Effects of BSA, PVA, Gonadotropins and Follicle Shell on *In Vitro* Maturation and *In Vitro* Fertilization of Porcine Oocytes

Pei-Qing Cong, Eun-Sook Song, Eui-Sook Kim, Zhao-Hua Li,
Yong-Hua Zhang, Young-Joo Yi and Chang-Sik Park†
Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon 305-764, Korea

ABSTRACT

This study was designed to evaluate effects of BSA, PVA, gonadotropins and follicle shell during IVM of porcine oocytes and subsequent development to the blastocyst stage after IVF. Cumulus oocyte complexes (COCs) were cultured in TCM-199 media containing 4 mg/ml BSA and 1 mg/ml PVA during IVM for 44 hr. To compare the effect of gonadotropins on oocyte maturation, COCs were cultured with FSH+LH, FSH, LH and FSH-LH-free media during IVM, respectively. Also, different number of follicle shells (0, 2, 4 and 6) was used to examine whether the presence of follicle shell in culture medium affects oocyte maturation. The percentages of fertilization and blastocyst formation, respectively, were higher in the medium containing the PVA (49.0 and 17.9%) than those containing the BSA (40.0 and 12.2%). Significantly higher rates of MII oocytes were in the presence of FSH+LH and FSH (88.6 and 85.1%) compared to other treatments (64.0 and 53.4% at LH and FSH-LH-free media). Co-culture with inverted follicle shells in 2 ml maturation medium enhanced the developmental competence of porcine oocytes. In conclusion, PVA could be used as a macromolecules instead of BSA, and FSH and follicle shell played important roles in maturation of porcine oocytes.

(Key words : *In vitro* maturation, Gonadotropins, Follicle shell, *In vitro* fertilization, Porcine oocytes)

INTRODUCTION

There are numerous reports describing successful maturation and fertilization of porcine oocytes under *in vitro* conditions using various culture media. In general, the oocytes maturation process can be broadly divided into two aspects, namely nuclear and cytoplasmic maturation. Although nuclear maturation seemed normal using earlier maturation conditions, as many recent reviews have highlighted, poor male pronuclear formation and polyspermy were major problems observed following *in vitro* fertilization (IVF) of porcine. This has been attributed to the result of incomplete cytoplasmic maturation of porcine oocytes matured *in vitro*. Funahashi *et al.* (1994a, 1996) compared tissue culture medium (TCM) 199 with modified Whitten's medium (mWM), and mWM with North Carolina State University (NCSU) 23 medium during oocyte maturation and found that cytoplasmic maturation of porcine oocytes was significantly affected by the maturation medium. However, TCM-199 has been used for oocyte maturation in most of laboratories (Mattioli *et al.*, 1989; Wang *et al.*, 1991; Funahashi *et al.*, 1994a, b).

In general, most of the IVM media is supplemented with gonadotropin and other growing factors. Major progress has been achieved in improving male pronucleus formation in porcine oocytes by supplementing the IVM medium with porcine follicular fluid (Naito *et al.*, 1988), cysteine (Yoshida *et al.*, 1992a; 1993), cysteamine (Grupen *et al.*, 1995), epidermal growth factor (Ding and Foxcroft, 1994; Grupen *et al.*, 1997) gonadotropins (Wang and Niwa, 1995), amino acid (Iwasaki *et al.*, 1999), or co-culture with extroverted follicles (Mattioli *et al.*, 1988). However, the effects of bovine serum albumin (BSA), polyvinyl alcohol (PVA), gonadotropins and follicle shell on IVM of porcine oocytes with TCM-199 medium have not been investigated in detail. This study was therefore designed to evaluate effects of BSA, PVA, gonadotropins and follicle shell during IVM of porcine oocytes, and embryos development after IVF.

MATERIALS AND METHODS

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† Corresponding author : Phone: +82-42-821-5873, E-mail: parkcs@cnu.ac.kr

Fig. 1. Whole antral follicle (arrow) with 4~6 mm in diameter for obtaining follicle shells was cut from ovaries using a fine scissors.

Oocyte Collection and *In Vitro* Maturation (IVM)

Ovaries were collected from prepubertal gilts at a local slaughter house and transported to the laboratory in 0.9% saline at 30~35℃. As shown in Fig. 1, whole antral follicle with 4~6 mm in diameter for obtaining follicle shells was cut from ovaries using a fine scissors. The follicle shells were then inverted using scissors and forceps and washed with the TL-HEPES-PVA medium. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated from follicles of 2~6 mm in diameter using an 18-gauge needle fixed to a 10 ml disposable syringe. The follicular contents were pooled into 50 ml tubes and allowed to sediment, and the sediment was placed into Tyrode-lactate-HEPES medium containing 0.1% (w/v) PVA (TL-HEPES-PVA). COCs were selected and washed with TL-HEPES-PVA and then washed twice with the maturation medium. The basic media used for IVM were three different modified TCM-199 (Table 1). COCs were cultured in 2 ml of maturation medium containing the 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml epidermal growth factor (EGF), 10% porcine follicular fluids (pFF) and 0.57 mM cysteine. After 22 hr of culture for maturation with the above three media, respectively, oocytes were cultured without FSH, LH and cysteine for 22 hr at 38.5℃, 5% CO_2 in air.

Semen Collection and Liquid Semen Processing

Semen was collected from one adult Yorkshire boar once weekly. The sperm-rich portion of ejaculates with greater than 85% motile sperm and normal apical ridge (NAR) acrosome was used in the experiments. Semen was slowly cooled to room temperature by 2 hr after collection. Semen was transferred into 15 ml tubes, centrifuged at room temperature for 10 min at 800 × g, and supernatant solution was poured off. The concentrated sperm was resuspended with 5 ml LEN (11.0 g lactose hydrate, 20 ml egg yolk and 0.05 g N-acetyl-D-glucosamine and 100 ml distilled water) diluent to provide 1.0×10^9 sperm/ml at room temperature. The resuspended semen was cooled in a refrigerator to 4℃ and preserved for 5 days (Park *et al.*, 2004).

Table 1. Composition of media for *in vitro* maturation of oocytes*

Components	MEDIUM 1	MEDIUM 2	MEDIUM 3
TCM-199 (mg/ml)	9.80	9.80	9.80
$NaHCO_3$ (mM)	26.19	26.19	26.19
Bovine apotransferrin (μg/ml)	10.00	10.00	-
Vitamin B_{12} (μg/ml)	2.00	2.00	-
Sodium pyruvate (mM)	0.91	0.91	0.91
Penicillin G (μg/ml)	75.00	75.00	75.00
Streptomycin (μg/ml)	50.00	50.00	50.00
Insulin (μg/ml)	10.00	10.00	-
D-glucose (mM)	-	-	3.05
HEPES (mM)	25.00	-	-
BSA (mg/ml)	4.00	4.00	-
Polyvinylalcohol (mg/ml)	-	-	1.00

* MEDIUM 1, 2 and 3 were supplemented with 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml EGF, 10% pFF and 0.57 mM cysteine before maturation of oocytes.

In Vitro Fertilization (IVF) and Culture of Oocytes

After 44 hr of maturation, cumulus cells of oocyte were removed by 0.1% hyaluronidase in TL-HEPES-PVA and washed twice with TL-HEPES-PVA and modified Tris-buffered media (mTBM; Abeydeera and Day, 1997), respectively. Thereafter, 30~40 oocytes were transferred into each well of a 4-well multidish containing mTBM that had been covered with mineral oil and equilibrated at 38.5℃, 5% CO_2 in air for 4 hr. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination. For IVF, 0.5 ml liquid semen was washed twice in TL-HEPES-PVA and then resuspended with mTBM to adjust a sperm concentration to 1×10^6 sperm/ml. Oocytes and sperm were co-incubated in 500 μl mTBM for 6 hr. After insemination, oocytes were transferred into 500 μl NCSU-23 containing 50 μM 2-mercaptoethanol, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate and 0.4% BSA. After 52 hr of culture, cleaved oocytes were transferred into 500 μl NCSU-23 containing 50 μM 2-mercaptoethanol, 5.55 mM D-glucose and 0.4% BSA.

Evaluation of Oocytes and Embryos Produced *In Vitro*

Oocytes were fixed for 48 hr with acetic alcohol (acetic acid:ethanol=1:3) at room temperature and stained with 1% orcein in 45% acetic acid to evaluate meiotic progression and fertilization parameters under a phase-contrast microscope at ×400 magnification. Blastocysts at 144 hr were stained with DAPI, and nuclei were counted under epifluorescent microscope (Olympus, Japan).

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental Designs

Experiment 1 was carried out to compare the three different mTCM-199 media for oocyte maturation.

Experiment 2 was carried out to evaluate the effects of BSA and PVA on maturation of oocyte *in vitro*. The maturation medium was the MEDIUM 3 supplemented with 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml EGF, 10% pFF and 0.57 mM cysteine containing 4 mg/ml BSA or 1 mg/ml PVA, respectively.

Experiment 3 was designed to investigate the effects of BSA and PVA on developmental ability of porcine embryos produced by IVF. The maturation media were MEDIUM 3 supplemented with 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml EGF, 10% pFF and 0.57 mM cysteine containing 4 mg/ml BSA and 1 mg/ml PVA, respectively.

Experiment 4 was performed to compare the different hormones for evaluating the effects on the development of oocyte maturation. The maturation medium was the MEDIUM 3 supplemented with 10% pFF, 10 ng/ml EGF and 0.57 mM cysteine. About 50 COCs were cultured in 500 μl medium containing both of 0.5 μg/ ml LH, 0.5 μg/ml FSH or either of them, respectively. The medium without FSH and LH was used as control. The oocytes cultured in these media are referred to as the FSH+LH, FSH, LH and FSH-LH-free groups.

Experiment 5 was conducted to determine whether the presence of follicular shell in culture medium affected the maturation of oocyte *in vitro*. The immature oocytes with 0, 2, 4 and 6 inverted follicle shells were cultured during the maturation.

Statistical Analysis

Analyses of variable (ANOVA) were carried out using the SAS package in a completely randomized design. Duncan's multiple range test and student's *t*-test were used to compare values of individual treatment, when the F-value was significant ($p<0.05$).

RESULTS

Effects of Different mTCM-199 Media on IVM

The effects of the three different mTCM-199 media on maturation of porcine oocytes are shown in Table 2. When oocytes were cultured in the three different mTCM-199 media for 44 hr, a large proportion of oocytes reached metaphase II (MII; 82.1~87.1%) and no differences were observed among the maturation media. Also, no significant differences were found in the rates of GVBD (4.2~6.0%) among the maturation media.

Effects of BSA and PVA on IVM

The effects of BSA and PVA on maturation of porcine oocytes are shown in Table 3. The percentage of germinal vesicle was higher in MEDIUM 3 containing BSA than in MEDIUM 3 containing PVA (12.5 and 2.7%, $p<0.05$). There were no significant differences in percentages of GVBD after maturation between the groups containing BSA or PVA. When oocytes were cultured in MEDIUM 3 containing PVA, higher percentage of MII oocytes was obtained ($p<0.05$).

Effects of BSA and PVA on IVF and Subsequent Development

As shown in Tables 4 and 5, the percentages of the oocytes with one male pronucleus and one female pronucleus at 12 hr of insemination and the blastocyst formation of IVF embryos derived from the oocytes

Table 2. Effects of three different mTCM-199 on maturation of porcine oocytes*

Maturation medium	No. of oocytes cultured	% of oocytes (mean±SE)		
		GV	GVBD	MII
MEDIUM 1	201	12.5±3.8	6.0±2.5	82.1±3.9
MEDIUM 2	190	10.2±2.6	4.2±2.7	87.1±2.8
MEDIUM 3	185	8.0±1.9	5.3±2.2	86.8±1.2

* GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II. Experiments were repeated 6 times.

Table 3. Effects of BSA and PVA on maturation of porcine oocytes in MEDIUM 3*

Maturation medium	No. of oocytes cultured	% of oocytes (mean±SE)		
		GV	GVBD	MII
MEDIUM 3 with 4 mg/ml BSA	87	12.5±1.8[a]	7.0±2.0	80.5±0.2[a]
MEDIUM 3 with 1 mg/ml PVA	76	2.7±1.3[b]	6.6±1.3	90.8±1.4[b]

* GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II. Experiments were repeated 3 times.

[a,b] Values in the same column with different superscripts differ significantly ($p<0.05$).

Table 4. The fertilization parameters of porcine oocytes matured in MEDIUM 3 containing BSA or PVA*

Treatment	No. of oocytes inseminated	% of oocytes (mean±SE)		
		Penetrated	With one male and female pronucleus	Polyspermic
MEDIUM 3 with 4 mg/ml BSA	156	50.7±3.2[a]	40.0±1.5[a]	10.7±2.4
MEDIUM 3 with 1 mg/ml PVA	157	61.2±3.0[b]	49.0±2.0[b]	13.0±2.7

* After 44 hr of maturation, oocytes and sperm were co-incubated in 500 μl mTBM for 6 hr. After insemination, oocytes were transferred into 500 μl NCSU-23 medium for culture. Experiments were repeated 6 times.

[a,b] Values in the same column with different superscripts differ significantly ($p<0.05$).

matured in medium containing 0.1% PVA were significantly enhanced compared to those matured in medium containing 0.4% BSA ($p<0.05$). The percentages of polyspermic oocytes and cleaved oocytes, and the cell number of blastocysts were not different between the two different maturation media.

Effects of Gonadotropins on IVM

The effects of gonadotropins maturation of oocytes are shown in Table 6. Significantly higher percentages of MII oocytes were obtained in the treatment groups of FSH+LH and FSH with significantly lower portions remaining at the germinal vesicle stage compared with the treatment groups of LH and FSH-LH-free, respectively ($p<0.05$).

Effects of Follicle Shell Co-culture on IVM, IVF and Embryos Development

The effect of follicle shell on maturation of porcine oocytes are shown in Table 7. In comparison with the presence of 0, 2, 4 and 6 follicular shells in the maturation media, the proportion of oocytes with MII stage

Table 5. Effects of BSA and PVA on developmental ability of porcine embryos*

Treatment	No. of embryos cultured	% (mean±SE) of		Cell no. (mean±SE) in blastocysts
		Cleaved	Blastocysts	
MEDIUM 3 with 4 mg/ml BSA	122	61.7±2.1	12.2±1.8[a]	31.3±3.1
MEDIUM 3 with 1 mg/ml PVA	125	64.9±2.4	17.9±1.5[b]	29.4±2.0

* After 44 hr of maturation, oocytes and sperm were co-incubated in 500 μl mTBM for 6 hr. After insemination, oocytes were transferred into 500 μl NCSU-23 medium for culture. Experiments were repeated 6 times.

[a,b] Values with different superscripts differ significantly ($p<0.05$).

Table 6. Effects of gonadotropins on maturation of porcine oocytes*

Treatment[1]	No. of oocytes cultured**	% of oocytes (mean±SE)		
		GV	GVBD	MII
FSH+LH	148	5.3±1.6[a]	6.1±1.4	88.6±2.3[a]
FSH	149	8.7±2.0[a]	6.2±1.5	85.1±1.9[a]
LH	155	29.1±4.7[b]	7.0±1.8	64.0±5.9[b]
FSH-LH-free	147	37.8±5.2[b]	8.9±2.7	53.4±5.4[b]

* GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II. Experiments were repeated 6 times.
** The gonadotropins were added into MEDIUM 3 supplemented with 10 ng/ml EGF and 10% pFF.
[a,b] Values in the same column with different superscripts differ significantly ($p<0.05$).

Table 7. Effects of number of follicle shell on maturation of porcine oocytes*

No. of follicle shell**	No. of oocytes cultured	% of oocytes (mean±SE)			pH of medium at	
		GV	GVBD	MII	22 hr	44 hr
0	156	11.5±2.5[a]	7.1±1.2	81.5±2.2[a]	7.39±0.03[a]	7.36±0.04[a]
2	156	4.4±1.1[b]	4.6±1.6	92.2±1.1[b]	7.25±0.02[b]	7.25±0.01[b]
4	162	11.2±2.1[ab]	7.7±2.5	81.0±3.5[a]	7.09±0.01[c]	7.09±0.01[c]
6	156	14.2±2.7[c]	4.6±1.7	80.0±1.7[a]	7.04±0.01[d]	7.03±0.01[d]

* GV, germinal vesicle; GVBD, germinal vesicle breakdown; MII, metaphase II. Experiments were repeated 6 times.
** The follicle shells were co-cultured with oocytes in MEDIUM 3 supplemented with 0.5 μg/ml FSH, 0.5 μg/ml LH, 10 ng/ml EGF and 10% pFF.
[a~d] Values in the same column with different superscripts differ significantly ($p<0.05$).

was significantly higher in the presence of 2 follicular shells than those in the 0, 4 and 6 follicle shells ($p<0.05$). After 22 and 44 hrs of culture, the pH of media with different number of follicle shells was significantly different ($p<0.05$). The pH declined according to increase of number of follicle shell. The pH of the medium without follicle shell was about 7.39. After adding 4 and 6 follicle shells, the pH of medium de-

Table 8. The fertilization parameters of porcine oocytes matured in MEDIUM 3 with follicle shell*

No. of follicle shell**	No. of oocytes inseminated	% of oocytes (mean±SE)		
		Penetrated	With one male and female pronucleus	Polyspermic
0	111	60.2±4.6[ab]	32.6±2.6[a]	25.9±3.3[a]
2	144	68.7±4.3[a]	50.8±1.7[b]	17.9±3.6[ab]
4	113	51.1±1.9[b]	38.5±1.9[a]	12.6±0.7[b]
6	144	55.3±4.4[b]	38.6±3.7[a]	14.2±1.2[b]

* After 44 hr of maturation, oocytes and sperm were co-incubated in 500 μl mTBM for 6 hr. After insemination, oocytes were transferred into 500 μl NCSU-23 medium for culture. Experiments were repeated 6 times.
** The follicle shells were co-cultured with oocytes in MEDIUM 3 supplemented with 0.5 μg/ml FSH, 0.5 μg/ml LH, 10 ng/ml EGF and 10% pFF.
[a,b] Values in the same column with different superscripts differ significantly ($p<0.05$).

Table 9. Effects of follicle shell on development of porcine embryos produced by IVF*

No. of follicle shell**	No. of embryos cultured	% (mean±SE) of		Cell no. (mean±SE) in blastocysts
		Cleaved	Blastocysts	
0	151	55.3±4.2	12.9±1.4[a]	23.1±1.9[a]
2	153	60.9±1.9	22.5±1.4[b]	31.9±2.7[b]
4	140	58.4±3.2	19.1±1.5[b]	30.0±2.2[ab]
6	136	56.0±2.7	18.3±1.5[b]	32.6±3.7[b]

* After 44 hr of maturation, oocytes and sperm were co-incubated in 500 μl mTBM for 6 hr. After insemination, oocytes were transferred into 500 μl NCSU-23 medium for culture. Experiments were repeated 6 times.

** The follicle shells were co-cultured with oocytes in MEDIUM 3 supplemented with 0.5 μg/ml FSH, 0.5 μg/ml LH, 10 ng/ml EGF and 10% pFF.

[a,b] Values in the same column with different superscripts differ significantly ($p<0.05$).

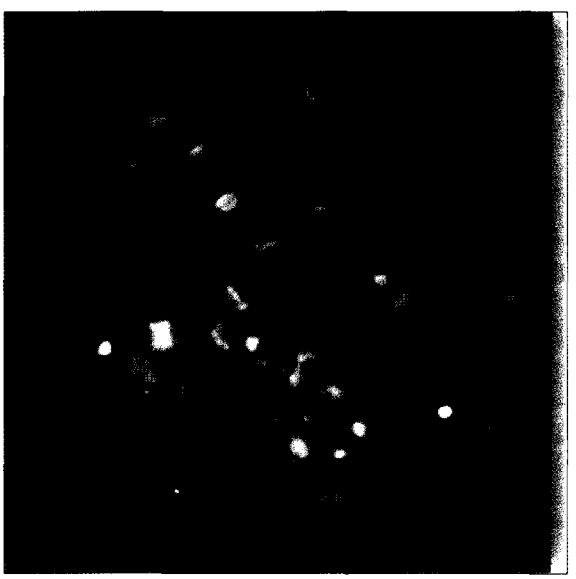

Fig. 2. A fluorescent picture of blastocyst stained with DAPI. The oocytes were cultured in MEDIUM 3 with 2 follicle shells for 44 hr, inseminated with 1×10^6 liquid boar sperm/ml for 6 hr, and then cultured in NCSU-23 medium for 7 days.

clined to 7.09 and 7.04 after 22 hr of maturation, respectively.

As shown in Table 8, oocytes cultured with two follicle shells effected sperm penetrations and pronucleus formations after IVF, and the polyspermy rates were significantly decreased in oocytes co-cultured with 4 and 6 follicle shells ($p<0.05$). The blastocyst formation rate and cell number in blastocysts derived from the oocytes co-cultured with follicle shells were higher than those matured in medium without follicle shell ($p<0.05$, Fig. 2 and Table 9).

DISCUSSION

Oocyte maturation includes both nuclear and cytoplasmic maturation. The developmental rate and nuclear maturation are generally used as simple indexes of the ability of oocytes to undergo subsequent development, although nuclear and cytoplasmic maturation of the porcine oocyte are relatively independent physiological events. Hagen *et al.* (1991) compared five media formations for the culture of 1- and 2-cell porcine embryos: TCM-199 with Eagle's salts and four formulations of modified Tyrode's medium. The results reported that porcine embryos could be cultured from the one-cell stage to blastocyst in a simple HEPES-buffered medium in air. The MEDIUM 1 of TCM-199 corresponded with that described by Park *et al.* (2004). The MEDIUM 2 was the MEDIUM 1 without HEPES. The MEDIUM 3 of mTCM-199 corresponded with that described by Park *et al.* (2002), which was supplemented PVA and D-glucose, and was deleted bovine apotransferrin, vitamin B_{12}, insulin, BSA and HEPES in the MEDIUM 1. In this study, the rate of oocytes at the MII stage after the IVM period was very close to those reported in previous studies (Ding and Foxcroft, 1992; Yoshida *et al.*, 1992b; Yi *et al.*, 2003), and the three maturation media were similarly effective.

PVA has proven to be extremely useful for the handling of embryos in protein-free medium. Embryos in medium containing PVA do not become sticky and hard to handle as they do in macromolecule-free media or in media containing other synthetic polymers such as polyvinylpyrrolidone or ficoll. PVA is also useful for culture of 1-cell rabbit embryos to early morulae but BSA is necessary for culture to the blastocyst stage (Bavister, 1981). Miyano *et al.* (1994) observed that supplementation of Whitten's medium with 0.5 mg/ml hyaluronic acid better supported blastocyst development of *in vivo* derived 1- to 2- cell porcine embryos. When culture medium contained 4 mg/ml BSA, blastocyst development were 70% and 45% in the presence and

absence of hyaluronic acid, respectively. However, addition of 15 mg/ml BSA in the presence and absence of hyaluronic acid resulted in 38% and 24% blastocyst development, respectively. It seems that at high concentrations, a factor in BSA has negative effects on the stimulatory action of hyaluronic acid. Kano *et al.* (1998) reported that addition of 0.5 mg/ml hyaluronic acid to Whitten's medium containing 0.4% BSA stimulated the development of IVM/IVF derived porcine embryos to the blastocyst stage.

It was reported by Mattioli *et al.* (1991) that the maturation to MII stage was significantly increased in the presence of LH and FSH. During the process of IVM, the cumulus cells of oocytes showed varying degree of expansion after 22 hr of culture. To a certain extent, the degree of cumulus expansion could serve as an indicator of successful nuclear and cytoplasmic maturation (Abeydeera, 2002). FSH and LH are important for progesterone production of cumulus cells, which is responsible for the acceleration of GVBD and the resumption of meiosis (Shimada and Terada, 2002; Yamashita *et al.,* 2003). The cumulus expansion in the hormone free group could be attributed to the presence of pFF and epidermal growth factor (EGF). It was found that pFF (Abeydeera *et al.,* 1998b) and EGF (Abeydeera, 2002) stimulated both hyaluronic acid production in COCs and its retention within the extracellular matrix of the expanding cumulus.

In the present study, we found that 92.2% of the oocytes cultured with 2 follicle shells developed to MII stage after 44 hr of maturation in MEDIUM 3, while only 81.5% of the oocytes cultured without follicle shell developed to MII stage. Hoshino *et al.* (2005) reported that 93% of oocytes matured with follicle shell reached the MII stage at 36 hr after maturation culture, while only 78% of oocytes matured without follicle shell reached MII after 40 hr after maturation culture. Supplementation of follicle shell has been shown to improve cytoplasmic maturation of porcine oocytes (Mattioli *et al,* 1989). Follicle cells in the presence of LH induced a transient increase of intra-cytoplasmic cAMP concentration (Mattioli *et al.,* 1991) and GSH concentration in porcine oocytes during maturation, which promoted the full maturation of pig oocytes and improved the developmental competence after parthenogenetic activation (Liu *et al.,* 1997) and enhanced the developmental competence of porcine oocytes after IVF (Abeydeera *et al.,* 1998a). Funahashi *et al.* (1997) reported that the presence of dibutyryl cAMP (dbcAMP) during the first 20 h of culture for maturation induced a more synchronous meiotic progress of porcine oocytes and improving the rate of early embryonic development to the blastocyst stage after IVF. High GSH levels also has shown to be correlated with increasing the efficiency of *in vitro* blastocyst production after IVF (De Matos *et al.,* 1995; Abeydeera *et al.,* 1998a; Brad *et al.,* 2003). Many groups were trying to improve the GSH synthesis by adding pFF (Brad *et al.,* 2003; Tatemoto *et al.,* 2004;), R-roscovitine (Coy *et al.,* 2005), follicle shell pieces (Abeydeera *et al.,* 1998a), cysteamine (De Matos *et al.,* 1995) in maturation medium. Liu *et al.* (1997) also reported that co-culture with two follicle shells during IVM resulted in a significantly higher cleavage rate after electrical activation in embryos produced by nuclear transfer compared to that in oocytes matured without co-culture. Furthermore, a high intracellular concentration of GSH also appeared to be essential for overcoming the *in vitro* developmental arrest in mice (Nasr-Esfahani and Johnson, 1992) and rats (Slott and Hales, 1987). All these results demonstrated that the level of GSH in oocyte might be as one of indicators of developmental capacity of oocytes on the cloned porcine research. When the oocytes were matured with 6 follicle shells in 2 ml of maturation medium, percentages of MII stage oocytes significantly decreased than those with 2 follicle shells, which may due to the pH of medium declined to about 7.0 after 22 hr of the culture and the lack of nutrition for oocyte maturation induced by follicle shell growing. These results suggested that 1 follicle shell per ml maturation medium was beneficial to maturation of oocytes.

In conclusion, the present study suggests that BSA was not necessary during the maturation of porcine oocytes and PVA could be used as macromolecule instead of BSA. Also, FSH and follicle shell played important roles in IVM of porcine oocytes.

REFERENCES

1. Abeydeera LR, Day BN (1997): Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol Reprod 57: 729-734.
2. Abeydeera LR, Wang WH, Cantley TC, Rieke A, Day BN (1998a): Coculture with follicular shell pieces can enhance the developmental competence of pig oocytes after *in vitro* fertilization: relevance to intracellular glutathione. Biol Reprod 58:213-218.
3. Abeydeera LR, Wang WH, Prather RS, Day BN (1998b): Maturation *in vitro* of pig oocytes in protein-free culture media: fertilization and subsequent embryo development *in vitro*. Biol Reprod 58:1316-1320.
4. Abeydeera LR (2002): *In vitro* production of embryos in swine. Theriogenology 57:257-273.
5. Bavister BD (1981): Analysis of culture media for *in vitro* fertilization and criteria of success. In: Mastroianni L, Biggera JD (eds.). Fertilization and Embryonic Development *In Vitro*. Plenum Press, New

York, USA. pp 41-60.
6. Brad AM, Bormann CL, Swain JE, Durkin RE, Johnson AE, Clifford AL, Krisher RL (2003): Glutathione and adenosine triphosphate content of *in vivo* and *in vitro* matured porcine oocytes. Mol Reprod Dev 64:492-498.
7. Coy P, Romar R, Ruiz S, Canovas S, Gadea J, Garcia VF, Matas C (2005): Birth of piglets after transferring of *in vitro*-produced embryos pre-matured with R-roscovitine. Reproduction 129:747-755.
8. De Matos DG, Furnus CC, Moses DF, Baldassarre H (1995): Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*. Mol Reprod Dev 43:432-436.
9. Ding J, Foxcroft GR (1992): Follicular heterogeneity and oocyte maturation *in vitro* in pigs. Biol Reprod 47:648-655.
10. Ding J, Foxcroft GR (1994): Epidermal growth factor enhances oocyte maturation in pigs. Mol Reprod Dev 39:30-40.
11. Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN (1994a): *In vitro* development of *in vitro*-matured porcine oocytes following chemical activation or *in vitro* fertilization. Biol Reprod 50:1072-1077.
12. Funahashi H, Stumpf TT, Terlouw SL, Cantley T, Rieke A, Day BN (1994b): Developmental ability of pig oocytes matured and fertilized *in vitro*. Theriogenology 41:1425-1433.
13. Funahashi H, Kim NH, Stumpf TT, Cantley TC, Day BN (1996): Presence of organic osmolytes in maturation medium enhances cytoplasmic maturation of porcine oocytes. Biol Reprod 54:1412-1419.
14. Funahashi H, Cantley TC, Day BN (1997): 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.
15. Grupen CG, Nagashima H, Nottle MB (1995): Cysteamine enhances *in vitro* development of porcine oocytes matured and fertilized *in vitro*. Biol Reprod 53:173-178.
16. Grupen CG, Nagashima H, Nottle MB (1997): Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development *in vitro*. Reprod Fertil Dev 9:571-575.
17. Hagen DR, Prather RS, Sims MM, First NL (1991): Development of one-cell porcine embryos to the blastocyst stage in simple media. J Anim Sci 69: 1147-1150.
18. Hoshino Y, Uchida M, Shimatsu Y, Miyake M, Nagao Y, Minami N, Yamada M, Mai H (2005): Developmental competence of somatic cell nuclear transfer embryos reconstructed from oocytes matured *in vitro* with follicle shells in miniature pig. Cloning Stem Cells 7:17-26.
19. Iwasaki T, Kimura E, Totsukawa K (1999): Studies on a chemically defined medium for *in vitro* culture of *in vitro* matured and fertilized porcine oocytes. Theriogenology 51:709-720.
20. Kano K, Miyano T, Kato S (1998): Effects of glycosaminoglycans on the development of *in vitro*-matured and -fertilized porcine oocytes to the blastocyst stage *in vitro*. Biol Reprod 58:1226-1232.
21. Liu L, Dai Y, Moor RM (1997): Role of secreted proteins and gonadotropins in promoting full maturation of porcine oocytes *in vitro*. Mol Reprod Dev 47:191-199.
22. Mattioli M, Galeati G, Seren E (1988): Effect of follicle somatic cells during pig oocyte maturation on egg penetrability and male pronuclear formation. Gamete Res 20:177-183.
23. Mattioli M, Bacci ML, Galeati G, Seren E (1989): Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 31:1201-1207.
24. Mattioli M, Bacci ML, Galeati G, Seren E (1991): Effects of LH and FSH on the maturation of pig oocytes *in vitro*. Theriogenology 36:95-105.
25. Miyano T, Hiro-oka RE, Kano K, Miyake M, Kusunoki H, Kato S (1994): Effects of hyaluronic acid on the development of 1- and 2-cell porcine embryos to the blastocyst stage *in vitro*. Theriogenology 41:1299-1305.
26. Naito K, Fukuda Y, Toyoda Y (1988): Effect of procine follicular fluid on male pronucleus formation in porcine oocytes matured *in vitro*. Gamete Res 21: 289-295.
27. Nasr-Esfahani MH, Johnson MH (1992): Quantitative analysis of cellular glutathione in early preimplantation mouse embryos developing *in vivo* and *in vitro*. Hum Reprod 7:1281-1290.
28. Park KW, Lai L, Cheong HT, Cabot R, Sun QY, Wu GM, Rucker EB, Durtschi D, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS (2002): Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. Biol Reprod 66:1001-1005.
29. Park CS, Kim MY, Yi YJ, Chang YJ, Lee SH, Lee JJ, Kim MC, Jin DI (2004): Liquid boar sperm quality during storage and *in vitro* fertilization and culture of pig oocytes. Asian-Aust J Anim Sci 17:1369-1373.
30. Shimada M, Terada T (2002): FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in porcine oocytes. Mol Hum Reprod 8:612-618.
31. Slott VL, Hales BE (1987): Effect of glutathione depletion by buthionine sulfoximineon rat embryonic development *in vitro*. Biochem Pharmacol 36: 683-688.

32. Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T (2004): Protection of porcine oocytes against cell damage caused by oxidative stress during *in vitro* maturation: Role of superoxide dismutase activity in porcine follicular fluid. Biol Reprod 71:1150-1157.
33. Wang WH, Nika K, Okuda K (1991): *In vitro* penetration of pig oocyts matured in culture by frozen-thawed ejaculated spermtozoa. J Reprod Fertil 93:491-496.
34. Wang WH, Niwa K (1995): Synergetic effects of epidermal growth factor and gonadotropins on the maturation of pig oocytes in a serum-free medium. Zygote 3:345-350.
35. Yamashita Y, Shimada M, Okazaki T, Maeda T, Terada T (2003): Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes. Biol Reprod 68:1193-1198.
36. Yi YJ, Kim MY, Lee SH, Min TS, Jin DI, Park CS (2003): Effect of cysteamine on *in vitro* maturation, fertilization and culture of porcine oocytes. Korean J Anim Reprod 27(4):275-280.
37. Yoshida M, Ishigaki K, Pursel VG (1992a): Effects of maturation media on male pronucleus formation in pig oocytes matured *in vitro*. Mol Reprod Dev 31:68-71.
38. Yoshida M, Ishizaki Y, Kawagishi H, bamba K, Kojima Y (1992b): Effects of pig follicular fluid on maturation and after fertilization of pig oocytes *in vitro* and on their subsequent fertilizing and developmental capacity *in vitro*. J Reprod Fertil 95:481-488.
39. Yoshida M (1993): Role of glutathione in the maturation and fertilization in pig oocytes *in vitro*. Mol Reprod Dev 35:76-81.

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