful technique for research into areas such as transgenesis, xenotransplantation, genetic improvement and recovery of endangered breeds and can be used in combination with sex-determining technologies. Although several research teams currently perform p-IVP, the success rate of the techniques remains low and this hinders advances in the aforementioned fields. For instance, the first transgenic pigs were obtained as early as 1985 (Brem *et al.* 1985; Hammer *et al.* 1985), but there have only been a few improvements in the field since then (Besenfelder *et al.* 1998). One of the reasons for this is that most of the time it is still necessary to obtain embryos from *in vivo* procedures, which is expensive and often impractical.

According to Moor (1994), the successful genetic modification of embryos in domestic species depends upon a reliable source of oocytes for manipulation, appropriate DNA constructs and a simple means of transferring manipulated embryos back into the uteri of foster mothers. Of these three criteria, the production of pig embryos in the laboratory by manipulating the source of oocytes has been one of the main obstacles to the advancement of this applied biotechnology. For many years, the *in vitro* production (IVP) of porcine embryos has been the major focus of numerous research projects. This has included *in vitro* maturation (IVM) techniques, sperm preparation, *in* vitro fertilization (IVF) and the *in vitro* culture of embryos up to transferable stages. The aim of the present review is to discuss the latest achievements in these methodologies, to review the key studies and to suggest areas for future research.

Collection of porcine oocytes and IVM

Large numbers of oocytes may be obtained from ovaries recovered from female pigs slaughtered at commercial weight. These animals are gilts and it is possible to find many follicles 3–6 mm in diameter (Fig. 1*a*) containing immature oocytes. Although mature oocytes from the oviducts of gilts that have just ovulated can be used, IVM has been shown to be much more effective in IVP because the age of the oviducal oocytes cannot be determined precisely (Yoshida 1987; Rath 1992). In addition, IVM of oocytes collected from immature follicles is more feasible than the recovery of oocytes from mature follicles shortly before ovulation. In many laboratories, the follicular wall is sectioned with a scalpel to allow follicular fluid and cumulus–oocyte complexes (COCs) to drop to the bottom of a Petri dish containing an enriched saline solution, such as Dulbecco's phosphate-buffered saline (PBS) at 37°C. In other cases, COCs are harvested by aspiration of the follicular content with a needle connected to a syringe or a vacuum pump. Thus far, there have not been many studies demonstrating which method is best. However, Liu and Moor (1997) showed that the method of oocyte preparation affects subsequent development, because both nuclear maturation and cleavage after activation were higher when oocytes were collected by follicle dissection rather than by aspiration.

The next step in the process is the examination of COCs under a stereomicroscope and the recovery of those COCs with uniform ooplasm and surrounded by several layers of compact cumulus cells (Fig. 1*b*). Finally, the selected oocytes are cultured in an incubator at 38.5°C in 5% CO_2 in maximally humidified air. Advances related to the maturation

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of COCs are mainly attributable to the results of studies conducted on folliculogenesis, the endocrine control of ovulation and intercellular communication between the cumulus oophorus and the oocyte during the maturation process (Mattioli *et al.* 1988*a*; Moor *et al.* 1990; Ding *et al.* 1992).

In vivo, oocytes mature 38–42 h after the pre-ovulatory luteinizing hormone (LH) peak (Hunter and Polge 1966; Hunter *et al.* 1976). Ovulation occurs approximately at this time, when most oocytes have reached metaphase II. The culture period for IVM is based on this and generally runs from a minimum of 36 h (Yoshida *et al.* 1990) to 48 (Iwasaki *et al.* 1999) or even 50 h (Kim *et al.* 1998). During this time, the cumulus cells also undergo their own maturation process. The morphological transformation consists of mucification and expansion, thus reducing the number of gap junctions and metabolic cooperation (Motlik *et al.* 1986). Hormone supplementation of the culture medium is necessary for cumuli oophori to synthesize a protein pattern that corresponds closely to that produced by cumuli oophori matured *in vivo* (Schroeter and Meinecke 1995).

Initial work by Mattioli *et al.* (1988*a*) showed the need for the oocyte to remain in contact with the follicular wall during the maturation period to yield good results. Meanwhile, this system has been replaced by systems using culture media that require no cellular support and, thereby, reduce the variability between replicates and minimize the risk of contamination. The most successfully used media for IVM of pig oocytes include TCM-199 (Mattioli *et al.* 1989; Yoshida *et al.* 1990; Coy *et al.* 1999), Waymouth medium (Yoshida *et al.* 1992*a*; Coy *et al.* 1999), Whitten's medium (Funahashi *et al.* 1994*a*; Coy *et al.* 1996); and Tyrode's lactate pyruvate medium (TLP; Yoshida *et al.* 1992*a*; Yoshida 1993). Some of these media need serum as a supplement. However, more defined media are currently

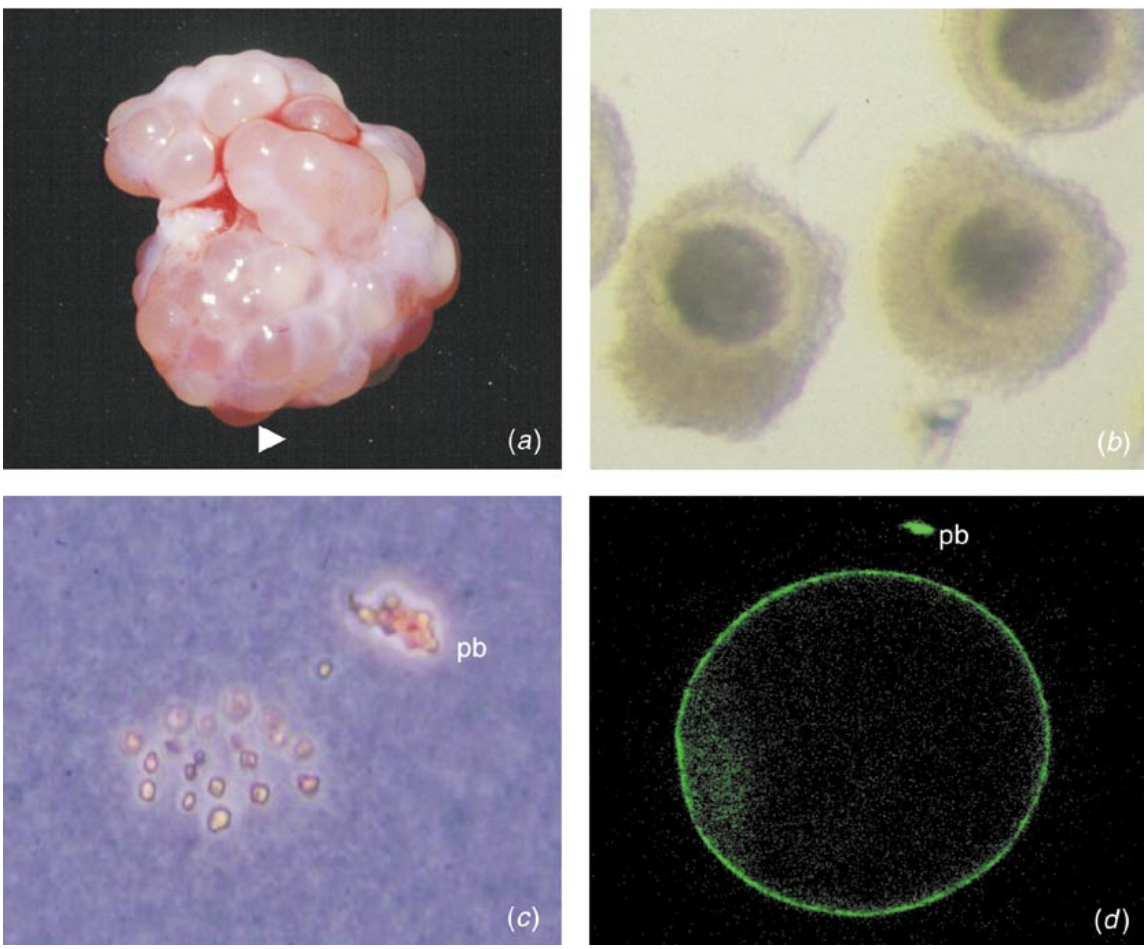

Fig. 1. (*a*) Ovary from a prepubertal gilt with several follicles ranging from 3 to 6 mm in diameter (arrowhead). (*b*) Immature cumulus–oocyte complexes with several cumuli cells layers and a homogeneous dark cytoplasm. (*c*) *In vitro*-matured oocyte showing the metaphase plate and the first polar body (pb) after staining with Lacmoid (Sigma-Aldrich Química SA, Madrid); viewed under phase-contrast microscopy at ×100. (*d*) Porcine oocyte observed after an *in vitro* maturation period and staining with peanut agglutinin (PNA) lectin, which shows the cortical granuli forming a monolayer under the plasmatic membrane and in the first pb (viewed with confocal microscopy at ×63.5).

under investigation (Abeydeera *et al.* 2000) that avoid supplements of variable composition and do not depend on batches of serum or bovine serum albumin (BSA). In this way, the use of Whitten's medium (Funahashi *et al.* 1994*a*) and North Carolina State University (NCSU) medium (Petters and Wells 1993) or BSA-free NCSU medium (Wang *et al.* 1997) has advanced IVM protocols because high rates of mature oocytes are achieved by the addition of porcine follicular fluid as the only protein supplement. According to the findings of Abeydeera *et al.* (1999*a*, 2000), the latest development in this area, aimed at obtaining a protein-free IVM medium supplemented with epidermal growth factor (EGF) but without requiring coculture with somatic cells or their secretions, may even already be feasible.

Considerable advances have also been made in the area of cytoplasmic maturation of the oocyte. Despite the fact that the proportion of oocytes at metaphase II (Fig. 1*c*,*d*; the stage at which nuclear maturation is reached) has been high since initial attempts at IVM, this is not true for cytoplasmic maturation. This problem has been attributed to the poor ability of the oocytes to transform the sperm head into the male pronucleus when these oocytes are not matured in contact with the follicular wall. Different studies have demonstrated that this ability is affected by, among other factors, hormone levels, follicular fluid addition (Naito *et al.* 1988) and the cysteine content of the medium (Day and Funahashi 1996).

In an effort to mimic *in vivo* events, hormonal supplements, such as follicle-stimulating hormone (FSH) or equine chorionic gonadotrophin (eCG), luteinizing hormone (LH) or human chorionic gonadotrophin (hCG) and oestradiol (Yoshida *et al.* 1989), have all been used since the early days of research. These supplements are added to the maturation medium, which is not renewed during the 40–44 h of the process. However, the work of Funahashi *et al.* (Funahashi and Day 1993; Funahashi *et al.* 1994*b*) has demonstrated that suppression of hormonal additives during the second half of the culture period is highly beneficial and most researchers have adopted this method.

Yoshida *et al.* (1992*b*) demonstrated that porcine follicular fluid contains an acidic substance(s) that promotes oocyte maturation. This team (Yoshida 1993) also showed that glutathione (GSH) synthesis occurs throughout the IVM of pig oocytes, this being an important cytoplasmic factor for the regulation of sperm nuclear decondensation and male pronucleus formation following sperm penetration. Glutathione is responsible for the disulphide bond reduction in sperm nuclei that is required for their decondensation. Other studies have demonstrated that the use of media with a reduced NaCl concentration and the addition of follicular fluid, cysteine and β-mercaptoethanol enhances male pronucleus development by increasing the intracellular content of GSH in the oocyte (Yoshida *et al.* 1992*a*; Yoshida 1993; Funahashi *et al.* 1994*c*). It has also been shown that the addition of amino acids to the maturation medium and the presence of cumulus cells improve the ability of the oocyte to develop the male pronucleus (Ka *et al.* 1997). However, it is important to point out that such an ability depends not only on intrinsic factors of the oocyte, but also on those related to the spermatozoon and it has been shown to vary with the particular boar (Xu *et al.* 1996*a*; Coy *et al.* 1999).

It appears that the IVM of pig oocytes is presently sufficiently developed in terms of nuclear and cytoplasmic maturation. What remains to be confirmed is the ability of such oocytes, once fertilized, to develop. In the past, IVM oocytes showed lower penetration rates, asynchronous pronuclear development and delayed and lower cleavage rates following IVF compared with oocytes matured *in vivo* (Laurincik *et al.* 1994). However, current findings indicate a beneficial effect on embryo development to the blastocyst stage following exposure of oocytes to dibutyryl cAMP (db-cAMP) for the initial 20–22 h (Funahashi *et al.* 1997) owing to the reversible inhibition of meiotic resumption, thus synchronizing the germinal oocyte stage. Furthermore, the addition of epidermal growth factor (EGF), insulin-like growth factor (IGF)-I or β-mercaptoethanol to the medium for the whole maturation period has a beneficial effect on subsequent embryo development (Xia *et al.* 1994; Abeydeera *et al.* 1998*a*,*b*, 1999*b*).

Sperm processing

The basic steps that sperm undergo before entering an oocyte are capacitation, penetration of the cumulus complex, binding to the zona pellucida (primary binding), the acrosome reaction, secondary binding to the zona pellucida, zona penetration and entry into the perivitelline space and, finally, binding and fusion with the oocyte plasma membrane, with subsequent oocyte activation. The system of recognition and sperm–oocyte fusion is mediated by a dynamic process of protein changes and interactions in the plasma membrane. An essential step in *in vitro* sperm processing is to achieve artificial sperm capacitation, as well as control of the time of the acrosome reaction, because only capacitated sperm can be recognized by the oocyte and respond to the oocyte signals in an appropriate manner (Töpfer-Petersen *et al.* 2000).

The capacitation concept was mentioned for the first time in the early 1950s (Austin 1951; Chang 1951). According to these authors, the spermatozoon needs to undergo a series of changes before acquiring the capacity to penetrate the oocyte. Under physiological conditions, these changes take place in the female tract (Fig. 2*a*) and the role of the oviducal isthmus is especially important in this process (Hunter and Nichol 1988). Based on this idea and on results from other species, the first successful *in vitro* penetration of a pig oocyte involved subjecting the sperm to a relatively complex procedure, including several washes by centrifugation and pre-incubation of high concentrations of spermatozoa (from

2 to 14 million mL^{-1}) in media with high calcium concentrations at a temperature approaching 37°C (Pavlok 1981). The objective was to destabilize the sperm membrane and, in some way, prepare the sperm for the acrosome reaction (Fig. 2*b*). Cheng (1985) was able to modify the technique of Pavlok technique and produce the first litter of piglets by IVF. The *in vitro* capacitation of spermatozoa involved a process including three washes in saline and pre-incubation in TCM-199 at pH 7.8 for 40 min, having previously subjected the spermatozoa to a storage period of 16 h at 20°C. A few years later, Mattioli *et al.* (1989) reported the birth of IVF piglets after subjecting sperm to a selection process through a Percoll (Pharmacia, Uppsala, Sweden) gradient similar to that used in human IVF. The Percoll medium is made of colloidal particles of silica gel, 15 to 30 nm in diameter, coated with polyvinylpyrolidone. Some years ago, Percoll fell into disuse in many human assisted reproduction laboratories. However, it is still used for the capacitation of animal spermatozoa because it allows for the rapid, efficient isolation of motile spermatozoa that are free from contamination by other seminal constituents (Ng *et al.* 1992).

To date, there are still many unanswered questions about the more suitable sperm treatment for IVF. Sperm penetration into the oocyte has been achieved with procedures including several washes, centrifugations or pre-incubation (Nagai *et al.* 1984; Yoshida *et al.* 1993) as well as with simpler methods avoiding rinsing and pre-incubation (Martinez *et al.* 1996). Both methods used for the entry of the spermatozoon into the oocyte *in vitro* have been succesful (Fig. 2*c*,*d*) using mature and immature oocytes (Mattioli *et al.* 1989; Kikuchi *et al.* 1999; Campos *et al.* 2001), although with very different sperm treatments. However, comparative studies on final productivity related to the different capacitation procedures are scarce (Jeong and Yang 2001). Capacitation is a very complex phenomenon involving changes in metabolism, motility, intracellular ion concentration and membrane fluidity and reorganization of the sperm surface (Töpfer-Petersen *et al.* 2000). As a consequence, it is not clear when capacitation actually takes place: either after sperm preparation with a simple system or with a more complex procedure (Maxwell and Johnson 1997).

Other important topics include the source of spermatozoa used in IVF and their preservation methods. First, it is much easier to capacitate spermatozoa obtained from the epididymal tail than from the ejaculate, because the former have not been in contact with decapacitating factors in the

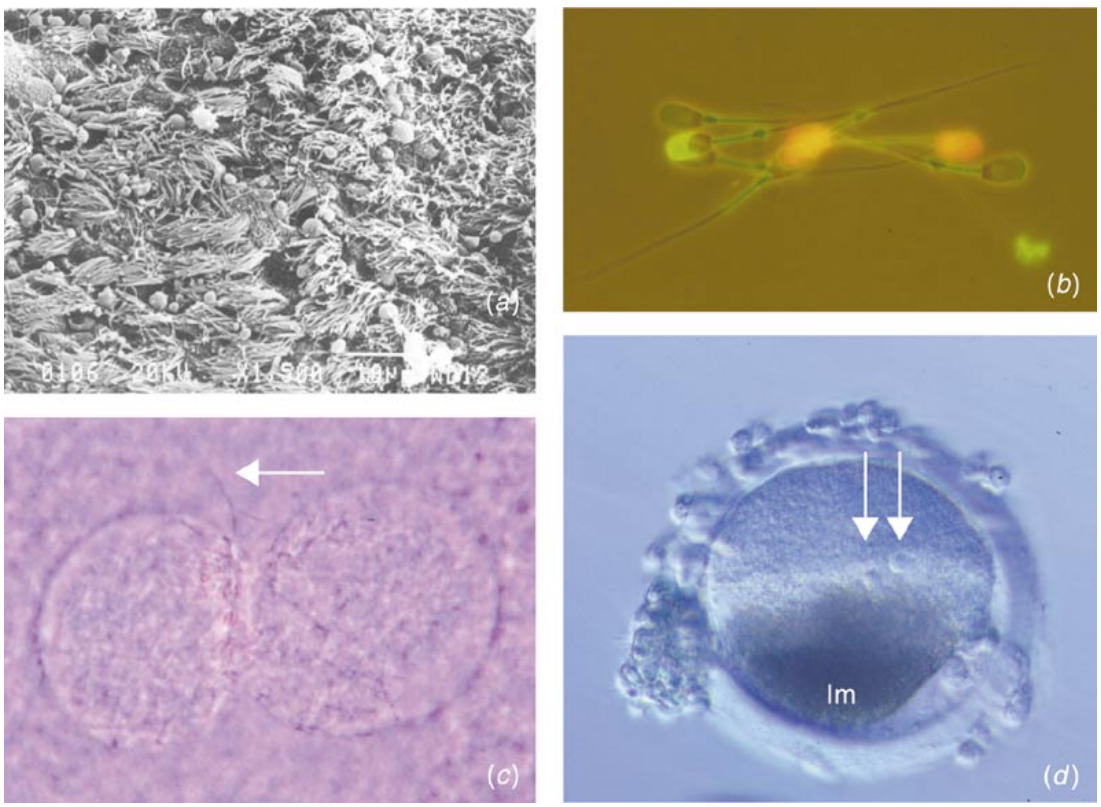

Fig. 2. (a) Scanning electron micrograph of the oviductal epithelial wall from a gilt showing the ciliated and secretory cells. (*b*) Boar spermatozoa stained with peanut agglutinin (PNA)–fluorescein isothiocyanate lectin and propidium iodide for evaluating the acrosome reaction. (*c*) Detail of the sperm tail (arrow) and the male and female pronuclei at the syngamy stage after *in vitro* fertilization (Lacmoid staining (Sigma-Aldrich Química SA, Madrid) at ×100). (*d*) Porcine oocyte recently centrifuged with the lipid material (lm) displaced and two pronuclei (arrows).

seminal plasma. Epididymal and ejaculated spermatozoa have been used since the first porcine IVF experiments (Cheng 1985). The drawbacks of using fresh epididymal spermatozoa are related to the fact that it is necessary to obtain the epididymis from the boar and that trials usually involve the use of males slaughtered in the abattoir, which makes it difficult to repeat experiments with spermatozoa from the same animal. For these reasons, several workers in the field have chosen to use ejaculated spermatozoa (Mattioli *et al.* 1989; Yoshida *et al.* 1993).

Currently, the number of teams using epididymal spermatozoa rather than ejaculated spermatozoa has increased owing to the development of cryopreservation techniques (Rath and Niemann 1997; Kikuchi *et al.* 1999). The first successful report of the use of frozen–thawed epididymal spermatozoa in porcine IVF was published in 1988 by Nagai *et al.*, who, using almost the same procedures as Cheng (1985), were able to achieve the birth of live offspring. Thawed spermatozoa have to be subjected to a shorter treatment for IVF than fresh semen because, during pre-incubation, the thawed spermatozoa undergo a rapid decrease in motility (Clarke and Johnson 1987; Nagai *et al.* 1988). In addition, the use of thawed spermatozoa leads to similar penetration rates as obtained using fresh ejaculated semen and is related to a higher monospermy rate (Zheng *et al.* 1992). If we add the fact that the IVF system is more easily standardized and straws from the same boar, or even from the same ejaculate, may be used in several experiments, it is understandable that it is now current practice to use frozen–thawed semen in porcine IVF (Abeydeera *et al.* 1998*d*).

Moreover, the development of cryopreservation techniques has allowed a return to the use of epididymal spermatozoa. According to Rath and Niemann (1997), the use of frozen–thawed epididymal sperm yields IVF returns of minimal variation and results in higher motility levels and penetration rates than the use of frozen–thawed ejaculated semen.

In conclusion, we would propose the use of frozen–thawed semen in order to standardize the male factor in IVF protocols and to freeze epididymal semen from tested boars. However, comparisons of early embryonic development after IVF using fresh and frozen semen are still scarce (Rath and Niemann 1997) and studies on this topic, together with those related to the capacitation system, would be of great interest and applicability to the production of pig embryos in the laboratory.

In vitro fertilization

Since the first report of the successful production of piglets from IVF (Cheng 1985), live offspring have been produced on several occasions (Mattioli *et al.* 1989; Yoshida *et al.* 1993; Abeydeera and Day 1997*a*; Rath *et al.* 1999). However, in terms of efficiency, the technique has not improved much. Cheng *et al.* (1986) pointed out that the main problem with porcine IVF was polyspermy and this continues to be a major difficulty (Fig. 3*a*). Whereas significant improvements have been achieved in the area of IVM, advances relating to the fertilization stage have lagged behind. Numerous *in vitro* assays have attempted to test different variables of IVF, including culture media, such as TCM-199, which was first used by Cheng (Mattioli *et al.* 1988*b*; Wang *et al.* 1991; Yoshida 1993; Funahashi *et al.* 1994*a*; Coy *et al.* 1999), Tyrode's albumin lactate pyruvate medium (TALP; Yoshida *et al.* 1993; Long *et al.* 1998; Rath *et al.* 1999; Coy *et al.* 2000); Brackett–Oliphant (BO) solution (Kikuchi *et al.* 1999; Funahashi *et al.* 2000) or Tris-buffered medium (TBM; Abeydeera and Day 1997*a*,*b*; Abeydeera *et al.* 1998*a*,*d*,*e*, 1999*b*; Han *et al.* 1999). Other variables studied have included sperm concentration (Cheng 1985; Coy *et al.* 1993*a*; Xu *et al.* 1996*b*), coincubation time (Cheng 1985; Coy *et al.* 1993*b*), source of spermatozoa (Rath and Niemann 1997) and the volume of the coculture medium (Coy *et al.* 1993*c*). Except for a series of outstanding papers by Hunter *et al.* (Hunter 1973, 1974, 1984, 1988, 1990, 1991; Hunter and Hall 1974; Hunter and Nichol 1988), there is a lack of studies that compare the precise conditions of *in vivo* and *in vitro* fertilization. For instance, there is a large gap in current knowledge of the precise composition of oviductal fluid. The most detailed analyses were undertaken in 1974 (Iritani *et al.* 1974) and, since then, there have been no significant improvements with current assay methods. Owing to the findings of Hunter, it is known that the number of spermatozoa that reach the site of fertilization, and, thereby, have access to the oocyte *in vivo*, is much lower than the number used in IVF. This is probably one of the main reasons for *in vitro* polyspermy and the results may be improved by reducing sperm concentration (Coy *et al.* 1993*a*) or using adenosine instead of caffeine in the fertilization medium because adenosine promotes capacitation but inhibits spontaneous acrosome reactions (Funahashi and Nagai 2001). We know that the composition of the oviductal fluid changes during the oestrous cycle and, close to ovulation, the temperature of the follicles is lower than that of the adjacent stroma (Hunter *et al.* 2000). Furthermore, changes in hormonal patterns are critical at this stage. Yet, these factors are not taken into account in IVF procedures. *In vitro* penetration rates are variable, depending on the boar used, but relatively standardized results have been attained using frozen semen samples (Rath and Niemann 1997). However, IVF output is still very low and polyspermy continues to be the major reason for this. It is not known whether this abnormality is due to inadequate conditions for the maturation of oocytes or for fertilization *in vitro* (Niwa 1993). In our opinion, the use of IVM oocytes cannot be mainly responsible for polyspermy, because IVF of ovulated oocytes renders high levels of polyspermy (Coy *et al.* 1993*a*,*b*,*c*) and production of viable piglets has been obtained following the transfer of IVM oocytes to the

oviduct of a recipient sow and subsequent artificial insemination (Coy *et al.* 1999).

There is an urgent need for research into basic phenomena, such as those described above, if we want to improve the IVP of pig embryos. Lines of particular interest would be the analysis of oviductal fluid composition aimed at establishing chemically defined culture media that resemble the physiological environment. In addition, it would be of great use to determine the changes occurring in calcium or protein content of oviductal fluid in the hours around fertilization, as well as modifications in the oocyte once it has reached the oviduct. There is some evidence that the zona pellucida of ovulated porcine oocytes contains oestrogen-dependent oviductal glycoproteins (Hedrick *et al.* 1987; Buhi *et al.* 1993) and these proteins may be responsible for the changes that occur in the oocyte to avoid polyspermy. Porcine oviducal cell cultures will prove useful for this type of experiment and, similarly, may help to improve our understanding of the changes that the spermatozoon undergoes when attached to these cells in the oviductal isthmus during the hours before fertilization. Finally, cell culture models could also be applied to determine whether the zona reaction in IVF oocytes is similar to that occurring *in vivo*. According to Cran and Cheng (1986), IVM oocytes undergo incomplete exocytosis of the cortical granule that could be related to polyspermy. The findings of recent studies using improved IVM media seem to indicate that IVM oocytes undergo a complete cortical reaction under certain conditions (Wang *et al.* 1997), although the effect of cortical granule content on the control of polyspermy does not seem to be the same as *in vivo* (Coy *et al.* 2002). Today, there are indications that point to a different morphology of the zona pellucida of IVM and ovulated oocytes at the time of sperm penetration, even though the zona reaction can appear similar (Funahashi *et al.* 2000). It is clear that future efforts need to focus on the *in vitro* zona reaction in the different IVF culture media, in the presence and absence of oviductal epithelial cells or other factor(s) with respect to the *in vivo* reaction.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) is a special kind of IVF whereby a single spermatozoon is injected into the oocyte with the help of a micromanipulator. Using this technique, the step at which the male gamete has to cross physiological barriers, such as the zona pellucida or the plasma membrane of the oocyte, is avoided. Intracytoplasmic sperm injection has certain advantages and drawbacks over IVF. One of the advantages of ICSI, is that it allows us to more precisely control the exact interaction of

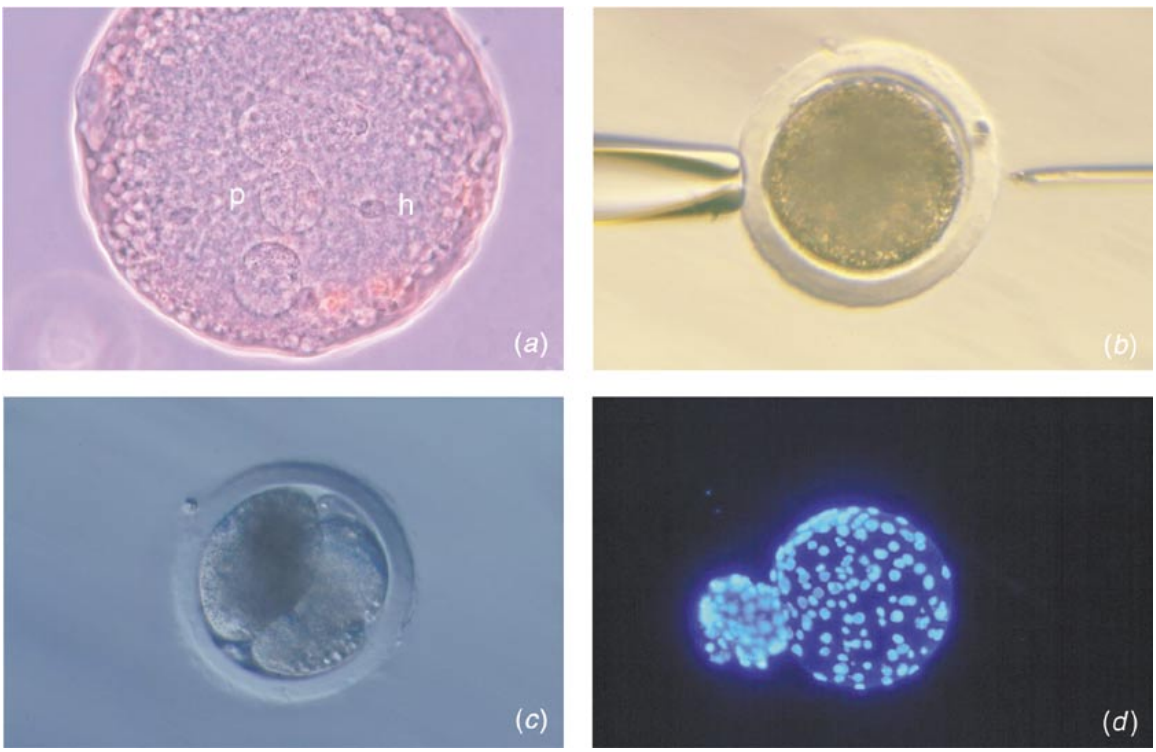

Fig. 3. (*a*) Classical polyspermic oocyte showing several spermatozoa as swollen heads (h) or pronuclei (p; Lacmoid staining at ×40). (*b*) Detail of the intracytoplasmic sperm injection procedure. (*c*) An *in vitro* mature/*in vitro* fertilized 2–4-cell stage embryo (Hoffman modulation contrast microscopy at ×20). (d) Hatching blastocyst showing a high number of nuclei after staining with Hoescht and viewed under ultraviolet light (original magnification ×20).

contact between gametes, because only one spermatozoon is introduced into the oocyte. This is of enormous benefit when working with high-quality semen and a low number of spermatozoa. For example, current studies on sorted semen are showing considerable success (Rath *et al.* 1999) and it is foreseen that the freezing of sorted porcine semen samples will be possible in a short time. Furthermore, the possibility of genetically modified sperm (i.e. their use as carriers of desirable genetic material) being introduced into an oocyte becomes ever-more feasible (Shim *et al.* 2000). Spermatozoa with highly specific characteristics (from boars of high genetic merit, of the desired sex, frozen or genetically modified) are not easily obtained in high numbers and, consequently, each is of high independent value. The ICSI procedure offers the advantage that each spermatozoon enters an oocyte (Fig. 3*b*). The disadvantages of ICSI include the necessity of relatively complex instrumentation (inverted microscope with an adapted micromanipulator), skilled personal and an effective protocol that allows the introduction of a sperm cell into an oocyte in a very short period (< 1 min) in order to obtain a considerable number of fertilized oocytes. Furthermore, several difficulties have been associated with the early embryonic development of these zygotes, with an extremely low or even null proportion reaching the blastocyst stage (Klocke 1999).

Catt and Rhodes (1995) performed the first ICSI trials in the pig and described two types of ICSI experiment, one using frozen–thawed semen and one using fresh semen. Only using fresh semen were Catt and Rhodes (1995) able to achieve a high rate of fertilization (60%; 15/25). In 1996, the same group reported apparently high fertilization rates, which they attributed to the addition of 25 mM $CaCl_2$ to the ICSI spermatozoon suspension and, in this manner, achieved fertilization after the microinjection of fresh sorted spermatozoa (O'Brien *et al.* 1996).

More recently, Kim *et al.* (1998, 1999) have described normal fertilization following ICSI of isolated sperm heads. Blastocysts were produced using complete sperm or sperm heads, although the microtubule matrix was found to be organized differently in the two groups and, in the case of isolated sperm heads, showed a similar pattern to that observed in the mouse (Kuretake *et al.* 1996).

Further interesting work has been described by Klocke *et al.* (Klocke 1999; Klocke *et al.* 1999), in which frozen–thawed spermatozoa were injected after flow cytometric sorting, yielding only slightly inferior results than those obtained using non-sorted semen. These authors also mention a reduced development capacity of embryos derived from ICSI compared with IVF.

It has been possible to obtain live offspring using ICSI since 1990 in cows (Goto *et al.* 1990) and since 1998 in horses (Cochran 1998). The farrowing of the first piglets by this technique was reported in the year 2000 (Kolbe and Holtz 2000; Martin 2000). However, the number of piglets produced was extremely low (one described in the paper by Kolbe and Holtz (2000) and three in the report of Martin (2000)) and the oocytes used in both cases were matured *in vivo*. More recently, the first litter produced using ICSI with semen sorted by flow cytometry has been achieved, with 13 piglets from four transfers (Probst *et al.* 2002).

It is expected that this technique will be improved to obtain larger litters. Another further goal is to obtain piglets by IVM/ICSI/embryo culture and subsequent transfer. Even supposing that this becomes feasible soon, we have yet to establish whether the final result will be a standardized, repeatable method that will allow us to produce enough viable porcine embryos in a regular way.

Applications for in vitro produced embryos

As indicated at the beginning of the present review, the main objective of techniques such as IVM, IVF and ICSI is the production of viable zygotes. Once all the obstacles described have been resolved, future research will focus on the use of these zygotes for desirable end-products. One of the main applications of this technology may be the production of transgenic animals and it seems obvious that obtaining zygotes at the pronuclear stage would be the last stage in this process bcause pronuclear DNA continues to be used for modification of the embryonic genome (Niemann and Rath 2001). However, assuming this point is reached, two clarifications should be made. First, reports of alternative ways of introducing exogenous DNA into the embryo are starting to emerge and, among the most feasible techniques, is the culture of spermatozoa in specific media such that they assimilate desired DNA constructs (Lavitrano *et al.* 1997). This would appear to emphasize the need to standardize IVF methods showing a high final output. Second, whether the new DNA is carried by the spermatozoon or whether it is injected into the pronucleus, the new embryo can be transferred directly or cultured *in vitro* for a variable period before transfer. In either case, the ultimate aim is to obtain a live animal with the desired characteristics that is able to transmit these to its offspring. The following sections provide an overview of the feasibility of these two proposals.

Embryo culture

During the past few years, the *in vitro* culture of pig embryos has shown rapid improvement and, as early as 1993, Petters and Wells published a large review on this subject. They updated the knowledge of findings of the time and provided guidelines for future research centred on trying to improve the success rate of the *in vitro* embryo culture (EC) system.

As these authors described, NCSU was the most commonly used medium because, although it was not as efficient as physiological systems, it was the medium related to the highest blastocyst rate (Macháty *et al.* 1998). An interesting point, which remains unclear, is the difference between the use of NCSU-23 (Abeydeera *et al*. 1998*a*,*b*,*c*; Macháty *et al.*

1998) and NCSU-37 (Hajdu *et al.* 1994; Kikuchi *et al.* 1999). It needs to be established whether taurine and hypotaurine in NCSU-23 should be replaced with sorbitol in NCSU-37 or whether it would perhaps be better to use all three supplements. Both the former (Rath *et al.* 1995; Abeydeera *et al.* 1998*c*) and latter (Petters and Wells 1993; Funahashi *et al.* 1996) compounds have been ascribed important physiological roles and may, therefore, exert synergistic effects when included in a medium in the appropriate ratios. Taurine and hypotaurine act as osmoregulators and pH stabilizers and the supplementation of culture media with taurine has been shown to enhance the development of porcine embryos (Petters and Reed 1991; Petters and Wells 1993), probably owing to a protective effect against peroxidative damage (Guerin *et al.* 1995). In addition, a beneficial effect of sorbitol on embryos has been shown in the mouse, whereby compaction is accelerated and blastocyst expansion improved (Wells *et al.* 1992).

A further interesting development involves the use of serum, starting from Day 5 of culture. According to Koo *et al.* (1997), serum-free media, such as NCSU, need to be completed by the addition of serum from the early morula stage or else the blastocyst will not hatch. Although most research groups have promoted the use of chemically defined media as a necessary step to standardize conditions among the different laboratories (Iwasaki *et al.* 1999), fetal calf serum (FCS), inactivated at 56°C for 30 min, continues to be the most common source of proteins and growth factors in embryo culture media. Owing to the biphasic effect of the serum, whereby early cleavage is inhibited but the compaction of morulae and blastocyst formation are stimulated (Lim *et al.* 1994), FCS should only be added from the 5th day of culture. In pig EC, inactivated serum has been used in different media at concentrations ranging from 10% (Dobrinsky *et al.* 1996) to 20% (Koo *et al.* 1997). The use of non-inactivated serum for EC would be very useful in order to compare results obtained in the different laboratories, because its properties can be uniformly maintained (Lim *et al.* 1994). Therefore, it would be interesting to determine the effects of the presence or absence of serum (inactivated or non-inactivated) in the medium.

Because the final objective is to yield results as similar as possible to those found *in vivo*, it should not be ignored that cell cultures, such as oviductal epithelial cells or endometrial cells, can provide a culture medium with essential substances. These, in many instances due to a lack of information, are not included in chemically defined media (revised by Rath *et al.* 1995). In porcine EC, the use of oviductal epithelial cells has shown a beneficial effect on final monospermy results after IVF (Nagai and Moor 1990; Romar *et al.* 2001) and the cells have been used with good results in bovine EC systems (Katska *et al.* 1995, 1998). In the same way, in an attempt to mimic *in vivo* conditions, coculture of embryos and endometrial cells has been performed in humans (Katsuragawa *et al.* 1995; Conway-Myers 1998) and in mice (Lai *et al.* 1996; Soong *et al.* 1998) and has been shown to improve embryonic development. In pigs, fertilization takes place in the oviduct and the embryo enters the uterus at the 2- to 4-cell stage (Harper 1988; Fig. 3*c*). This means that, during early development, the embryo is first in contact with oviductal cells and subsequently in contact with endometrial cells. Simulating this microenvironment in the Petri dish may represent an improvement, not only in the number of embryos reaching the blastocyst stage, but also in their quality (Fig. 3*d*), which is still too poor to support regular fetal development upon transfer to recipients (Niemann and Rath 2001).

Finally, another interesting observation is the fact that some embryos from polyspermic oocytes can develop to the blastocyst stage and even produce live offspring (Han *et al.* 1999). In this situation, a female and male pronucleus of a polypronuclear zygote could participate in cleavage and the other male pronucleus may divide separately, giving rise to a mosaic of one haploid and two diploid cells. Han *et al.* (1999) propose that the developmental mechanism of these polypronuclear pig eggs may be analogous to that of yolky eggs and that not all pronuclei of polypronuclear eggs participate in syngamy, resulting in diploid cells in the conceptus. This observation could explain, at least in part, why the outputs of embryo production systems, when measured 48 h after IVF, are higher than 24 h after IVF: in the first case, the observer counts the number of cells that have reached the 2- to 4-cell stage (including 3-cell embryos from a zygote with three pronuclei), whereas in the second case only zygotes showing two pronuclei are taken as viable. However, it is also true that many embryos can present a 'normal' morphology at 48 h and, in reality, they have not been produced by cleavage but by cytoplasmic fragmentation, contributing to the increased rate of embryos wrongly classified as viable. Wang *et al.* (1999) stated that 27% of 2-cell and 51% of 4-cell *in vitro*-derived embryos were abnormal in morphology. In addition, the *in vivo*-derived blastocysts had significantly more cells than *in vitro*-produced blastocysts. Once again, this emphasizes the need to establish standardized criteria if we want to adequately compare output rates. Finally, transfer of IVM/IVF zygotes to the oviduct of recipient animals (Funahashi *et al.* 1994*d*) and culture of *in vivo* derived zygotes (Macháty *et al.* 1998) for 5–6 days suggested that suboptimal EC conditions, but not the IVM process itself, are responsible for poor embryo quality in terms of low cell numbers (Abeydeera 2002).

Embryo transfer

An excellent review of recent developments in pig embryo transfer techniques may be found in Hazeleger and Kemp (1999). Although the details of this topic are outside the scope of the present review, we would like to point out the

importance of research into new approaches designed to achieve a high rate of success with non-surgical procedures. A potential ideal system would be the *in vitro* production of blastocysts (IVM/ICSI/EC or IVM/IVF/EC) followed by a transcervical transfer procedure. However, currently the most feasible method is the surgical transfer of 2- to 4-cell *in vitro*-produced embryos.

Summary

Assisted reproductive techniques in swine offer a promising future of indispensable tools for developing biotechnologies such as transgenesis and xenotransplantation. However, progress is limited at different steps in the *in vitro* production of embryos and further research is required to improve these techiques. For example, the development of maturation media enriched with new supplements has finally resulted in oocytes with a fully matured cytoplasm. However, the ability of such oocytes, once fertilized, to develop needs to be confirmed by achieving the same synchronous pronuclear development, sperm penetration and cleavage rates as obtained with oocytes matured *in vivo*. A high incidence of polyspermy is a frequently encountered problem in porcine embryos and a controlled microenvironment with careful selection of the source of spermatozoa and their treatment, as well as more indepth studies on oviductal fluid and other supplements used in IVF media, are necessary to eliminate or drastically reduce polyspermy. There are still many unanswered questions about the most suitable source of sperm for IVF and studies comparing the effect of sperm processing on the early embryonic development are scarce. In the same way, in order to establish chemically defined media that resemble the physiological environment, there is an urgent need for research into the basic phenomenum of oocyte maturation, including changes occurring in the calcium and protein content of oviductal fluid in the hours around fertilization and those occurring in the oocyte itself once it has reached the oviduct (e.g. with respect to the zona pellucida). Further research efforts also need to be directed towards obtaining larger litters using standardized, repeatable methods of IVM, ICSI and EC to allow subsequent transfer of enough viable embryos for commercial use. Finally, optimization of EC conditions remains, of course, a priority to ensure development of *in vitro*-derived blastocysts of maximum viability with cell numbers comparable to those derived *in vivo* and healthy metabolic activity.

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