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# Advances in *in vitro* production of pig embryos

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A series of integrated, effective techniques is required to produce pig embryos from follicular oocytes *in vitro*. The failure to form a male pronucleus and polyspermy have been serious problems in efforts to produce embryos efficiently *in vitro* from pig oocytes. The former problem is now considered to be due to oxidative stress and the latter has been partially solved by reducing the number of capacitated spermatozoa reaching the oocytes. By the use of new technology for *in vitro* production of embryos, an acceptable rate of blastocyst formation and the birth of live piglets has been achieved. However, even with the use of these improved *in vitro* maturation (IVM) and fertilization (IVF) conditions, the efficiency of production of *in vitro* blastocysts and offspring still remains relatively low. More recently the developmental competence of embryos matured and fertilized *in vitro* has been investigated through modification of culture conditions of oocytes during the germinal vesicle stage. Oocyte competence for early embryonic development appears to be achieved by active communication between the oocyte and follicular cells. Since the ovarian oocytes available for IVM are primarily those present in mid-size antral follicles of prepubertal gilts, more research is needed to gain an improved understanding of the culture conditions required to induce developmental competence in oocytes from both preantral and antral follicles as well as additional modifications in IVF systems to overcome the problem of polyspermic penetration.

## Introduction

The pig ovary contains a relatively large number (approximately 210 000) of primordial follicles compared with other mammals (Gosden and Telfer, 1987). An objective of *in vitro* production of pig embryos is to use the large number of follicular oocytes from ovaries in abattoirs to reduce the cost of procurement of embryos for various research purposes. Since successful culture techniques for early development of pig embryos from the one-cell to blastocyst stages have recently been developed (reviewed by Petters and Wells, 1993), efforts to produce embryos *in vitro* have focused on the development of a system for efficient *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes, with particular attention on the historical problems of a reduced incidence of male pronuclear formation and a high incidence of polyspermy. Continuous progress towards the solution of traditional problems is being made (see reviews by Day and Funahashi, 1996; Niwa, 1993; Mattioli, 1994; Nagai, 1994). The incidence of blastocyst formation following IVM, IVF and *in vitro* culture has been improved significantly by modification of conditions during IVM (Funahashi *et al.*, 1997a; Funahashi *et al.*, 1997b). However, the simple integration of these improved IVM–IVF techniques and the successful culture system for early embryonic development still does not appear to result in a high incidence of viable blastocyst formation *in vitro*. Piglets from oocytes matured and fertilized *in vitro* have been produced only in a few laboratories (Mattioli *et al.*, 1989; Yoshida *et al.*, 1993c; Funahashi *et al.*, 1996a). Therefore, the present review aims to discuss not only recent technological developments in IVM and IVF but also the establishment of competence of pig oocytes for early embryonic and fetal development.

## Historical Problems: Male Pronuclear Formation

Although an abnormally low incidence of male pronuclear formation had frequently been observed in earlier studies when IVM oocytes were fertilized *in vitro* (Nagai *et al.*, 1984; Mattioli *et al.*, 1988), research in several laboratories has solved this problem. As discussed in our previous review (Day and Funahashi, 1996), factors affecting the ability of oocytes to form a male pronucleus are hormonal conditions, follicular secretions, intracellular ionic strength and especially oxidative stress. The incidence of male pronuclear formation is highly correlated with the glutathione content of the oocyte (Yoshida *et al.*, 1993a; Funahashi *et al.*, 1994b) which is associated with a required reduction in the disulfide bond cross-linking of sperm protamine to initiate sperm decondensation (Perreault, 1990). Both the incidence of male pronuclear formation and glutathione content in pig oocytes is increased during IVM when cysteine, which is a thiol, is added to the maturation medium, whereas the content decreases in the absence of cysteine (Yoshida *et al.*, 1993a). The incidence of male pronuclear formation is also increased by supplementation of maturation medium with other thiols such as cysteamine (Grupen *et al.*, 1995) and β-mercaptoethanol (H. Funahashi and B. N. Day, unpublished observation). Therefore, the previous problem of failure in male pronuclear formation appears to have been at least partially due to oxidative stress. Although the presence of cumulus cells surrounding the oocyte is required throughout IVM to maintain a high oocyte glutathione concentration (Funahashi and Day, 1995), the presence of cysteine from 36 h after the start of IVM will maintain both a high oocyte glutathione content and a high incidence of male pronuclear formation, and the absence of cysteine from 36 h after the start of IVM induces a significant reduction in both of these (Sawai *et al.*, 1997). Thus, the oxidative stress may be especially detrimental at relatively late stages of IVM when active intercellular coupling between the oocyte and cumulus cells is significantly reduced (Motlik *et al.*, 1986; Mattioli *et al.*, 1988).

## Persistent Problem: Polyspermic Penetration

In mammals, polyspermic penetration results in the developmental failure of the zygote (Hunter, 1996). Since nearly all mammals inherit their centrosomes from their fathers (Schatten, 1994; Navara *et al.*, 1995) and the paternal centrosome is known to organize the microtubules to form a sperm aster in pigs (Kim *et al.*, 1996a), polyspermic zygotes have an abnormal cleavage during early embryonic development (Fig. 1). Many IVF conditions that have been developed for pig oocytes result in a high incidence of polyspermic penetration (Niwa, 1993).

### *Possible methods to prevent polyspermic penetration* in vivo

In normal gilts, polyspermic fertilization appears to be reduced to a very low incidence by two major mechanisms, namely by the female reproductive tract controlling the number of capacitated spermatozoa reaching the site of fertilization and the direct exocytotic reaction of oocytes to prevent further sperm attachment and penetration. The female reproductive tract, especially the ovary, utero–tubal junction, and fallopian tube isthmus, exerts an important role in the control of the number of spermatozoa that reach the site of fertilization (Hunter, 1991; Hunter, 1996). During transport of spermatozoa through the female reproductive tract, spermatozoa are capacitated and the number that finally reach the site of fertilization number less than a hundred cells per oocyte (Hunter, 1982; Mburu *et al.*, 1996). The direct reactions of oocytes to sperm penetration are cortical granule exocytosis and secondarily the reactions to exocytosis in the perivitelline space and zona pellucida (Cran and Esper, 1990; Jones, 1990). During fertilization in mammals, exocytosis of the cortical granule contents containing hydrolytic enzymes and saccharide components to the perivitelline space leads to the formation of a cortical granule envelope (Dandekar and Talbot, 1992), modification of zona pellucida proteins and the inactivation of sperm receptors.

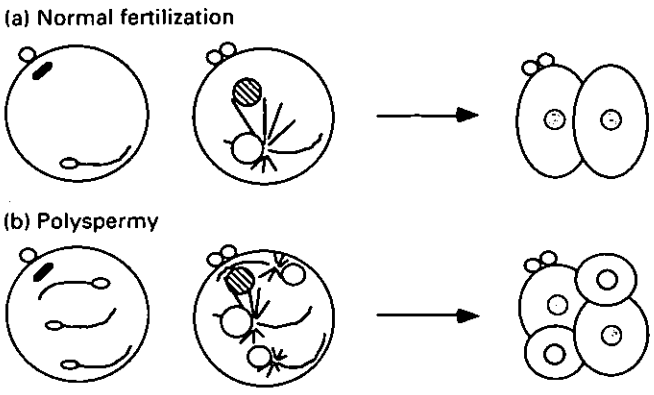


**Fig. 1.** Schematic diagram of the first cleavage of pig oocytes fertilized normally (a) or polyspermy (b). Sperm aster microtubules are formed around the paternal centrosome. Multiple sperm asters are formed in association with each male chromatin in polyspermy oocytes, resulting in mosaicism.

*Is sperm capacitation induced efficiently* in vitro*?*

In most IVF systems, a relatively large number of spermatozoa has been added to the fertilization medium containing mature pig oocytes (Nagai, 1996). Furthermore, even the current IVF systems may be unsuitable for inducing sperm capacitation because there is a large variation among boars in the ability of spermatozoa to penetrate oocytes (Martinez *et al.*, 1993; Wang *et al.*, 1995). Although a Tris-buffered medium (Abeydeera *et al.*, 1996a) as well as bicarbonate-buffered medium (Suzuki *et al.*, 1994) supports sperm penetration of pig oocytes *in vitro*, most IVF systems use a bicarbonate-buffered medium. Bicarbonate causes a loss of sperm surface coating materials (Ashworth *et al.*, 1995) and a major alteration in sperm plasma membrane lipid architecture (Harrison *et al.*, 1996) which may induce an initial permissive event in the capacitation process. However, in a traditional IVF system that uses modified Medium 199, frozen and thawed spermatozoa seem to undergo the capacitation process leisurely, and consequently the population of acrosome-reacted spermatozoa increases very slowly during co-culture of oocytes with spermatozoa compared with the pattern in modified Tris-buffered medium (Fig. 2; H. Funahashi, Y. Kajiwara, K. Sawai and K. Niwa, unpublished). Similar results have been obtained in a capacitation medium that used freshly ejaculated spermatozoa (Mattioli *et al.*, 1996). In most current IVF systems, therefore, pig oocytes may be exposed to a number of freshly capacitated spermatozoa over a long period after insemination. Finding a suitable IVF system that will induce sperm capacitation of nearly all spermatozoa at the same time may reduce the number of newly formed penetrable spermatozoa during IVF. Determining the nature of oviductal secretions around the time of ovulation more completely would help define the conditions needed to induce efficient sperm capacitation. Hyaluronic acid, which is detected in oviductal fluid, seems to induce the sperm capacitation but not the acrosome reaction (H. Rodriguez-Martinez, Y. Han, X. Song, H. Funahashi and K. Niwa, unpublished).

*Attempts to reduce the number of spermatozoa binding to oocytes*

A short co-preincubation of spermatozoa with pig oviductal epithelial cells (Nagai and Moor, 1990) or preincubation in the presence of a low concentration of pig follicular fluid (Funahashi and Day, 1993) reduces the incidence of polyspermic penetration in pig oocytes, although a long co-culture with oviductal cells or a high concentration of follicular fluid also reduces the incidence of sperm penetration. Similar observations have been demonstrated when oocytes were co-cultured with spermatozoa in the presence of 1% oviductal fluid collected from day 20 and 21 oestrous gilts

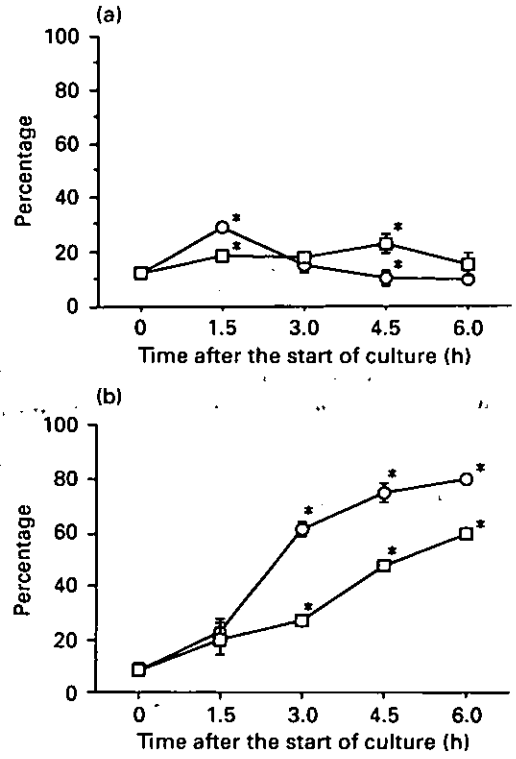


Fig. 2. The incidence of capacitated (a) and acrosome-reacted (b) spermatozoa after the start of culture in modified Tris-buffered medium (○) or modified medium 199 (□). After washing, frozen–thawed spermatozoa were cultured in 500 μl droplets of each medium at $1 \times 10^6$ cells $ml^{-1}$, fixed every 1.5 h and the functional state of the cells was examined using a chlortetracycline fluorescence assay. Values with asterisks are significantly different ($P < 0.05$) within each observation time.

(Kim *et al.*, 1996a) or in a medium conditioned with secretions of pig oviductal epithelial cells (Kano *et al.*, 1994). The pig zona pellucida is composed of three glycoprotein families, pZP1, pZP3-α and pZP3-β. pZP1 is divided into pZP2 and pZP4 by reduction of S-S bonds (Hedrick, 1993; Hasegawa *et al.*, 1994). pZP3-α is a primary receptor for boar spermatozoa (Sacco *et al.*, 1989; Yurewicz *et al.*, 1993) and binds to the acrosome region of acrosome-damaged or partially acrosome-reacted spermatozoa, but not to acrosome-intact or fully acrosome-reacted spermatozoa (Yonezawa *et al.*, 1995). The presence of sulfated polymers such as dextran sulfate, fucoidan, polyvinylsulphate and heparin are known to compete with ZP3 on the zona pellucida to bind the zona-binding glycoproteins of boar spermatozoa and to induce the acrosome reaction (Jones, 1991; Parry *et al.*, 1992). Therefore, oviductal and follicular sulfate compounds in preincubation and(or) fertilization media probably compete with sperm receptors for binding to zona pellucida ligands, stimulate the rate of sperm acrosome reaction, and consequently reduce the number of capacitated spermatozoa attaching to the surface of pig oocytes.

*Can the oocyte cortical reaction prevent polyspermic penetration in the current IVF systems?*

In pigs, the timing of cortical granule migration in IVM oocytes and the reorganization of microfilaments to the oocyte cortex area (Kim *et al.*, 1996c) are similar to those of oocytes matured *in vivo* (Yoshida *et al.*, 1993b). Even so, the cortical reaction does not seem to be sufficiently effective in pig IVF systems to prevent polyspermy. The amount of pZP1, which is a second receptor for boar spermatozoa, decreases substantially during IVF of pig oocytes (Hatanaka *et al.*, 1992). Sperm penetration is not blocked after electrical stimulation of IVM oocytes (Funahashi *et al.*, 1993; Funahashi *et al.*, 1995) with the incidence of polyspermic oocytes and the number of penetrated spermatozoa increasing gradually with time after insemination (Funahashi *et al.*, 1995), even though cortical granules are released following electrical stimulation (Sun *et al.*, 1992) or microinjection of the G-protein stimulator guanosine-5′-*O*-(3′-thiotriphosphate) (Machaty *et al.*, 1995). Although the cortical granule reaction seems to be slower and incomplete in some oocytes matured and fertilized in historical *in vitro* systems as compared with ovulated oocytes fertilized *in vivo* (Kim *et al.*, 1996b) or *in vitro* (Kim *et al.*, 1996b), the degree of exocytosis in oocytes matured and fertilized in a modified *in vitro* system appears not to be different from that in ovulated oocytes fertilized in the same system (Fig. 3; W. H. Wang and B. N. Day, unpublished). However, since pig oocytes seem to need several hours *in vivo* to complete the release of the cortical granules from the ooplasma (Laurincik *et al.*, 1995; Soede *et al.*, 1995), the preventive mechanism of the cortical reaction may be inadequate to prevent polyspermy in the current IVF system in which pig oocytes are exposed to a number of waves of capacitated spermatozoa. Furthermore, the volume fraction and size of mitochondria and cortical granules of IVM oocytes from prepubertal sheep differ from those of IVM oocytes from adult sheep (O'Brien *et al.*, 1996). Oocytes from prepubertal animals may have an inadequate amount of cortical granule substance to induce effective reaction(s). In summary, the best possible means to reduce the incidence of polyspermic penetration in oocytes from prepubertal gilts may be to reduce the number of capacitated spermatozoa reaching the oocytes.

## Developmental Competence of Pig Embryos Matured And Fertilized *In Vitro*

*Culture of pig embryos matured and fertilized* in vivo

As described by Petters and Wells (1993), recent technological progress in culturing one-cell pig embryos matured and fertilized *in vivo* has been achieved by the use of simple media such as modified Whitten's medium, NCSU-23 or NCSU-37 media and modified Tyrode's medium. Recently, it has been reported that replacement of BSA with FBS in BECM-3 medium by late day 5 (Dobrinsky *et al.*, 1996) or transferring embryos from CZB medium to modified Eagle's minimal essential medium containing 20% FBS (Pollard *et al.*, 1995) improve the incidence of hatched blastocysts.

*Attempts to culture IVM–IVF embryos for early embryonic development*

Available methods for the successful culture of pig embryos to the blastocyst stage have made it possible to examine the developmental ability of IVM/IVF pig embryos. However, although IVM and IVF pig oocytes develop to the blastocyst stage in simple media, the efficiency is very low, even with improved methods that promote male pronuclear formation and monospermic penetration (Funahashi *et al.*, 1994a; Funahashi *et al.*, 1994c). An increased incidence of the development of IVM/IVF embryos to the blastocyst stage has been shown by culture in the amniotic fluid of developing chick embryos (Ocampo *et al.*, 1994) and in co-culture systems with pig cumulus cells (Nagai and Takahashi, 1992), trophoblastic cells (Nagai and Takahashi, 1992) or oviduct epithelial cell aggregates (Choi *et al.*, 1995). Furthermore, adding IGF-I to the culture medium enhances the cleavage rate of IVM and IVF pig embryos (Xia *et al.*, 1994), and has a stimulating effect on metabolism and cellular proliferation of pig blastocysts (Lewis *et al.*, 1992). The cleavage rate of pig

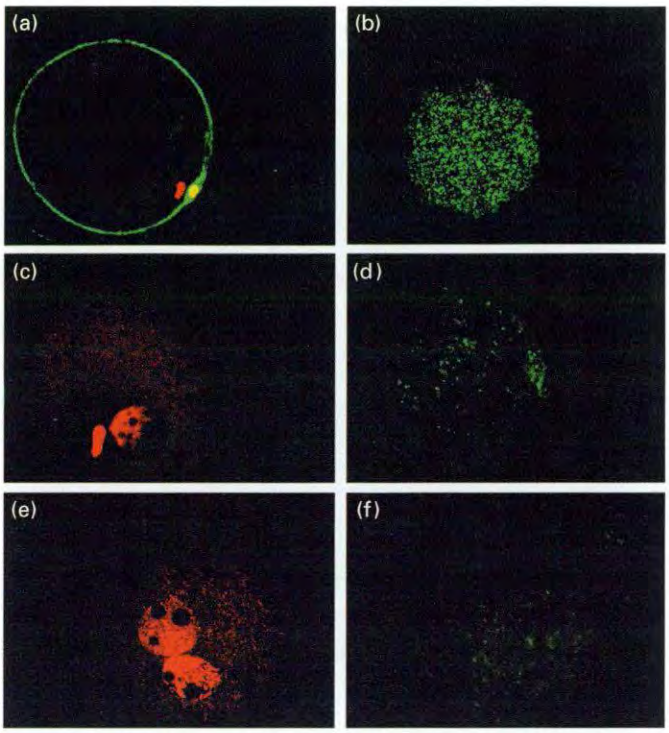


**Fig. 3.** Confocal micrographs of cortical granules in pig oocytes matured in NCSU23 for 44 h, and then inseminated in modified Tris-buffered medium. Oocytes were examined 6 h (a–d) and 12 h (e and f) after insemination. Green and red images show cortical granules and DNA, respectively. (a,b) Oocyte that was not penetrated by spermatozoa at 6 h after insemination; the oocyte was still at the stage of metaphase II. No cortical granule exocytosis was found. (c,d) An oocyte that was penetrated at 6 h after IVF by a spermatozoon; this oocyte had been activated to form a female pronucleus (c). Some cortical granules were still intact in the cortex (d). (e,f) A penetrated oocyte at 12 h after IVF: both male and female pronuclei had been formed (e) and very few residual cortical granules were present in the cortex (f).

IVM/IVF embryos is also increased if glycine and oviductal cells are present in the medium during early embryonic development (Xia *et al.*, 1995).

The ability of IVM/IVF embryos to develop to the blastocyst stage in simple media has also been improved by modification of the maturation medium to reduce the concentration of sodium chloride (Funahashi *et al.*, 1994a) or supplementation of maturation medium with cysteamine (Grupen *et al.*, 1995) or organic osmolytes (Funahashi *et al.*, 1996a). The presence of glycine in the maturation medium also improves the incidence of cleaved IVM/IVF embryos (Xia *et al.*, 1995). However, there is a significantly smaller number of cells in the trophectoderm of pig IVF embryos compared with those produced *in vivo* (Rath *et al.*, 1995a). Since the presence of BSA in the culture media after day 5 of culture decreases the number of blastomeres of embryos matured and fertilized *in vivo* (Dobrinsky *et al.*, 1996), the smaller number of cells in the trophectoderm of pig IVM/IVF embryos may be due to a detrimental effect of BSA in culture media.

*Embryonic development following embryo transfer*

The transfer of the IVM and IVF pig embryos at the four-cell stage has often resulted in extended oestrous cycles and failure of pregnancy during later stages of gestation (Funahashi *et al.*, 1996a). Furthermore, piglets from oocytes matured and fertilized *in vitro* have been produced in only a few laboratories (Mattioli *et al.*, 1989; Yoshida *et al.*, 1993c; Funahashi *et al.*, 1996a). In gilts, embryo survival seems to be associated with a close synchrony between the peak concentration of oestradiol and the onset of the LH surge (Blair *et al.*, 1994; Soede *et al.*, 1994) or oestrus (Blair *et al.*, 1994). It has also been suggested that the longer time interval between the onset of oestrus and ovulation is important for the high rate of embryo survival in Meishan pigs (Hunter and Picton, 1995). Furthermore, in polytocous animals such as pigs, it is proposed that asynchronous embryonic development reduces the number of embryos that develop to full term because oestrogen secretion from the more advanced blastocysts adversely affects the development of smaller blastocysts (Pope *et al.*, 1990; Greenwald and Roy, 1994). Therefore, the quality control of oocytes before the start of IVM may be an important factor for synchronized embryonic development and consequently the survival of IVM and IVF pig embryos following embryo transfer.

## Oocyte Competence For Early Embryonic Development

*Developmental competence of oocytes matured* in vivo *and* in vitro

Maternal materials for *in vitro* production of pig embryos have usually been collected from antral follicles of slaughtered prepubertal gilts. Recently, it has been reported that glutamine metabolism of IVM oocytes from prepubertal sheep is lower than that of oocytes from adult sheep and that the mitochondria and cortical granules of IVM oocytes from prepubertal sheep differ from those of IVM oocytes from adult sheep (O'Brien *et al.*, 1996). Furthermore, it has been shown that the low developmental competence of calf oocytes compared with cow oocytes would appear not to be due to a difference in oocyte protein patterns (Khatir *et al.*, 1996), but to a low sensitivity of the inositol 1,4,5-trisphosphate receptor (Damiani *et al.*, 1996). Therefore, the mechanism for signal transduction in oocytes of pigs as well as those of sheep and cattle may not be completed until about the time of puberty or during the follicular phase of the oestrous cycle.

The developmental competence of oocytes matured *in vitro* as determined by *in vitro* development to the blastocyst stage and the number of cells in blastocysts are lower than those matured *in vivo* (Nagashima *et al.*, 1996). However, since culture conditions used for the maturation of pig oocytes *in vitro* affect their subsequent response to oocyte activation (Yamauchi *et al.*, 1996), there may still be a major need for further improvements in the culture conditions during IVM of oocytes. For example, the ability of IVM and IVF pig embryos to cleave and develop to the blastocyst stage *in vitro* has been improved by modification of various conditions between oocyte collection and germinal vesicle breakdown (Table 1; Funahashi *et al.*, 1997a, b).

*Variation in the morphology of oocytes at the germinal vesicle stage*

Extensive use has been made in IVM and IVF systems of cumulus–oocyte complexes (COCs) with uniform ooplasm and a compact cumulus cell mass collected from antral follicles of slaughtered prepubertal gilts. However, the size of follicles that are selected for *in vitro* production of pig embryos differs among investigators (Day and Funahashi, 1996). There is a large variation in the dictyate stage of the first meiotic prophase among oocytes collected from follicles of slaughtered gilts for *in vitro* production of embryos (Hirao *et al.*, 1995; Funahashi *et al.*, 1997a). Furthermore, this variation in the germinal vesicle morphology seems to cause an increased range in the meiotic stage at the end of the maturation culture (Funahashi *et al.*, 1997b). Since histone H1 kinase activity of aged oocytes is significantly lower with time in culture (Kikuchi *et al.*, 1995), extension of the culture duration to obtain a higher maturation rate in a meiotically asynchronized population of oocytes

may reduce the oocyte competence for early embryonic development. In contrast, the germinal vesicle stage of COCs collected from gilts 72 h after injection of equine chorionic gonadotrophins (eCG) is closely synchronized (Funahashi *et al.*, 1996b). For efficient embryo production *in vitro*, therefore, oocytes to be used for IVM may need to be meiotically synchronized and of high quality. Although variation in oocyte quality may also be affected by the method (for example dissection versus aspiration) used to collect COCs (Nagai, 1994) and by the presence of corpora lutea in ovaries (Ocampo *et al.*, 1993), an oocyte population that is harvested from follicles of different ages may be expected to have an increased variation in range of quality of the oocytes. The ability of follicles to secrete steroids and support cytoplasmic maturation of the oocyte is more dependent on age than size of follicles (Ding and Foxcroft, 1994).

Reducing morphological variation appears to enhance the developmental competence of pig oocytes. Preincubation of COCs in maturation medium without gonadotrophins for 12 h before exposing them to gonadotrophins reduces the variation in the morphology of germinal vesicle and enhances the developmental competence of pig oocytes following IVM–IVF (Funahashi *et al.*, 1996a). Furthermore, exposure of COCs to dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP) for the first 20 h of culture for maturation does not affect the maturation of oocytes during a 44 h culture period or sperm penetration but does increase the homogeneity of oocyte nuclear maturation (Funahashi *et al.*, 1997b). This treatment also improves the efficiency of *in vitro* production of pig embryos, and piglets have been produced with a high pregnancy rate and acceptable litter size following surgical embryo transfer of the IVM–IVF embryos at the two–four-cell stages (Funahashi *et al.*, 1997b). Treatment with hypoxanthine may be expected to produce similar effects during IVM because hypoxanthine (Miyano *et al.*, 1995) as well as dbcAMP (Petr *et al.*, 1991; Funahashi *et al.*, 1997b) inhibits meiotic resumption reversibly, and oocytes treated with these inhibitors have a mass of filamentous chromosomes around the nucleolus (Miyano *et al.*, 1995; Funahashi *et al.*, 1997b). Hypoxanthine appears to maintain the oocyte arrest by modulating cAMP concentration through its inhibitory action on cAMP-phosphodiesterase (Downs *et al.*, 1989).

However, since increasing and decreasing cAMP per se also stimulates oocyte phosphorylation via a signal transduction pathway, the stimulatory effect of cAMP may also enhance the competence of oocytes to develop to the blastocyst stage. Inhibiting all tyrosine phosphorylation with the tyrosine-specific protein inhibitor prevents changes in the morphology of the germinal vesicle (Jung *et al.*, 1993). A 42 kDa protein in pig oocytes that increases in amount after 12 h of maturation culture is localized to condensing and condensed chromosomes (Miyano *et al.*, 1996). A further understanding of the mechanisms required to induce developmental competence in oocytes is needed in our efforts to improve the viability of IVM–IVF blastocysts.

### *Oocyte competence after the germinal vesicle stage*

The competence of oocytes to develop to the blastocyst stage also seems to be improved by modification of IVM conditions after the germinal vesicle stage. The presence of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) from 20 to 44 h of culture for *in vitro* maturation of pig oocytes enhances the oocyte competence to the blastocyst stage without affecting factors associated with fertilization (Table 1; Funahashi *et al.*, 1997c). A combination of techniques using dbcAMP during the first 20 h of IVM and TIMP-1 from 20 to 44 h improves the ability of IVM–IVF embryos to develop to the blastocyst stage to a more acceptable level (34%) for *in vitro* production of embryos (Funahashi *et al.*, 1997c). TIMP-1 is a major secretory protein of pig preovulatory granulosa cells following treatment with hCG (Smith *et al.*, 1994). After the preovulatory gonadotrophin surge, the concentration of mRNA encoding TIMP-1 is known to be increased and localized to the granulosa cells (Smith *et al.*, 1994). Therefore, TIMP-1 may be required for oocytes to obtain the full ability for early embryonic development. However, an understanding of how TIMP-1 promotes the ability of pig oocytes to develop to the blastocyst stage without affecting fertilization is still not known. Cyclin B1 content in pig oocytes is relatively low during the GV stage and increases between 20 and 30 h after the start of IVM (Naito *et al.*, 1995). Mitogen-activated protein kinase is involved in the

**Table 1.** Recent improvement in blastocyst formation of IVM/IVF porcine embryos

| Authors | Percentage of blastocyst formation | Improved technology |
|---|---|---|
| Simple culture systems | | |
| Funahashi *et al.* (1994a) | 3 | Using modified Whitten's medium during IVM and embryo culture |
| Grupen *et al.* (1995) | 12 | Supplementation of maturation medium (modified Medium 199) with 500 μmol cysteamine $l^{-1}$. IVM/IVF embryos were cultured in modified Whitten's medium |
| Funahashi *et al.* (1997a) | 23 | Preincubation of cumulus–oocyte complexes in gonadotrophin-free medium (modified NCSU37 medium) before the start of IVM. Culture medium was NCSU37 medium. |
| Funahashi *et al.* (1997b) | 22 | No preincubation but adding dibutyryl cAMP and gonadotrophic supplements to maturation medium (modified NCSU37 medium) during the first 20 h period of IVM. Culture medium was modified NCSU37 medium. |
| Funahashi *et al.* (1997c) | 34 | A combination of techniques adding dibutyryl cAMP and gonadotrophic supplements to modified NCSU37 medium during the first 20 h of IVM and tissue inhibitor of metalloproteinase 1 from 20 to 44 h of IVM. Culture medium was modified NCSU37 medium. |
| Co-culture systems and *in vivo* culture system | | |
| Nagai and Takahashi (1992) | 10 | Co-culture of IVM–IVF embryos with cumulus cells in modified BMOC-II. Maturation medium was modified Waymouth MB752/1 medium. |
| Ocampo *et al.* (1994) | 26 | Culture of IVM–IVF embryos in the amniotic fluid in chick embryo. Maturation medium was modified Medium 199. |
| Abeydeera *et al.* (1996b) | 36 | Co-culture of cumulus–oocyte complexes with follicular shell piece in modified NCSU23 medium during IVM. IVM–IVF embryos were cultured in NCSU23 medium. |

regulation of meiotic maturation of pig oocytes, especially after germinal vesicle breakdown (Inoue *et al.*, 1995). It is of interest that the effective time of adding TIMP-1 is close to the time of initial synthesis of cyclin B1 and mitogen-activated protein kinase in pig oocytes. Further experiments will be required to clarify the mechanism of the stimulating effect by TIMP-1.

A recent study has also demonstrated that co-culture of pig cumulus–oocyte complexes with follicular shell pieces improved early embryonic development to the blastocyst stage after *in vitro* fertilization (Table 1; Abeydeera *et al.*, 1996b). In this IVM system (Abeydeera *et al.*, 1996b), piglets were produced at a relatively high efficiency following surgical embryo transfer of the IVM–IVF embryos. It is also possible that the effect of the follicular shell pieces is mainly due to TIMP-1 secretion from granulosa cells.

The developmental competence of pig oocytes matured *in vivo* is lower following IVF than following *in vivo* fertilization (Rath *et al.*, 1995b). Therefore, there are inadequacies not only in the IVM system but also in the IVF system that impair the development of pig oocytes.

## Future Studies

There are large numbers of primordial follicles in a pig ovary (210 000), compared with those of other mammals such as humans (151 000), cattle (105 000) and mice (2 135) (Gosden and Telfer, 1987). Use of primordial oocytes for *in vitro* embryo production would bring about a major reduction in the cost and effort required to produce large numbers of embryos. However, the materials used for *in vitro* embryo production have largely been limited to a few fully grown oocytes from mid-size antral follicles. The development of mature mouse oocytes *in vitro* from the primordial follicle has been reported and it was shown that offspring could be produced from these oocytes (Eppig and O'Brien, 1996). Oocyte maturation and sperm penetration of pig oocytes following culture of preantral follicles was reported by Hirao *et al.* (1994). However, the incidence (40%) of oocytes grown *in vitro* that matured is still low (Hirao *et al.*, 1994) and developmental ability of this type of oocyte following *in vitro* fertilization has not been reported. Isolation and culture of preantral follicles from pig ovaries have been reviewed (Telfer, 1996). Further research with oocytes derived from primordial and growing follicles needs to be undertaken to increase the number of oocytes available from a single ovary for IVM–IVF.

## Conclusion

Failure in male pronuclear formation has been overcome by reducing the oxidative stress during IVM. However, there is a considerable need for improved methods to reduce further the rate of polyspermic penetration even though it has been decreased significantly in recent years. The developmental competence of oocytes matured and fertilized *in vitro* has been enhanced through modification of culture conditions of oocytes during *in vitro* maturation. Oocyte competence for early embryonic development appears to be achieved by mimicking active communications between the oocyte and follicular cells. In the most current IVM–IVF system, more than 80% of pig oocytes that were matured and fertilized normally developed to the blastocyst stage. Further research is needed to improve our understanding of the culture conditions required for pig oocytes of both preantral and antral follicles to achieve efficient growth, maturation, fertilization and early development *in vitro*, especially to induce developmental competence in oocytes, as well as to determine how to overcome the problem of polyspermic penetration.

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