Effects of oocyte maturation media on development of pig embryos produced by *in vitro* fertilization

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Few embryos derived from pig oocytes matured and inseminated *in vitro* are able to develop to blastocysts in culture. The present study was conducted to examine the effects of oocyte maturation media on the developmental ability of pig oocytes matured and inseminated *in vitro*. Follicular oocytes collected from ovaries of prepubertal gilts were cultured in NCSU23 medium, tissue culture medium 199 or a modified Whitten's medium. All of the media were supplemented with 0.57 mmol cysteine l⁻¹ and 10% pig follicular fluid. After maturation, some of the oocytes were used for examination of intracellular glutathione content, nuclear maturation and cortical granule distribution. The other oocytes were inseminated *in vitro* in a modified Tris-buffered medium with cryopreserved, ejaculated spermatozoa for examination of cortical reaction, sperm penetration, male pronuclear formation and blastocyst development. No differences ($P > 0.05$) were observed in nuclear maturation, cortical granule distribution, sperm penetration, male pronuclear formation, polyspermy and cleavage in oocytes matured in the three media. However, significant ($P < 0.05$) differences were observed in glutathione content, cortical granule exocytosis, blastocyst development and number of cells in blastocysts. NCSU23 medium gave the best results of the three media, resulting in 5.8 pmol glutathione per oocyte, 97% of cortical granule exocytosis, 30% blastocyst development and 36.8 ± 17.0 cells per blastocyst. These results clearly indicate that cytoplasmic maturation of pig oocytes was significantly affected by oocyte maturation media even in the presence of cysteine and pig follicular fluid. In addition, it was demonstrated that a large proportion of pig oocytes can develop to blastocysts under *in vitro* conditions.

Introduction

Pig oocytes matured and inseminated *in vitro* can develop to blastocysts after transfer of fertilized oocytes to the oviduct (Mattioli *et al.*, 1989; Yoshida *et al.*, 1990; Funahashi *et al.*, 1994a). Only recently have some of the embryos that resulted from *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) developed to blastocysts under *in vitro* conditions (Nagashima *et al.*, 1993; Funahashi *et al.*, 1994b, 1996; Rath *et al.*, 1996). However the developmental rates are very low when compared with those of oocytes matured and fertilized *in vivo* (Beckmann and Day, 1993; Nagashima *et al.*, 1993; Petters and Wells, 1993; Rath *et al.*, 1996; Dobrinsky *et al.*, 1996). This low developmental ability was thought to be the result of incomplete cytoplasmic maturation of pig oocytes matured *in vitro* and high polyspermic penetration of oocytes inseminated *in vitro* (Niwa, 1993). However, considerable improvement has been made in the IVM and IVF of pig oocytes (Niwa, 1993; Nagai, 1996). Low male pronuclear (MPN) formation has almost been overcome by the removal of fetal calf serum from the oocyte maturation medium (Naito *et al.*, 1988; Funahashi and Day, 1993a), supplementation of pig follicular fluid (pFF)

(Naito *et al.*, 1988) and cysteine (Yoshida *et al.*, 1993a) to the maturation medium, and the adjustment of hormones (Funahashi and Day, 1993b) during the maturation of oocytes. However, different media have been used in different laboratories. Tissue culture medium (TCM)-199, a common medium for oocyte maturation, has been used in most of the laboratories (Mattioli *et al.*, 1989; Yoshida *et al.*, 1990; Wang *et al.*, 1991; Funahashi *et al.*, 1994a, b). Blastocyst development (Mattioli *et al.*, 1989; Yoshida *et al.*, 1990; Nagashima *et al.*, 1993; Funahashi *et al.*, 1994a, b) and birth of piglets (Mattioli *et al.*, 1989) resulting from oocytes matured in TCM-199 have been reported, although the efficiency is low. A modified Whitten's medium (mWM) (Funahashi *et al.*, 1994c) and a bovine serum albumin (BAS)-free NCSU23 medium (Funahashi *et al.*, 1996), which were primarily developed for the culture of mouse (Whitten and Biggers, 1968) and pig (Petters and Wells, 1993) preimplantation embryos, have been shown to support pig oocyte maturation *in vitro*. Funahashi *et al.* (1994b, 1996) compared TCM-199 with mWM, and mWM with NCSU23 medium during oocyte maturation and found that cytoplasmic maturation of pig oocytes is significantly affected by the maturation medium. Some modifications to the composition of medium, such as a reduction in the concentration of NaCl (Funahashi *et al.*, 1994c) and the addition of organic osmolytes

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Table 1. Composition of media used for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC)

Concentration (mmol l ⁻¹)	IVM medium			IVF medium mTBM	IVC medium NCSU23
	TCM-199 ^a	NCSU23	mWM		
NaCl	116.36	108.73	68.49	113.1	108.73
KCl	5.36	4.78	4.78	3.0	4.78
CaCl ₂	1.80	1.70	—	7.5	1.70
KH ₂ PO ₄	—	1.19	1.19	—	1.19
MgSO ₄ · 7H ₂ O	0.81	1.19	1.19	—	1.19
NaHCO ₃	26.19	25.07	25.07	—	25.07
Glucose	5.55	5.55	5.56	11.0	5.55
Sodium lactate	—	—	25.20	—	—
Sodium pyruvate	—	—	0.33	5.0	—
Calcium lactate	—	—	1.71	—	—
Glutamine	0.68	1.0	—	—	1.0
Taurine	—	7.0	—	—	7.0
Hypotaurine	—	5.0	—	—	5.0
Tris	—	—	—	20.0	—
Penicillin G (i.u. ml ⁻¹)	100	100	100	100	100
Streptomycin (µg ml ⁻¹)	50	50	50	50	50
pFF (% v/v)	10	10	10	—	—
BSA (mg ml ⁻¹)	—	—	—	2 ^b	4 ^c
Cysteine	0.57	0.57	0.57	0.57	—
Caffeine	—	—	—	—	—

^aPartial listing of components of TCM199 purchased from Gibco, Cat. No. 31100-035.

^bFraction V, Sigma, Cat. No. A-7888.

^cFraction V, Sigma, Cat. No. A-8022.

mWM, modified Whitten's medium; mTBM, modified Tris buffer medium; pFF, porcine follicular fluid.

(Funahashi *et al.*, 1996), significantly improve oocyte cytoplasmic maturation. However, the blastocyst developmental rate is still low (< 10%).

Supplementation of maturation medium with cysteine enhances the amount of glutathione in pig oocytes and improves MPN formation after sperm penetration (Yoshida *et al.*, 1993a). Greater amounts of glutathione improve the developmental ability of preimplantation embryos in cattle (Takahashi *et al.*, 1993; Lim *et al.*, 1996). It is likely that a supplement of cysteine during oocyte maturation not only increases MPN formation but also improves embryo development.

One of the reasons that developmental competence of pig oocytes has not been widely studied is that high proportions of IVM/IVF oocytes are polyspermic. Polyspermy results in early embryo death, which limits the study on preimplantation embryo development under *in vitro* conditions. A modified Tris buffer medium (mTBM) has been found to not only support sperm penetration but also to reduce polyspermic penetration of pig oocytes *in vitro* when frozen-thawed semen is used (Abeydeera and Day, in press). It would appear that mTBM can also support oocytes to undergo normal cortical reaction during IVF.

Putting the recent progress in IVM and IVF of pig oocytes together, it is possible to develop a system for the production of pig embryos under *in vitro* conditions. In the present study, using BSA-free NCSU23 medium, TCM-199 and mWM,

supplemented with cysteine and pFF, as the oocyte maturation media and mTBM as the IVF medium, the *in vitro* developmental ability of pig embryos that resulted from IVM/IVF oocytes was examined, with special reference to the effects of maturation media on developmental competence.

Materials and Methods

Media

Three maturation media were used in this study: TCM-199 (with Earle's salts, Gibco, Grand Island, NY), BSA-free NCSU23 medium (Petters and Wells, 1993) and mWM (Funahashi *et al.*, 1994c). All media were supplemented with 0.57 mmol cysteine l⁻¹ (Sigma Chemical Co., St Louis, MO) and 10% (v/v) pFF. The pFF was collected from follicles (3–5 mm diameter) of prepubertal pig ovaries. After centrifugation at 1500 g for 30 min at 4°C, the suspension was filtered through 1.2 µm syringe filters and stored at –20°C until use. The same central pool of pFF was used for all three maturation media. The media used for IVF was a Tris buffer medium (Berger and Horton, 1988) modified by this laboratory (Abeydeera and Day, in press). Embryo culture medium was NCSU23 medium supplemented with 4 mg BSA (Fraction V, A-8022) ml⁻¹ (Petters and Wells, 1993). The detailed compositions of these media are shown (Table 1).

Collection and culture of oocytes

The collection and culture of oocytes were based on procedures reported by Funahashi *et al.* (1996). Briefly, ovaries collected from prepuberal gilts at a local abattoir were put in 0.9% (w/v) NaCl at 25°C and maintained at this temperature while being transported to the laboratory. Three to four hours after exsanguination, follicular oocytes were aspirated from antral follicles (3–5 mm diameter) using an 18 gauge needle fixed to a 10 ml disposable syringe. After washing four times with Hepes-buffered Tyrodes medium containing 0.1% (w/v) polyvinyl alcohol, oocytes were washed three times with a specified maturation medium and each group of 50 oocytes surrounded by compact cumulus were transferred to a 500 µl drop of the same medium which had been previously covered with warm paraffin oil (Fisher Scientific, Pittsburgh, PA) in a polystyrene culture dish (Falcon 1008, Beckton Dickinson Labware, Lincoln Park, NJ) and equilibrated in an atmosphere of 5% CO₂ in air overnight. Five microlitres of water containing 1000 i.u. equine chorionic gonadotrophin (eCG) ml⁻¹ (Intervet America Inc., DE) and 1000 i.u. hCG ml⁻¹ (LyphoMed Inc., Rosemont, IL) were added to the culture drops containing oocytes, resulting in a final concentration of 10 i.u. hormones ml⁻¹. After culture for 22 h, oocytes were washed three times and then cultured in maturation medium without hormones for another 22 h at 39°C, 5% CO₂ in air (Funahashi and Day, 1993b).

In vitro fertilization of oocytes

After culture, all oocytes were freed from cumulus and corona cells by treatment with maturation media containing 0.1% (w/v) hyaluronidase, obtained from bovine testis (Type I-S, H-3506; Sigma), and then washed three times with the insemination medium (mTBM). Thereafter, each 30 oocytes were transferred into a 50 µl droplet of mTBM covered with paraffin oil. The dishes were kept in a CO₂ incubator until spermatozoa were added for insemination. For IVF, one 0.2 ml frozen semen pellet, made as described by Wang *et al.* (1991), was thawed at 39°C in 10 ml PBS containing 1 mg BSA ml⁻¹ (Fraction V, A-8022; Sigma) and antibiotics. After being washed three times, spermatozoa were resuspended with mTBM containing 2 mmol caffeine l⁻¹ (Sigma), to give a concentration of to 1×10^6 cells ml⁻¹, and 50 µl of the sample was added to 50 µl of the fertilization drops containing the oocytes. Six hours after insemination at 39°C in an atmosphere of 5% CO₂ in air, oocytes were removed from fertilization drops, washed four times and cultured in 500 µl of culture medium in a four-well culture plate (Nunc, Roskilde) until examination.

Assessment of cortical granules

The methods for assessment of cortical granules were based on those described by Yoshida *et al.* (1993b) with a few modifications. Zona pellucidae of oocytes were removed by putting the oocytes in PBS containing 0.1% (w/v) pronase (Type XIV, P-8811; Sigma). After washing three times, oocytes were fixed with 3.7% (w/v) paraformaldehyde (Sigma) in PBS for at least 30 min at room temperature, and then washed three

times in PBS containing 3 mg BSA ml⁻¹ and 100 mmol glycine l⁻¹ (Sigma) for 5 min each time. After treating for 5 min in PBS containing 0.1% (v/v) Triton X-100 (Sigma), oocytes were washed two additional times for 5 min. Oocytes were then cultured in 100 µg fluorescein isothiocyanate labelled peanut agglutinin ml⁻¹ (Sigma) in PBS for 30 min in a dark box. After staining, the oocytes were washed three times in PBS containing 3 mg BSA ml⁻¹ and 0.01% (v/v) Triton X-100. The nuclear status of oocytes was determined after staining with 10 µg propidium iodide ml⁻¹ (Sigma) in PBS for 1 h at 39°C. These oocytes were then mounted on slides and observed by laser confocal microscopy.

Confocal microscopy was performed using a BioRad MRC-600 equipped with a Krypton–argon ion laser, and mounted on an Optiphot II Nikon microscope equipped with $\times 40$ and $\times 60$ objectives. Two sections in each oocyte were observed at magnifications of $\times 400$ or $\times 600$. The largest optical section is referred to as the equator of oocyte and the top surface section is referred to as the cortex of oocyte. In each section, the image was obtained by repeated laser scanning (eight times during 8 s) to improve the signal: noise ratio. The numbers of cortical granules in the cortex were counted in squares of 100–1000 µm², and then the average number per 100 µm² of cortex in each oocyte was calculated. After evaluation of the number of cortical granules, oocytes were examined for nuclear stage and sperm penetration.

Assay of glutathione

The assay of glutathione was based on the procedures reported by Funahashi *et al.* (1996). Briefly, after culture, cumulus cells were removed completely from oocytes and oocytes were washed three times with a stock buffer (0.2 mol sodium phosphate l⁻¹ containing 10 mmol sodium EDTA l⁻¹, pH 7.2). Five microlitres of stock buffer containing 10–30 oocytes was transferred to a 1.5 ml microfuge tube. The oocytes were ruptured by agitation with a narrow-bore glass pipette, and 5 µl of 1.25 mmol phosphoric acid l⁻¹ (Sigma) was added to the tube. The tubes containing samples were kept in a freezer at –30°C until assay. The concentration of glutathione in oocytes was determined by the 5,5'-dithiobis-(2-nitrobenzoic acid) and glutathione disulfide reductase recycling assay (Anderson, 1985).

Assessment of nuclear maturation, sperm penetration, pronuclear formation and number of cells in blastocyst

Matured, inseminated oocytes or day 7 blastocysts were mounted on slides, fixed in 25% (v/v) acetic alcohol, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope. Oocytes containing one polar body and a metaphase plate were regarded as matured. Oocytes were considered as penetrated when they had at least one swollen sperm head or a male pronucleus with the second polar body released and the corresponding sperm tail in the vitellus. Because oocytes were examined 12 h after insemination, sperm heads were found to be present as either swollen heads or male pronuclei. Only oocytes that contained a male pronucleus(ei) with an intact nuclear membrane were classified

Table 2. Effects of different maturation medium on nuclear maturation and fertilization of pig oocytes *in vitro*^a

Medium	Number of oocytes examined	Number of oocytes matured (%)	Number of oocytes inseminated	Number of oocytes penetrated		Number of polyspermic oocytes (%) ^c	Mean number of spermatozoa in penetrated oocytes
				Total (%) ^b	With male pronucleus (%) ^b		
NCSU23	120	112 (93)	91	65 (71)	60 (92)	20 (31)	1.43
TCM-199	105	91 (87)	92	70 (76)	58 (83)	21 (30)	1.71
mWM	94	84 (89)	85	63 (74)	54 (86)	19 (30)	1.33

^aOocytes used for examination of nuclear maturation (44 h after culture) and for insemination (12 h after insemination) were different subgroups. Experiments were repeated seven times.

^bPercentage of the number of oocytes inseminated.

^cPercentage of the total number of oocytes penetrated.

mWM, modified Whitten's medium.

as having formed a male pronucleus. The number of cells in blastocysts was determined by counting the number of orcein-stained nuclei.

Assessment of developmental ability of embryos

At 48 h after insemination, cleavage of oocytes was evaluated under a stereomicroscope and blastocyst formation was examined 144 h (day 6) and 168 h (day 7) after insemination.

Statistical analysis

Seven replicate trials for examination of oocyte nuclear maturation, fertilization and development were conducted. All percentage data were subjected to arc sine transformation before statistical analysis. Comparisons among maturation media were analysed by ANOVA. Three replicate trials for glutathione content and five replicate trials for cortical granules were conducted. The data were expressed as mean $\pm$ SD and analysed by Student's *t* test.

Results

Effect of maturation media on nuclear maturation, sperm penetration and male pronuclear formation

When oocytes were cultured in NCSU23, TCM-199 and mWM for 44 h, a large proportion of oocytes (87–93%) reached metaphase II and no differences ($P > 0.05$) were observed among the maturation media (Table 2). After being

inseminated in mTBM, 71–76% of oocytes were penetrated by spermatozoa and 83–92% of oocytes formed MPN. Polyspermic oocytes were observed in 30–31% of oocytes, and the number of spermatozoa in penetrated oocytes was 1.33–1.71. No significant differences ($P > 0.05$) were found in these parameters among the maturation media.

Quantification of cortical granule distribution and exocytosis in oocytes

After maturation, cortical granules in all oocytes examined were located just beneath the oolemma and formed a monolayer irrespective of the maturation medium used. When observed in the top surface, cortical granules were distributed evenly in the cortex with 42.9 ± 12.5 to 46.3 ± 6.3 cortical granules per $100 \mu\text{m}^2$ of the cortex. No differences in cortical granule density were observed in the oocytes cultured in the three maturation media. At 6 h after IVF, neither nuclear status (Fig. 1a) nor cortical granule distribution (Fig. 1a, b) changed in the oocytes without sperm penetration. However, in oocytes penetrated by spermatozoa, most of the spermatozoa were still at the stage of swollen head (Fig. 1c), and 78–84% of the cortical granules were released from oocytes with residual numbers of 7.0 ± 3.1 to 9.8 ± 5.7 cortical granules per $100 \mu\text{m}^2$ of cortex (Fig. 1d), which was not different among the media used. However, at 12 h after IVF, almost all of the sperm heads had been transformed to MPN (Fig. 1e, g) and cortical granule density was significantly ($P < 0.01$) lower than at 6 h after IVF in oocytes matured in NCSU23, and 97% of cortical granules were released from the ooplasm (Fig. 1f, h). In contrast, the cortical granule density was similar at 6 h and 12 h after IVF for

Fig. 1. Confocal micrographs of cortical granules in pig oocytes matured in NCSU23 for 44 h, and then inseminated in modified Tris buffer medium. Oocytes were examined (a–d) 6 h and (e–h) 12 h after insemination. Green and red images show cortical granules and DNA, respectively. (a, b) An oocyte that was not penetrated by spermatozoa at 6 h after insemination, still at the stage of metaphase II (M, metaphase; pb, polar body). No cortical granule exocytosis was observed. (c, d) An oocyte that was penetrated at 6 h after IVF by one spermatozoon (sp). The oocyte has been activated to form (c) a female pronucleus (F), and (d) some cortical granules are still intact in the cortex. (e, f) A penetrated oocyte at 12 h after IVF in which both male and female pronuclei have formed (e) and in which very few residual cortical granules were present in the cortex (f). (g, h) A polyspermic oocyte, observed 12 h after IVF, displaying three male pronuclei and one female pronucleus (g), and very few cortical granules in the cortex (h). Scale bar represents $20 \mu\text{m}$.

Table 3. Changes in cortical granule density of pig oocytes matured in different medium before and after *in vitro* fertilization (IVF)

Medium	Mean $\pm$ SD number of cortical granules per 100 μm^2 of cortex (<i>n</i>)		
	Before IVF	6 h after IVF	12 h after IVF
NCSU23	45.3 $\pm$ 10.3 ^A (42)	9.8 $\pm$ 5.6 ^B (32)	1.5 $\pm$ 1.3 ^{aC} (32)
TCM-199	42.9 $\pm$ 11.0 ^A (46)	7.0 $\pm$ 3.3 ^B (24)	6.1 $\pm$ 4.8 ^{abB} (30)
mWM	46.2 $\pm$ 6.5 ^A (44)	9.1 $\pm$ 5.7 ^B (36)	8.1 $\pm$ 4.0 ^{bB} (28)

^{ab}Values with different superscripts within the same column are significantly different, $P < 0.001$.

^{ABC}Values with different superscripts within the same row are significantly different, $P < 0.01$.

oocytes matured in TCM-199 and mWM (Table 3). The cortical granule density was similar for monospermic (7.8 ± 3.0 to 10.0 ± 6.4 per 100 μm^2 at 6 h, and 1.8 ± 1.4 to 8.7 ± 4.3 per 100 μm^2 at 12 h) and polyspermic (5.5 ± 3.6 to 9.3 ± 3.5 per 100 μm^2 at 6 h, and 1.0 ± 1.0 to 6.9 ± 2.9 per 100 μm^2 at 12 h) oocytes, although the latter tended to be lower at both 6 h and 12 h in all three maturation media.

Intracellular glutathione content

Glutathione concentration of oocytes matured in NCSU23 (5.8 ± 0.4 pmol per oocyte, from 71 oocytes) was significantly higher ($P < 0.05$) than those matured in TCM-199 (3.6 ± 0.6 pmol per oocyte, from 82 oocytes) or mWM (2.3 ± 0.8 pmol per oocyte, from 63 oocytes). Oocytes matured in TCM-199 showed a higher ($P < 0.05$) concentration of glutathione compared with those cultured in mWM.

Effect of maturation media on developmental ability of oocytes after *in vitro* fertilization

At 48 h after IVF, 61–70% of the oocytes inseminated had cleaved and no differences were observed among the three maturation media, although NCSU23 showed a trend towards a higher rate of cleavage (70%) than TCM-199 (61%) or mWM (61%). At day 6 (144 h after IVF), of 473 inseminated oocytes

matured in NCSU23, 128 (27%) developed to blastocysts, and at day 7, this had increased to 30% (Table 4). These rates were significantly ($P < 0.01$) higher than those observed in TCM-199 (15–19%) or in mWM (4–6%). Oocytes matured in NCSU23 that developed into blastocysts had significantly ($P < 0.05$) more nuclei than oocytes matured in mWM. Although a greater ($P < 0.01$) percentage of oocytes matured in TCM-199 developed into blastocysts than in mWM, the number of nuclei per blastocyst did not differ (Table 4).

Discussion

Supplementation of maturation media of pig oocytes with pFF (Naito *et al.*, 1988), cysteine (Yoshida *et al.*, 1993a), and adjustment of hormonal conditions (Funahashi and Day, 1993b) during oocyte maturation enhances cytoplasmic maturation as measured by MPN formation after IVF. In this study, these conditions were combined and large proportions of oocytes formed MPN irrespective of the maturation medium used. However, when these oocytes were cultured further, the oocyte maturation medium used significantly affected the rate of development to blastocysts. More oocytes developed to blastocysts when matured in NCSU23 compared with TCM-199 or mWM. A positive effect was observed for intracellular concentration of glutathione and the ability of oocytes to release cortical granules on blastocyst development. These results indicate that NCSU23 is a superior maturation medium for pig oocytes than TCM-199 and mWM, even in the presence of cysteine and pFF.

Funahashi *et al.* (1996) reported that organic osmolyte components of NCSU23 were beneficial to cytoplasmic maturation of pig oocytes. When an organic osmolyte, such as sorbitol, is added to mWM during oocyte maturation, it increases blastocyst development, but the rate is still low ($< 10\%$). Cytoplasmic maturation in that study was not fully developed. In the present study, when NCSU23, supplemented with cysteine and pFF, was used as the maturation medium, 30% of oocytes developed to blastocysts by day 7. This was almost the same as the proportion of monospermic oocytes forming both male and female pronuclei. Therefore, it appeared that most of the normal zygotes that resulted from NCSU23 developed to blastocysts. Conditions that result in higher sperm penetration and lower polyspermy need to be developed

Table 4. Developmental ability of pig oocytes matured in different media, inseminated in modified Tris buffer medium (mTBM) and cultured in NCSU23

Medium	Number of oocytes inseminated	Number of cleaved oocytes (%)	Number of blastocysts		Mean number of nuclei in day 7 blastocysts
			Day 6 (%)	Day 7 (%)	
NCSU23	473	332 (70)	128 (27) ^a	142 (30) ^a	36.8 $\pm$ 17.0 ^a
TCM-199	509	308 (61)	76 (15) ^b	97 (19) ^b	30.7 $\pm$ 13.3 ^{ab}
mWM	529	321 (61)	21 (4) ^c	32 (6) ^c	29.4 $\pm$ 12.0 ^b

^{abc}Values with different superscripts within the same column are significantly different, $P < 0.05$. mWM, modified Whitten's medium.

to increase further the proportion of oocytes developing to blastocysts. In addition, although a high rate of blastocyst development was obtained in this study, the number of cells in the blastocysts was lower than in blastocysts developed in the oviduct after transfer (Funahashi *et al.*, 1994a). Modifications in *in vitro* culture conditions are still necessary to increase the quality of embryos. A medium, BECM-3, that supports both the rate of blastocyst formation and a large number of cells in blastocysts has been developed for the culture of pig embryos produced *in vivo* (Dobrinisky *et al.*, 1996). Whether this medium can also support development of IVM/IVF embryos needs to be examined further.

From this experiment, improved MPN formation and development of embryos may be the result of supplementation of cysteine in maturation. Supplementation of cysteine is important for glutathione synthesis (Yoshida *et al.*, 1993a) and glutathione is necessary for sperm chromatin decondensation and, hence, for the formation of MPN in mouse (Calvin *et al.*, 1986), hamster (Perreault *et al.*, 1988) and pig (Yoshida *et al.*, 1993a) oocytes. In the present study, when cysteine was added to three maturation media, high rates of MPN formation resulted and most (83–92%) oocytes formed MPN. However, the ability of oocytes to develop to blastocysts was significantly different among media. Glutathione concentration is significantly higher in oocytes matured in NCSU23 than in TCM-199 or mWM. On the basis of the differences observed in the three media, it seems possible that the synthesis of glutathione by cumulus-oocytes through cysteine is affected by other components in the medium. Components in the medium, such as taurine or hypotaurine in NCSU23, may also improve the synthesis of glutathione during oocyte maturation. Although the effectiveness of glutathione in enhancing embryo development has also been reported for bovine embryo development (Takahashi *et al.*, 1993; Lim *et al.*, 1996), the mechanism is not clear. Glutathione is an major intracellular free thiol that regulates cell proliferation, amino acid transport, synthesis of protein and DNA, and reduction of disulfides and other chemicals (Kosowa and Kosowa, 1978; Meister and Anderson, 1983), and all of these effects may be involved in embryo development.

Cortical granules are important organelles that play a central role in the zona block to polyspermic penetration during fertilization (Yanagimachi, 1994). Polyspermy is unusually high when pig oocytes are matured and inseminated *in vitro* (Niwa, 1993; Nagai, 1996). Delayed cortical granule exocytosis may be the main reason for polyspermy under *in vitro* conditions (Sathananthan and Trounson, 1982; Cran and Cheng, 1986). However, in the present study, 6 h after IVF (1–3 h after sperm penetration) approximately 80% of the cortical granules had been released from oocytes and no difference was found between monospermic and polyspermic oocytes. This finding suggests that pig oocytes cultured under the present conditions possess the ability to release cortical granules. The fertilization medium (mTBM) used in this study supported both sperm capacitation and oocyte cortical reaction. Polyspermy may be due to simultaneous sperm penetration (Hunter, 1991) or nonfunction of cortical granule exudate (Cran and Cheng, 1986). Conditions that support not only cortical granule exocytosis but also the activation of cortical granule exudate during IVF, such as increase of the availability of calcium (Cran and Cheng, 1986), should be examined further.

However, in oocytes 12 h after IVF, 97% of cortical granules had been released from oocytes matured in NCSU23, but there was no obvious decrease from 6 h in oocytes cultured in TCM199 or mWM. It has been suggested that cortical granules released at sperm penetration are critical for blocking polyspermy, and that the subsequent exocytosis is necessary to form a micro-environment around the oocyte, such as a complete cortical granule envelope (Dandekar and Talbot, 1992), which is beneficial for subsequent embryo development (Hoodbhoy and Talbot, 1994).

In conclusion, the present study indicates that cytoplasmic maturation of pig oocytes was significantly affected by maturation medium even in the presence of cysteine and pFF. When pig oocytes were cultured in BSA-free NCSU23 supplemented with pFF and cysteine, and then inseminated in mTBM by cryopreserved ejaculated spermatozoa, a large proportion of the fertilized oocytes developed to blastocysts in culture. The ability of oocytes to synthesize glutathione and release cortical granules are two additional important criteria for assessment of cytoplasmic maturation in pig oocytes.

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