Pig embryo production by *in vitro* maturation and fertilization of ovarian oocytes. A review

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(Received 27 March 2006; revised version 31 July 2006; accepted 6 November 2006)

**ABSTRACT**

In addition to their major role in food production, pigs have become an increasingly important species in biomedical applications involving the production of pharmaceutical products and as donors of organs for xenotransplantation. They are also used as a model for studies of human diseases. In pigs, as in other mammals, immature oocytes released from ovarian follicles resume meiosis and complete maturation in culture. Although several systems have been established to generate embryos *in vitro*, the quality of embryos produced *in vitro* is inferior to those produced *in vivo*. This review focuses on recent achievements in the development and identification of defined conditions for the *in vitro* production of porcine embryos. It also discusses the effects of oocyte-donor age, size of follicles used for oocyte recovery, synchronization of meiosis before IVM, supplementations of media for IVM, IVF and embryo culture, oxygen tension during culture and the ways for overcoming polyspermy.

**KEY WORDS:** pig, oocyte, IVM, IVF, embryo culture

**INTRODUCTION**

The development and identification of defined *in vitro* conditions for oocyte maturation and fertilization is required by biotechnological researches since most of the new reproductive technologies rely on these basic techniques. *In vitro* embryo production (IVP) in pigs will contribute to reproductive biotechnology including cloning and transgenesis and will generate multiple opportunities for the
use of these modified animals as bioreactors of pharmaceutical products, donors of organs for xenotransplantation, donors of embryonic stem cell lines or as a model for studies of human diseases. Furthermore, the development of efficient \textit{in vitro} techniques would allow the production of large numbers of mature oocytes and embryos in shorter time and with lower costs than from those produced \textit{in vivo}.

The \textit{in vitro} developmental competence of pig IVM/IVF oocytes was first reported by Matiolli et al. (1989). Then, piglets were born in 1993 from IVP porcine embryos transferred to donors at 2 to 4-cell stage (Yoshida et al., 1993). Since then, several laboratories have developed and improved porcine IVP systems. However, in spite of the progress, the quality of \textit{in vitro} mature oocytes and \textit{in vitro} produced embryos is inferior to those produced \textit{in vivo}. The major problems include improper oocyte maturation, both nucleus and cytoplasmic, high polyspermy and unsatisfactory quality of \textit{in vitro} developed blastocysts.

This review will focus on recent advances in the identification and development of defined \textit{in vitro} conditions for porcine IVP technology.

**OOCYTE IN VITRO MATURATION**

Many of the events that prepare the female gamete for fertilization and make it capable of supporting the initiation and continuation of embryonic development take place during oocyte maturation. Maturation of the oocyte is connected with achievement of the competence to undergo three aspects of maturation, i.e. nuclear, cytoplasmic and genomic (Opiela and Kątska-Książkiewicz, 2004, 2005). Generally, looking into the basic aspects of oocyte meiotic maturation, fully-grown follicular oocytes of most mammals are arrested at G2 phase of the first meiosis (germinal vesicle stage - GV), and resume meiosis \textit{in vivo} in response to specific signals, such as the preovulatory peak in LH, or \textit{in vitro} after being released from the follicular environment and cultured \textit{in vitro} for 20 to 24 h. This resumption is characterized by germinal vesicle breakdown (GVBD), chromosome condensation, and spindle formation. Oocytes then proceed toward metaphase I, anaphase I, telophase I, and without any chromosome decondensation, they enter meiosis II. Oocytes, reaching the metaphase stage of the second meiotic division (MII), accompanied by extrusion of the first polar body, are then arrested again. Resumption of meiosis in pig oocytes (Sun and Nagai, 2003) is controlled by a complex cascade of phosphorylation and dephosphorylation events that lead to activation of the M-phase promoting factor (MPF). The MPF induces M-phase in eukaryotic cells, including oocytes. The MPF is a composite formed by a catalytic subunit (p34cdc2), and a regulatory subunit (cyclin B) and this complex displays a serine/threonine kinase activity. During S and G2 phases,
association of these two subunits leads to the formation of pre-MPF, the inactive form of MPF, which is converted into the active complex by phosphorylation and dephosphorylation events. In the natural oestrous cycle, nuclear, cytoplasmic and genomic maturation are three series of events that occur simultaneously during oocyte maturation. Therefore, a nuclear matured oocyte normally means that it achieves full developmental potential if fertilized. However, in oocyte maturation in vitro these series of maturation events may be dissociated, resulting in the loss of developmental potential due to impaired cytoplasmic maturation and/or asynchrony of nuclear and cytoplasmic maturation (Opiela and Kątska-Książkiewicz, 2004, 2005). Despite the fact that more than 20 years have been dedicated to optimizing oocyte maturation in vitro in a number of species, in vitro matured oocytes still have an overall developmental competence that is far from normal and the kind of developmental abnormalities that are most frequently recorded, such as the large offspring syndrome in ruminants, point to aberrant epigenetic changes as the probable underlying cause (Niemann et al., 2002).

In the pig, meiotic competence of oocytes is reached in ovarian follicles with a diameter of 3 mm or more (Marchal et al., 2002). Oocytes from larger follicles usually are more competent than those from smaller ones (Liu et al., 2002; Marchal et al., 2002), and those from sows develop better than those from prepubertal gilts (Marchal et al., 2001; Ikeda and Takahashi, 2003; Sherrer et al., 2004). The heterogeneity of oocytes from different sources leads to asynchronous meiotic progression during IVM, especially because pig oocytes need a longer culture period (42 to 48 h) than those of other species. Reducing nuclear morphological variation, i.e. meiotic synchronization, before maturation by preincubation without gonadotropins (Funahashi et al., 1997a) or with dibutyryl cAMP (Funahashi et al., 1997b; Somfai et al., 2003) appears to enhance pig oocyte development. Butyrolactone I and roscovitine, which are specific inhibitors of Cdc2 (a universal G2/M-phase regulator) have been found to arrest meiosis in vitro (Motlik et al., 1998; Wu et al., 2002; Le Beux et al., 2003). These substances reversibly block meiosis resumption and may be used to synchronize subsequent nuclear maturation (Hirao et al., 2003; Le Beux et al., 2003). However, there is little evidence to suggest any significant improvement in oocyte developmental competence and no proof of full-term development in any species has been reported (Fair, 2003; Lonergan et al., 2003). Protein synthesis is essential for meiotic resumption of oocytes in vitro in the pig, as in other mammals (Figure 1). It has been shown (Le Beux et al., 2003; Ye et al., 2005) that cycloheximide (CHX), a nonspecific protein synthesis inhibitor, can reversibly block meiotic resumption in porcine oocytes, improving their developmental competence.
Recent IVM methods allow achievement in pig, as in other mammalian species, 80 to 90% of oocytes in MII stage (Figure 2). Similarly as in other mammals, a large percentage of in vitro matured pig oocytes do not reach cytoplasmic maturity. In vitro conditions may cause several disruptions. These include movement of mitochondria to the inner cytoplasm (Sun et al., 2001; Torner et al., 2004), protein synthesis (Ellederova et al., 2006), transport of signals, ions (especially calcium) and other substances (Petr et al., 2000; Sun et al., 2001; Sun and Nagai, 2003). As a consequence of these disruptions incomplete maturation may occur due to the deficiency in some factors needed for full cytoplasmic maturation. To synchronize meiotic resumption, IVM conditions have been improved in recent years, through the realization of the importance of the redox state and glutathione content in relation to cysteine in the maturation medium (Nagai, 2001).

For pig oocyte maturation several culture media have been successfully used, such as North Carolina State University (NCSU) containing taurine and...
hypotaurine - NCSU-23 (Yonedo et al., 2004) or sorbitol - NCSU-37 (Kikuchi et al., 2002; Yoshioka et al., 2003; Karja et al., 2004; Suzuki et al., 2004). Some research groups use Tissue Culture Medium 199 - TCM 199 (Qian et al., 2003; Booth et al., 2005; Skrzyszowska et al., 2005) or modified Krebs-Ringer medium TALP (Yoshida et al., 1993). Maturation medium is usually supplemented with small quantities of additional substances, that have appeared to be beneficial for oocyte maturation and subsequent embryo development. Supplementation of culture medium with cysteine, cysteamine, cycloheximide, glutamine, hormones: FSH, HCG, eCG, leptin, epidermal growth factor, β-mercaptoethanol, follicular fluid, serum or serum albumin improve cytoplasmic maturation as indicated by the higher developmental competence (Grupen et al., 1995; Singh et al., 1997; Abeydeera et al., 1998; Bing et al., 2001; Jeong and Yang, 2001; Marchal et al., 2001; Yoshioka et al., 2003; Craig et al., 2004; Ye et al., 2005).

The process of cumulus cell expansion that occurs both in vivo and in vitro is one of the major indicators of oocyte maturation. It has been shown that supplementation of maturation medium with porcine follicular fluid increases cumulus expansion and cytoplasmic maturation in in vitro matured pig oocytes (Yoshida et al., 1993; Rath et al., 1995; Funahashi et al., 1997a). Indeed, supplementation of IVM medium with follicular fluid has been applied by several authors (Yoshida et al., 1993; Marchal et al., 2001; Kikuchi et al., 2002; Qian et al., 2003; Yoshioka et al., 2003; Suzuki et al., 2004; Skrzyszowska et al., 2005).

Oxygen tension during IVM is also an important factor in cytoplasmic maturity for in vitro development to blastocysts (Kikuchi et al., 2002; Park et al., 2005). When cumulus-oocyte complexes were matured in vitro in the medium NCSU-37 under 5% O2 or 20% O2, fertilized under 5% O2, and subsequently cultured under 5% O2, there were no significant differences in blastocyst rates (Kikuchi et al., 2002). However, the quality of blastocysts, as evaluated by total cell number, was better after IVM under 5% O2 than under 20% O2. Contrary to this observation, the investigations of Macháty et al. (1998) have indicated that embryos developed from oocytes that matured in medium NCSU-23, under 20% O2 showed a higher number of nuclei than those developed under 5% O2. Recent investigations of Park et al. (2005) have shown that high oxygen tension (20%) during IVM significantly improved blastocyst formation (23 vs 13%) after IVF than low oxygen (5%) but did not improve the rates of nuclear maturation, sperm penetration, monospermic fertilization, pronuclear formation, cleavage and blastocyst cell number. On the basis of these investigations we can conclude that oxygen content during IVM should be correlated with type of culture medium used.

Nuclear maturation of oocytes is easily assessed morphologically by the presence of the first polar body, but there is a lack of non-invasive methods to
evaluate cytoplasmic maturation. Until now, the only reliable method for assessing the competence of an oocyte is its post-fertilization development.

**IN VITRO FERTILIZATION**

Fertilization is the cascade of the cellular mechanisms that pass the genome from one generation to the next and initiate development of a new organism. A typical mammalian egg freshly ovulated or *in vitro* matured is enclosed by two layers: an outer layer of corona cells and inner, extracellular matrix, the zona pellucida. To reach the egg plasma membrane, sperm must penetrate both layers in steps requiring sperm motility, sperm surface enzymes, and sperm secreted enzymes. Binding to the oocyte zona pellucida induces the sperm cell to undergo the acrosomal reaction in which the outer acrosomal membrane fuses with the overlying plasma membrane (Primakoff and Myles, 2002). Sperm bind transiently to the oocyte zona pellucida and the plasma membrane and then fuse. This exocytotic event results in the release of hydrolytic enzymes, principally the trypsin like acrosin, and in the exposure of new membrane domains, both of which are essential for the fertilization process. Signaling in the sperm is induced by sperm adhesion to the zona pellucida, and signaling in the oocyte by gamete fusion (Primakoff and Myles, 2002).

However, mammalian spermatozoa are unable to fertilize the oocyte immediately after ejaculation. They require a period of incubation in the female reproductive tract or in the appropriate *in vitro* conditions in order to acquire the capacity to fertilize. During this time, the spermatozoa undergo a poorly defined process of maturation known as capacitation (Breitbart, 2003). Sperm capacitation includes a cascade of biochemical changes that must occur before spermatozoa can effectively interact with an oocyte. This involves activation of adenylyl cyclase, protein tyrosine phosphorylation and actin polymerization, cholesterol efflux, increases in calcium ions and changes in sperm motility (Breitbart, 2003). It had been generally accepted that spermatozoa are translationally and transcriptionally silent; however, the latest investigations of Gur and Breitbart (2006) have demonstrated, for the first time, incorporation of labeled amino acids into polypeptides during sperm capacitation. These authors also demonstrated that protein translation in sperm involves mitochondrial but not cytoplasmic ribosomes and that inhibition of protein translation significantly reduced sperm motility, capacitation and *in vitro* fertilization rate. The importance of the mitochondrion for pig fertilization has also been shown by El Shourbagy et al. (2006). Their data suggest that mitochondrial number is important for fertilization outcome and embryonic development. Furthermore, a mitochondrial pre-fertilization threshold
may ensure that, as mitochondria are diluted out during post-fertilization cleavage, there are sufficient copies of mtDNA per blastomere to allow transmission of mtDNA to each cell of the embryo after the initiation of mtDNA replication during the early postimplantation stages (El Shourbagy et al., 2006).

Following sperm penetration into the cytoplasm of a mature oocyte, the highly condensed chromatin of the sperm nucleus first decondenses and the protamines are replaced by histones. After a short period of chromatin recondensation a final phase of decondensation and the formation of the interphase male pronucleus, surrounded by a new organized pronuclear membrane, occurs. During this sperm chromatin structure reorganization, and particularly during protamine-histone replacement, profound DNA epigenetic modifications take place. At the same time the maternal genome is also modified and prepared for integration with the paternal genome (Gioia et al., 2005).

During pig in vitro fertilization, sperm penetration begins at 3 h post-insemination (Ding et al., 1992). Sperm penetration quickly induces the resumption of meiosis and cortical reaction that blocks polyspermy. By 5 h, decondensing sperm head and anaphase II plate are observed in half of the oocytes, and by 8 h, both female and male pronuclei are formed (Ding et al., 1992). In appropriate in vitro conditions 60 to 70% of in vitro mature pig oocytes may be penetrated by spermatozoa; however, the rate of normally fertilized oocytes, i.e. monospermic fertilization is rather low while exceptionally high incidence of polyspermic fertilization has been observed. Polyspermy is considered to be one of the persistent and most difficult problems to overcome in pig IVF. Incidence of polyspermy in porcine eggs in vivo can reach 30 to 40%, and polyspermy rate in the in vitro fertilized eggs can be as high as 65% (Xia et al., 2001). The reasons for the occurrence of polyspermy in pig oocytes are not clear, and our knowledge about the exact mechanisms for preventing polyspermy in this species is relatively poor. Low developmental rates of in vitro produced porcine embryos may, therefore, be caused not only by inadequate culture conditions but by a high incidence of polyspermy, a lethal condition in mammals (Hunter and Nicol, 1988). Although polyspermic porcine embryos can develop to blastocysts, they have fewer numbers of inner cell mass cells compared with monospermic embryos (Han et al., 1999). Polyspermic penetration in vitro is caused by a delayed zona reaction and/or the simultaneous penetration by a number of spermatozoa with a reacted acrosome (Wang et al., 1999; Funahashi and Nagai, 2001; Yoshioka et al., 2003).

To reduce the incidence of polyspermic penetration several systems of sperm capacitation have been developed. It has been shown that methylxanthines, such as caffeine and theophylline, can enhance the ability of sperm to penetrate in vitro matured porcine oocytes stimulating and maintaining sperm motility by acting as phosphodiesterase inhibitors, presumably by elevating cAMP levels
The replacement of caffeine with adenosine in a porcine IVF system increased the incidence of monospermic penetration (Funahashi and Nagai, 2001; Yoshioka et al., 2003). Addition of cysteine to the maturation medium, the precursor of glutathione, can increase intracellular glutathione levels resulting in an enhancement of male pronucleus formation and the percentage of zygotes developing to blastocysts (Yoshioka et al., 2003). It has also been reported that follicular fluid may decrease the incidence of polyspermic penetration of porcine oocytes (Abeydeera, 2002). Furthermore, incubation of spermatozoa with oviductal epithelial cells during capacitation prior to IVF and fertilization may reduce polyspermy by 40 to 50% (Nagai and Moor, 1990). Supplementation of culture medium with bovine serum albumin (BSA) accelerated the ability of porcine spermatozoa to penetrate in vitro mature oocytes (Suzuki et al., 1994). Serum albumin has numerous properties that can play a role in capacitation, including removal or alteration of sperm membrane surface components and alteration of cholesterol:phospholipid ratios in sperm membranes (Suzuki et al., 1994). According to Suzuki et al. (1994) replacement of BSA with polyvinyl alcohol decreases the number of polyspermic oocytes and the number of spermatozoa per penetrated oocyte. Also anti-hyaluronidase oligosaccharide derived from chondroitin sulphate A effectively reduces the incidence of polyspermy during IVF of porcine oocytes promoting the normal fertilization process as has recently been shown by Tatemoto et al. (2005). However, according to Rath et al. (2005) the only way to avoid polyspermic penetration is to reduce the number of spermatozoa per oocyte used for IVF. The amount of spermatozoa depends on the treatment of the sperm and has to be set for each individual boar (Rath et al., 2005). Recent investigations of Koo et al. (2005) have also shown that the spermatozoa concentration during in vitro fertilization may be important for developmental competence and quality of pig embryos.

All three steps of in vitro embryo production, i.e. maturation, fertilization and embryo culture are closely mutually dependent. The capability of reprogramming the male chromatin after fertilization is dependent on the quality of oocyte maturation (Gioia et al., 2005). In fact, as has been shown by Gioia et al. (2005), while in about 80% of in vivo matured and in vitro fertilized pig oocytes the male pronucleus underwent a process of active demethylation and showed a condition of histone H4 hyperacetylation, only 40% of IVM/IVF zygotes displayed a similar pronucleus remodeling asymmetry. However, oocytes that carried out the first part of maturation in vivo (up to GVBD) and then completed the process in vitro, displayed the same pronucleus asymmetry as oocytes matured entirely in vivo (Gioia et al., 2005). Therefore, in addition to the improvement of IVM/IVF procedures, improvement of the in vitro embryo culture procedure seems to be the more important factor needed to obtain viable blastocysts.
EMBRYO CULTURE

The development of in vitro produced porcine embryos from the two-cell to the hatched blastocyst stage (Figure 3a-f) requires adequate culture conditions.

In spite of intensive efforts of several investigators, there are very few satisfactory protocols for embryo culture in vitro. The main problem in pig embryo culture is relatively low quality in terms of total cell number compared to in vivo embryos of the same chronological age (Macháty et al., 1998). For example, the total cell number of blastocysts was reported to range from 30 to 38 on day 6 after IVF, while the mean number of cells in 6-day-old expanded blastocysts developed in vivo was twice as high, i.e. 74 cells (Abeydeera and Day, 1997; Wang et al., 1997). Furthermore, blastocyst development is a poor indicator of embryo viability. The most valid criterion of embryo viability is in vivo development to term following embryo transfer to a synchronized recipient.
In many studies oriented on in vitro culture of pig embryos, several modifications of the culture conditions were used. These modifications included different media (Wang et al., 1997; Macháty et al., 1998; Gajda and Smorag, 2004; Im et al., 2004), differentiated volumes of culture medium, medium covered with mineral oil or without covering (Yoshioka et al., 2002, 2003; Gil et al., 2003; Im et al., 2004, 2005), group embryo culture with differentiated distances between embryos (Stokes et al., 2005) and supplementation of medium with different ingredients (Ka et al., 1997; Swain et al., 2002; Karja et al., 2004; Booth et al., 2005; Craig et al., 2005; Lee et al., 2005).

For pig embryo culture several media have been successfully used: North Carolina State University containing taurine and hypotaurine - NCSU 23 (Macháty et al., 1998; Lee et al., 2003; Gajda and Smorag, 2004; Im et al., 2004; Yoneda et al., 2004; Skrzyszowska et al., 2005) or NCSU containing sorbitol - NCSU 47 (Kikuchi et al., 2002; Gajda and Smorag, 2004; Karja et al., 2004), Carles Rosenkrans 2 - CR2 (Bettauser et al., 2000), potassium simplex optimized medium - KSOM (Macháty et al., 1998), modified Calot, Ziómek, Bavister-CZB (Gajda and Smorag, 2004), Beltsville embryo culture medium - BECM 3 (Onishi et al., 2000; Im et al., 2004), porcine zygote medium - PZM 4 (Yoshioka et al., 2003) or modified Whitten’s medium (Onishi et al., 2000).

The comparative study of Gajda and Smorag (2004) on in vitro development of in vivo produced 2 to 4-cell pig embryos cultured in different media (NCSU-23, NCSU-37 and CZB) has revealed that the highest percentage of embryos reached the blastocyst stage in NCSU-23 (89.2%) followed by NCSU-37 (78.9%) and CZB (67.8%) medium. The average total number of cells in the blastocyst developed in NCSU-23, NCSU-37 and CZB media was 139.5±32.8, 71.9±36.6 and 58.3±8.6, respectively (Gajda and Smorag, 2004).

The developmental competence of porcine embryos can be markedly affected by protein supplementation (Ka et al., 1997). Preimplantation embryos can resume and produce amino acids in a manner dependent upon the stage of development that may be predictive of subsequent viability. The investigations of Booth et al. (2005) have shown that the net rates of depletion and uptake of amino acids by pig embryos vary between amino acids, the day of embryo development and the type of embryos present at a given stage of development.

The in vitro development of porcine zygotes to the blastocyst stage may be facilitated by culture in groups, suggesting a role for autocrine/paracrine factors. Recent reports of Stokes et al. (2005) on culture of in vitro produced and in vivo derived porcine embryos have suggested a role for, as yet unknown diffusible paracrine/autocrine factors released by early porcine embryos, in promoting the growth of neighbouring embryos in vitro. The development of individual zygotes to the blastocyst stage was optimal when they were cultured at a distance of
between 81 and 160 μm. As the distance between the embryos was increased, blastocyst rates declined significantly. Blastocyst volume and cell number (both inner cell mass and trophectoderm) were also increased when the distance apart was between 81 and 160 μm. Culturing embryos in groups at different stages of development suggested that group culture confers a greater advantage to development after the activation of the genome. Group culture of in vivo derived embryos showed a weak distance effect. This advantage was observed to a lesser extent by in vivo derived zygotes which are likely to have been better conditioned for development in vitro by being conceived in the female reproductive tract.

Recently Lee et al. (2005) have pointed to a favourable effect of supplementing pig oocyte and embryo culture medium with insulin and metformin. When added during the entire IVM and IVC, insulin increased the developmental potential of porcine oocytes and embryos, and metformin enhanced the action of insulin. The effects of insulin and metformin were associated with oocyte GSH content and tyrosine kinase activity. Insulin significantly increased oocyte GSH content and metformin significantly enhanced the action of insulin on GSH content and tyrosine kinase activity compared to insulin alone. Recent studies have also suggested that leptin plays an important role in embryo development (Craig et al., 2005). As has already been mentioned, leptin increases oocyte maturation in vitro, and inclusion of leptin in both IVM and embryo culture medium further increased blastocyst development compared to when leptin was included in the embryo culture alone. These results have suggested that lepin has a synergistic role on both oocyte maturation and preimplantation embryo development (Craig et al., 2005).

In addition, the oxygen concentration has been shown to be a major factor causing a difference in the developmental rates of porcine IVM/IVF embryos between in vivo and in vitro environments. The oxygen concentration in the mammalian oviduct and uterus is about 5% (Fisher and Bavister, 1993), whereas in vitro cultured embryos are usually maintained under 5% CO₂ and 95% air, i.e. 20% O₂. Reduction of the O₂ concentration from 20 to 5% has been shown to enhance embryonic development in humans (Dumoulin et al., 1995; Catt and Henman, 2000), cattle (Liu and Foote, 1995; Lim et al., 1999), sheep (Thompson et al., 1990) and mice (Goto et al., 1992; Dumoulin et al., 1995). The developmental rate to the blastocyst stage of IVM/IVF porcine embryos cultured under 5% O₂ was significantly higher than that of embryos cultured under 20% O₂ (Yoneda et al., 2004). On the other hand, there was no difference in the developmental rate to the blastocyst stage between in vivo fertilized oocytes cultured under 5% O₂ and 20% O₂ (Yoneda et al., 2004).

The metabolism of porcine embryos produced both in vivo and in vitro is different from that of other species, as they metabolize glucose throughout
preimplantation development (Swain et al., 2002; Karja et al., 2004). Some reports showed that glucose inhibits embryo development before compaction or before the blastocyst stage in mice (Gardner and Leese, 1988) and cattle (Kim et al., 1993). Investigations of Swain et al. (2002) showed that pig embryos use glucose via glycolysis in significant amounts at all stages examined, regardless of embryo origin. In vitro derived embryos have significantly increased glycolytic activity after the eight cell stage, whereas in vivo derived embryos have increased glycolysis at the blastocyst stage. In vivo derived embryos have higher rates of glycolysis compared with in vitro derived embryos. Glucose usage through the Krebs cycle for in vitro- and in vivo derived embryos increased significantly at the blastocyst stage. Pig embryos produced in vitro used constant amounts of glutamine throughout development, whereas in vivo derived embryos increased glutamine usage after the eight-cell stage. Pyruvate use was minimal at all stages examined for both in vitro- and in vivo derived pig embryos, showing significant increases at the blastocyst stage. Krebs cycle metabolism of pyruvate, glutamine and glucose by in vivo derived embryos was higher than that by in vitro derived embryos (Swain et al., 2002). Therefore it can be concluded that in vitro culture conditions produce pig embryos with altered metabolic activity, which may compromise embryo viability (Swain et al., 2002). It has been shown by Karja et al. (2004) that the replacement of pyruvate and lactate with glucose at 58 h of culture significantly enhanced the rate of blastocyst production. Successful culture conditions allowing the expanded blastocysts to be obtained on Day 6 of embryo culture with the mean cell number of 80 cells were reported by Kikuchi (2004). Embryo culture was carried out for the first 2 days in the conditioned medium obtained from the culture of porcine oviductal epithelial cells, then medium was supplemented with glucose (Kikuchi, 2004). Moreover, some of these blastocysts possessed the ability to develop to term. When Day 5 expanded blastocysts (50 blastocysts per recipient) were transferred to an oestrus-synchronized recipient they farrowed 8 normal piglets, and out of Day 6 expanded blastocysts transferred to two recipients a total of 11 piglets were obtained (Kikuchi, 2004).

In spite of obtaining satisfactory results of pig embryo development in the conditioned medium (Kikuchi, 2004), there are intensive efforts aimed to develop chemically defined porcine culture medium (Yoshioka et al., 2002, 2003; Cui et al., 2004; Kishida et al., 2004; Booth et al., 2005). Because a chemically defined medium eliminates undefined factors present in biological materials such as follicular fluid, serum or serum albumin and/or co-culture with somatic cells, application of a chemically defined medium to IVP of embryos has certain advantages. For instance, the use of defined media facilitates the analysis of the physical action of substances on the development of preimplantation embryos, improves reliability of formulations, yields a higher reproducibility of results, and
ensures biosafety of culture media by elimination of protein preparations, which may be contaminated with pathogens (Bavister, 1995). However, up to now the complete chemically defined systems for porcine IVP have not been developed. For example, although there was no significant difference in cleavage rates between newborn calf serum and polyvinyl alcohol supplemented media containing both cysteine and epidermal growth factor, the rate of blastocyst development was significantly lower in the defined medium than in the serum containing medium (Kishida et al., 2004). In spite of these lower results, it may be expected that in the near future chemically defined media for oocyte and embryo culture will be used in practical applications of pig IVP.

In conclusion, application of advanced embryo production technologies is essential for the progression of animal breeding and for the use of animals in the production of pharmaceuticals and for xenotransplantation purposes. However, the associated in vitro techniques have inherent problems, which from a mechanistic viewpoint, are not well understood. For widespread use of IVP technology it is essential to remove all the side effects observed following culture. The greatest concern is related to the production loss as a consequence of lowered embryo quality, high rates of conceptus loss and to the deviations in pregnancy, parturition and the development of offspring. It should be emphasized however that many normal offspring have been born using IVP and that the method may be successfully used, albeit with low efficiency and with concerns regarding the well being of the offspring.

ACKNOWLEDGEMENTS

The figures presented in the article were done by my colleagues: Dr. Maria Skrzyszowska (Figure 1) and Dr. Barbara Gajda (Figures 3a-f) from the National Research Institute of Animal Production in Balice (Poland) and Figure 2 was done by Dr. Hannelore Alm from the Research Institute for the Biology of Farm Animals, Dummerstorf/Rostock (Germany).
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