Journal of Animal and Feed Sciences, 15, 2006, 525–542

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

L. Kątska-Książkiewicz[1]

Rzeszów University, Branch Campus of the Faculty of Biotechnology,
Department of Bioengineering
36-100 Kolbuszowa, Poland

(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

[4] Corresponding author: e-mail: lkatska@izoo.krakow.pl

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 *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 *in vivo*.

The *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 *in vitro* mature oocytes and *in vitro* produced embryos is inferior to those produced *in vivo*. The major problems include improper oocyte maturation, both nucleus and cytoplasmic, high polyspermy and unsatisfactory quality of *in vitro* developed blastocysts.

This review will focus on recent advances in the identification and development of defined *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 *in vivo* in response to specific signals, such as the preovulatory peak in LH, or *in vitro* after being released from the follicular environment and cultured *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 G_2/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.

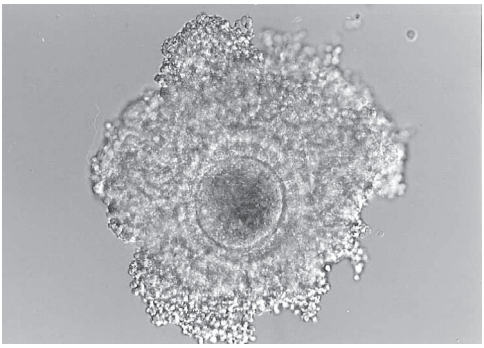

Figure 1. An immature pig oocyte surrounded by compact, dense cumulus cell layers, suitable for *in vitro* maturation (x100)

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

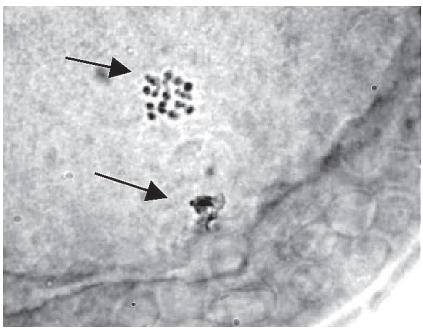

Figure 2. *In vitro* matured pig oocyte at metaphase II stage. The chromosomes arranged in metaphase plate and first polar body are indicated (x1000)

hypotaurine - NCSU-23 (Yoneda 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% O_2 or 20% O_2, fertilized under 5% O_2, and subsequently cultured under 5% O_2, 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% O_2 than under 20% O_2. 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% O_2 showed a higher number of nuclei than those developed under 5% O_2. 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

(Yoshioka et al., 2003; Funahashi and Romar, 2004). 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.

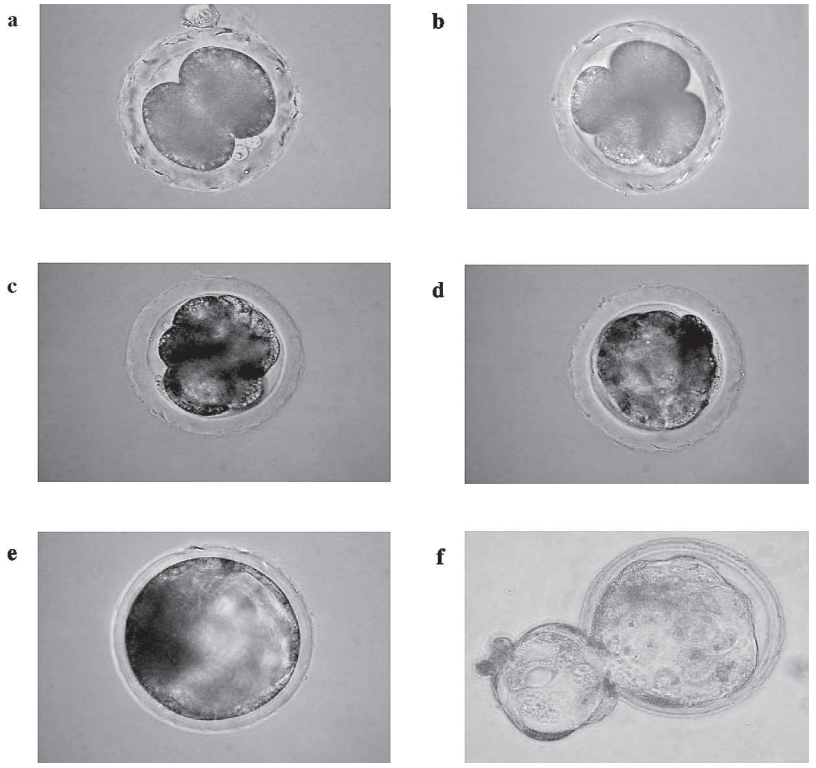

Figure 3. Pig embryos at: a. 2-cell stage; b. 4-cell stage; c. 8-cell stage; d. morula; e. the late blastocyst; f. hatching blastocyst

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 Smorąg, 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 Smorąg, 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 Smorąg, 2004; Karja et al., 2004), Carles Rosenkrans 2 - CR2 (Bettauser et al., 2000), potassium simplex optimized medium - KSOM (Macháty et al., 1998), modified Calot, Ziomek, Bavister-CZB (Gajda and Smorąg, 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 Smorąg (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 Smorąg, 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% CO_2 and 95% air, i.e. 20% O_2. Reduction of the O_2 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% O_2 was significantly higher than that of embryos cultured under 20% O_2 (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% O_2 and 20% O_2 (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).

REFERENCES

Abeydeera L.R., 2002. In vitro production of embryos in swine. Theriogenology 57, 256-273

Abeydeera L.R., Day B.N., 1997. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 48, 537-544

Abeydeera L.R., Wang W.H., Cantley T.C., Prather R.S., Day B.N., 1998. Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. Theriogenology 50, 747-756

Bavister B.D., 1995. Culture of preimplantation embryo: facts and artifacts. Hum. Reprod. Update 1, 91-148

Bettauser J., Forsberg E., Augenstein M., Childs L., Eilertsen K., Enos J., Forsythe T., Golueke P., Jurgella G., Koppang R., Lesmeister T., Mallon K., Mell G., Misica P., Pace M., Pfister-Genskow M., Strelchenko N., Voelker G., Watt S., Thompson S., Bishop M., 2000. Production of cloned pigs from in vitro systems. Nat. Biotechnol. 18, 1055-1059

Bing Y.Z., Rodriguez-Martinez H., Nagai T., 2001. Effects of cysteamine, FSH, and estradiol-17β on in vitro maturation of porcine oocytes. Theriogenology 55, 867-876

Booth P.J., Humpherson P.G., Watson T.J., Leese H.J., 2005. Amino acid depletion and appearance during porcine preimplantation embryo development in vitro. Reproduction 130, 655-668

Breitbart H., 2003. Signaling pathways in sperm capacitation and acrosome reaction. Cell. Mol. Biol. 49, 321-327

Catt J.W., Henman M., 2000. Toxic effects of oxygen on human embryo development. Hum. Reprod. 15, Suppl. 2, 199-206

Craig J., Zhu H., Dyce P.W., Wen L., Li J., 2004. Leptin enhances oocyte nuclear and cytoplasmic maturation via the mitogen-activated protein kinase pathway. Endocrinology 145, 5355-5363

Craig J., Zhu H., Dyce P.W., Wen L., Li J., 2005. Leptin enhances porcine preimplantation embryo development in vitro. Mol. Cell Endocrinol. 229, 141-147

Cui X.S., Lee J.Y., Choi S.H., Kwon M.S., Kim T., Kim N.H., 2004. Mouse granulocyte-macrophage colony-stimulating factor enhances viability of porcine embryos in defined culture conditions. Anim. Reprod. Sci. 84, 169-177

Ding J., Clarke N., Nagai T., Moor R.M., 1992. Protein and nuclear changes in pigs at fertilization. Mol. Reprod. Dev. 31, 287-296

Dumoulin J.C., Vanvuchelen R.C., Land J.A., Pieters M.H., Geraedts J.P., Evers J.L., 1995. Effect of oxygen concentration on in vitro fertilization and embryo culture in the human and the mouse. Fert. Steril. 63, 115-119

El Shourbagy S.H., Spikings E.C., Freitas M., St. John J.C., 2006. Mitochondria directly influence fertilization outcome in the pig. Reproduction 131, 233-245

Ellederova Z., Kovarova H., Melo-Sterza F., Livingstone M., Tomek W., Kubelka M., 2006. Suppression of translation during in vitro maturation of pig oocytes despite enhanced formation of cap-binding protein complex eIF4F and 4E-BP1 hyperphosphorylation. Mol. Reprod. Dev. 73, 68-76

Fair T., 2003. Follicular oocyte growth and acquisition of developmental competence. Anim. Reprod. Sci. 78, 203-216

Fisher B., Bavister B.D., 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J. Reprod. Fertil. 99, 673-679

Funahashi H., Cantley T.C., Day B.N., 1997a. Preincubation of cumulus-oocyte complexes before exposure to gonadotropins improves the developmental competence of porcine embryos matured and fertilized in vitro. Theriogenology 47, 679-686

Funahashi H., Cantley T.C., Day B.N., 1997b. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. Biol. Reprod. 57, 49-53

Funahashi H., Nagai T., 2001. Regulation of in vitro penetration of frozen-thawed boar spermatozoa by caffeine and adenosine. Mol. Reprod. Dev. 58, 424-431

Funahashi H., Romar R., 2004. Reduction of the incidence of polyspermic penetration into porcine oocytes by pretreatment of fresh spermatozoa with adenosine and transient co-incubation of the gametes with caffeine. Reproduction 128, 789-800

Gajda B., Smorąg Z., 2004. Cell number in pig blastocysts cultured in different media. Ann. Anim. Sci. 4, 315-320

Gardner D.K., Leese H.J., 1988. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. Development 104, 423-429

Gil M.A., Abeydeera L.R., Day B.N., Vazquez J.M., Roca J., Martinez E.A., 2003. Effect of the volume of medium and number of oocytes during in vitro fertilization on embryo development in pigs. Theriogenology 60, 767-776

Gioia L., Barboni B., Turriani M., Capacchietti G., Pistilli M.G., Berardinelli P., Mattioli M., 2005. The capability of reprogramming the male chromatin after fertilization is dependent on the quality of oocyte maturation. Reproduction 130, 29-39

Goto Y., Noda Y., Narimoto K., Umaoka Y., Mori T.,1992. Oxidative stress on mouse embryo development in vitro. Free Radial. Biol. Med. 13, 673-679

Grupen C.G., Nagashima H., Nottle M.B., 1995. Cysteamine enhances in vitro development of porcine oocytes matured and fertilized in vitro. Biol. Reprod. 53**,** 173-178

Gur Y., Breitbart H., 2006. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. Genes Dev. 20, 411-416

Han Y.M., Abeydeera L.R., Kim J.H., Moon H.B., Cabot R.A., Day B.N., Prather R.S., 1999. Growth retardation of inner cell mass cells in polyspermic porcine embryos produced in vitro. Biol. Reprod. 60, 1110-1113

Hirao Y., Nishimoto N., Kure-bayashi S., Takenouchi N., Yamauchi N., Masuda H., Nagai T., 2003. Influence of meiotic inhibition by butyrolactone I during germinal vesicle stage on the ability of porcine oocytes to be activated by electric stimulation after nuclear maturation. Zygote 11, 191-197

Hunter R.H.F., Nicol R., 1988. Capacitation potential of the fallopian tube: a study involving surgical insemination and the subsequent incidence of polyspermy. Gamete Res. 21, 255-266

Ikeda K., Takahashi Y., 2003. Comparison of maturational and developmental parameters of oocytes recovered from prepubertal and adult pigs. Reprod. Fert. Develop. 15, 215-221

Im G.S., Lai L., Liu Z., Hao Y., Wax D., Bonk A., Prather R.S., 2004. In vitro development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology 61, 1125-1135

Im G.S., Yang B.S., Lai L., Liu Z., Hao Y., Prather R.S., 2005. Fragmentation and development of preimplantation porcine embryos derived by parthenogenetic activation and nuclear transfer. Mol. Reprod. Dev. 71, 159-165

Jeong B.S., Yang X., 2001. Cysteine, glutathione, and Percoll treatments improve porcine oocyte maturation and fertilization in vitro. Mol. Reprod. Dev. 59, 330-335

Ka H.H., Sawai K., Wang W.H., Im K.S., Niwa K., 1997. Amino acids in maturation medium and presence of cumulus cells at fertilization promote male pronuclear formation in porcine oocytes matured and penetrated in vitro. Biol. Reprod. 57, 1478-1483

Karja N.W.K., Medvedev S., Onishi A., Fuchimoto D.I., Iwamoto M., Otoi T., Nagai T., 2004. Effect of replacement of pyruvate/lactate in culture medium with glucose on preimplantation development of porcine embryos in vitro. J. Reprod. Dev. 50**,** 587-592

Kikuchi K., 2004. Developmental competence of porcine blastocysts produced in vitro. J. Reprod. Dev. 50, 21-28

Kikuchi K., Onishi A., Kashiwazaki N., Iwamoto M., Noguchi J., Kaneko H., Akita T., Nagai T., 2002. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biol. Reprod. 66, 1033-1041

Kim J.H., Niwa K., Lim J.M., Okuda K., 1993. Effects of phosphate, energy substrates, and amino acids on development of in vitro-matured, in vitro-fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol. Reprod. 40, 1320-1325

Kishida R., Lee E.S., Fukui Y., 2004. In vitro maturation of porcine oocytes using a defined medium and developmental capacity after intracytoplasmic sperm injection. Theriogenology 62, 1663-1676

Koo D.B., Kim Y.J., Yu I., Kim H.N., Lee K.K., Han Y.M., 2005. Effects of in vitro fertilization conditions on preimplantation development and quality of pig embryos. Anim. Reprod. Sci. 90, 101-110

Le Beux G., Richard F.J., Sirard M.A., 2003. Effect of cycloheximide, 6-DMAP, roscovitine and butyrolactone I on resumption of meiosis in porcine oocytes. Theriogenology 60, 1049-1058

Lee J.W., Wu S.C., Tian X.C., Barber M., Hoagland T., Riesen J., Lee K.H., Tu C.F., Cheng W.T.K., Yang X., 2003. Production of cloned pigs by whole-intracytoplasmic microinjection. Biol. Reprod. 69, 995-1001

Lee M.S., Kang S.K., Lee B.C., Hwang W.S., 2005. The beneficial effects of insulin and metformin on in vitro developmental potential of porcine oocytes and embryos. Biol. Reprod. 73, 1264-1268

Lim J.M., Reggio B.C., Godke R.A., Hansel W., 1999. Development of in vitro-derived bovine embryos cultured in 5% O_2, 5% CO_2 and 90% N_2. Hum. Reprod. 14, 458-464

Liu R.H., Li Y.H., Jiao L.H., Wang X.N., Wang H., Wang W.H., 2002. Extracellular and intracellular factors affecting nuclear and cytoplasmic maturation of porcine oocytes collected from different sizes of follicles. Zygote 10, 253-260

Liu Z., Foote R.H., 1995. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O_2. Biol. Reprod. 53, 786-790

Lonergan P., Faerge I., Hyttel P.M., Boland M., Fair T., 2003. Ultrastructural modifications in bovine oocytes maintained in meiotic arrest in vitro using roscovitine or butyrolactone. Mol. Reprod. Dev. 64, 369-378

Macháty Z., Day B.N., Prather R.S., 1998. Development of early porcine embryos in vitro and in vivo. Biol. Reprod. 59, 451-455

Marchal R., Feugang J.M., Pereau C., Venturi E., Terqui M., Mermillod P., 2001. Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 56, 17-29

Marchal R., Vigneron C., Pereau C., Bali-Papp A., Mermillod P., 2002. Effect of follicular size on meiotic and developmental competence of porcine oocytes. Theriogenology 57, 1523-1532

Mattioli M., Bacci M.L., Galeati G., Seren E., 1989. Developmental competence of pig oocytes matured and fertilized in vitro. Theriogenology 31, 1201-1207

Motlik J., Pavlok A., Kubelka M., Kalous J., Kalab P., 1998. Interplay between cdc2 kinase and map kinase pathway during maturation of mammalian oocytes. Theriogenology 49, 461-469

Nagai T., 2001. The improvement of in vitro maturation systems for bovine and porcine oocytes. Theriogenology 55, 1291-1301

Nagai T., Moor R.M., 1990. Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilized in vitro. Mol. Reprod. Dev. 57, 256-273

Niemann H., Wrenzycki C., Lucas-Hahn A., Brambrink T., Kues W.A., Carnwath J.W., 2002. Gene expression patterns in bovine in-vitro produced and nuclear transfer-derived embryos and their implications for early development. Cloning Stem Cells 4, 29-38

Onishi A., Iwamoto M., Akita T., Mikawa S., Takeda K., Awata T., Hanada H., Perry A.C.F., 2000. Pig cloning by microinjection of fetal fibroblast nuclei. Science 289, 1188-1190

Opiela J., Kątska-Książkiewicz L., 2004. Characterization of mammalian oocyte competence to undergo fertilization and embryonic development. I. Nuclear maturation and molecular aspects of its regulation (in Polish). Biotechnologia 66, 107-118

Opiela J., Kątska-Książkiewicz L., 2005. Characterization of mammalian oocyte competence to undergo fertilization and embryonic development. II. Regulation of cytoplasmic and genomic maturation (in Polish). Biotechnologia 69, 151-162

Park J.I., Hong J.Y., Yong H.Y., Hwang W.S., Lim J.M., Lee E.S., 2005. High oxygen tension during in vitro maturation improves in vitro development of porcine oocytes after fertilization. Anim. Reprod. Sci. 87, 133-141

Petr J., Rozinek J., Jilek F., Urbankova D., 2000. Activation of porcine oocytes using cyclopiazonic acid, an inhibitor of calcium-dependent ATPases. J. Exp. Zool. 287, 304-315

Primakoff P., Myles D.G., 2002. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. Science 296, 2183-2185

Qian Y., Shi W.Q., Ding J.T., Sha J.H., Fan B.Q., 2003. Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilized in vitro. J. Reprod. Dev. 49, 167-174

Rath D., Niemann H., Tao T., 1995. In vitro maturation of porcine oocytes in follicular fluid with subsequent effects on fertilization and embryo yield in vitro. Theriogenology 44, 529-538

Rath D., Topfer-Petersen E., Michelmann H.W., Schwartz P., Ebeling S., 2005. Zona pellucida characteristics and sperm-binding patterns of in vivo and in vitro produced porcine oocytes inseminated with differentially prepared spermatozoa. Theriogenology 63, 352-362

Sherrer E.S., Rathbun T.J., Davis D.L., 2004. Fertilization and blastocyst development in oocytes obtained from prepubertal and adult pigs. J. Anim. Sci. 82, 102-108

Singh B., Meng L., Rutledge J.M., Armstrong D.T., 1997. Effects of epidermal growth factor and follicle stimulating hormone during in vitro maturation on cytoplasmic maturation of porcine oocytes. Mol. Reprod. Dev. 46, 401-407

Skrzyszowska M., Samiec M., Smorąg Z., 2005. Effect of in vitro culture conditions on the developmental potential of porcine nuclear transfer embryos reconstructed with cumulus cells analyzed on apoptosis. Ann. Anim. Sci. 5, 99-109

Somfai T., Kikuchi K., Onishi A., Iwamoto M., Fuchimoto D., Papp A.B., Sato E., Nagai T., 2003. Meiotic arrest maintained by camp during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes. Zygote 11, 199-206

Stokes P.J., Abeydeera L.R., Leese H.J., 2005. Development of porcine embryos in vivo and in vitro; evidence for embryo 'cross talk' in vitro. Develop. Biol. 284, 62-71

Sun Q.Y., Nagai T., 2003. Molecular mechanisms underlying pig oocyte maturation and fertilization. J. Reprod. Dev. 49, 347-359

Sun Q.Y., Wu G.M., Lai L., Park K.W., Cabot R., Cheong H.T., Day B.N., Prather R.S., Schatten H., 2001. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. Reproduction 122, 155-163

Suzuki C., Iwamura S., Yoshioka K., 2004. Birth of piglets through the non-surgical transfer of blastocysts produced in vitro. J. Reprod. Dev. 50, 487- 491

Suzuki K., Mori T., Shimizu H., 1994. In vitro fertilization of porcine oocytes in chemically defined medium. Theriogenology 42, 1357-1368

Swain J.E., Bormann C.L., Clark S.G., Walters E.M., Wheeler M.B., Krisher R.L., 2002. Use of energy substrates by various stage preimplantation pig embryos produced in vivo and in vitro. Reproduction 123, 253-260

Tatemoto H., Muto N., Yim S.D., Nakada T., 2005. Anti-hyaluronidase oligosaccharide derived from chondroitin sulfate A effectively reduces polyspermy during in vitro fertilization of porcine oocytes. Biol. Reprod. 72, 127-134

Thompson J.G., Simpson A.C., Pugh P.A., Donnelly P.E., Tervit H.R., 1990. Effect of oxygen concentration on in vitro development of preimplantation sheep and cattle embryos. J. Reprod. Fertil. 89, 573-578

Torner H., Brüssow K.P., Alm H., Rátky J., Pöhland R., Tuchscherer A., Kanitz W., 2004. Mitochondrial aggregation pattern and activity in porcine oocytes and apoptosis in surrounding cumulus cells depends on the stage of pre-ovulatory maturation. Theriogenology 61, 1675-1689

Wang W.H., Abeydeera L.R., Cantley T.C., Day B.N., 1997. Effect of oocyte maturation media on development of pig embryos produced by in vitro fertilization. J. Reprod. Fert. 111, 101-108

Wang W.H., Macháty Z., Abeydeera L.R., Prather R.S., Day B.N., 1999. Time course of cortical and zona reaction of pig oocytes upon intracellular calcium increase induced by thimerosal. Zygote 7, 79-86

Wu G.M., Sun Q.Y., Mao J., Lai L.X., McCauley T.C., Park K.W., Prather R.S., Didion B.A., Day B.N., 2002. High developmental competence of pig oocytes after meiotic inhibition with a specific M-phase promoting factor kinase inhibitor, butyrolactone I. Biol. Reprod. 67, 170-177

Xia P., Wang Z., Yang Z., Tan J., Qin P., 2001. Ultrastructural study of polyspermy during early embryo development in pigs, observed by scanning electron microscope and transmission electron microscope. Cell Tissue Res. 303, 271-275

Ye J., Campbell K.H.S., Craigon J., Luck M.R., 2005. Dynamic changes in meiotic progression and improvement of developmental competence of pig oocytes in vitro by follicle-stimulating hormone and cycloheximide. Biol. Reprod. 72, 399-406

Yoneda A., Suzuki K., Mori T., Ueda J., Watanabe T., 2004. Effects of delipidation and oxygen concentration on in vitro development of porcine embryos. J. Reprod. Dev. 50, 287-295

Yoshida M., Mizoguchi Y., Ishigaki K., Kojima T., Nagai T., 1993. Birth of piglets derived from in vitro fertilization of pig oocytes matured in vitro. Theriogenology 39, 1303-1311

Yoshioka K., Suzuki C., Itoh S., Kikuchi K., Iwamura S., Rodriguez-Martinez H., 2003. Production of piglets derived from in vitro-produced blastocysts fertilized and cultured in chemically defined media: effects of theophylline, adenosine, and cysteine during in vitro fertilization. Biol. Reprod. 69, 2092-2099

Yoshioka K., Suzuki C., Tanaka A., Anas I.M.K., Iwamura S., 2002. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol. Reprod. 66, 112-119