Effects of FBS(Fetal Bovine Serum) and pFF(Porcine Follicular Fluid) on *In Vitro* Maturation and Development of Porcine Parthenogenetic and Nuclear Transfer Embryos

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ABSTRACT

In this study, *in vitro* maturation system using fetal bovine serum (FBS) or porcine follicular fluid (pFF) was investigated to produce comparable oocytes to those derived from *in vivo*. Control group of oocytes was cultured in TCM 199 supplemented with 0.1% polyvinyl alcohol (PVA). Other three groups of oocytes were cultured in TCM 199 supplemented with 10% FBS, 10% pFF or 5% FBS + 5% pFF, respectively. After 44 h maturation, oocytes with the first polar body were activated with two electric pulses (DC) of 1.2 kv/cm for 30 μsec. Also, matured oocytes of four groups were reconstructed and fused. Reconstructed embryos were cultured in PZM-3 under 5% CO_2 in air at 38.5℃ for 6 days. The oocytes matured in the medium supplemented with FBS or/and pFF showed significantly higher maturation rates (64.0 vs. 73.9 to 85.2%). In PA embryos, cleavage rates (89.7 vs. 77.1 to 86.6%) and blastocysts rates (30.0 vs. 16.2 to 26.2%) were significantly higher in pFF group (p<0.05). In NT embryos, there was no difference among treatments in cleavage rate, but the blastocyst rates (28.5 vs. 15.5 to 24.6%) were significantly higher in pFF group (p<0.05). The apoptosis rate was significantly higher (p<0.05) in the control than other groups (10.8 vs. 4.9 to 8.2% for PA, 3.1 vs. 0.5 to 1.3% for NT). In order to select the comparable oocyte to *in vivo* oocytes, each group of oocytes was stained with Brilliant cresyl blue (BCB) after 42h maturation. The matured oocytes were separated according to color of cytoplasm; stained group (BCB+) and unstained group (BCB-). The oocytes matured in the presence of FBS or/and pFF showed significantly higher staining rates (70.3 to 72.7 vs. 35.1%) (p<0.05). To verify the fact that the supplementation of FBS or/and pFF can increase the maturation rates, cdc2 kinase activity, the catalytic subunit of MPF, was determined. The cdc2 kinase activity of the oocytes matured in the medium supplemented with FBS or/and pFF was significantly higher than control group (6.7 to 9.3 vs. 3.8). In conclusion, the supplementation of FBS or/and pFF can support *in vitro* maturation rate of porcine oocytes through the increment of cdc2 kinase activity level in the cytoplasm.

(Key words : FBS, pFF, Porcine embryos, BCB, Cdc2 kinase)

INTRODUCTION

There has been an increasing interest in the production of large quantities of porcine oocytes through *in vitro* maturation (IVM)/*in vitro* fertilization (IVF) techniques, because of the physiological similarities of pig oocytes to those of humans (Prather *et al*., 2003). Since the first successful production of cloned piglet using *in vitro* matured oocyte, it has been frequently used in pig cloning. Various methods for IVM of porcine oocytes have been developed, but their developmental potential is still lower compared to *in vivo* production (Dobrinsky *et al*., 1996).

The ability of oocytes to undergo IVM and subsequent embryo development is influenced by maturation media (Marques *et al*., 2007) and supplements of hormone and serum (Lonergan *et al*., 2003). In general, porcine embryos are cultured in media supplemented with serum. Several studies have shown that serum has a biphasic effect. The presence of serum does inhibit the early cleavage divisions, while it will have an accelerating effect in later development (Algriany *et al*., 2004; Hunter *et al*., 2000).

When cumulus oocyte complexes (COCs) were matured in the medium containing fetal bovine serum (FBS), it increased the tight compaction of cumulus cells, the migration of neighbor cells into a mass, or attachment of cumulus cells to the bottom of the dish (Suzuki *et al*., 2006). Men *et al*. (2005) reported that the supple-

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mentation of 10% FBS (starting at Day 4 of embryo culture) significantly increased the ability of *in vitro* produced Day 6 blastocysts. FBS is generally used to supplement the medium for not only oocyte or embryo culture in mammalian IVP systems, but also for cell and tissue cultivation.

Also IVM media are usually supplemented with porcine follicular fluid (pFF) to promote developmental competence of oocytes (Funahashi *et al*., 1994; Yoshida *et al*., 1992). Based on observations of maturation media supplemented with pFF, several group concluded that pFF contains substances that improve the expansion of the cells of the cumulus oophorus, and increases the percentage of germinal vesicle breakdown (GVBD) oocytes reaching MII stage and subsequent formation of male pronucleus and cleavage rates (Yoon *et al*., 2000; Marchal *et al*., 2001). Tatemoto *et al*. (2004) reported that pFF contains high concentrations of superoxide dismutase (SOD), it also plays an important role in the protection of oocytes against oxidative stress. Nevertheless, pFF introduces a series of unknown factors to the medium, which cause difficulties in technical standardization and in the exact identification of the substances that are essential to the regulation of maturation (Yoshida *et al*., 1992). As an alternative, researchers have been developing new maturation media supplemented with polyvinyl alcohol (PVA), as a substitute for pFF (Hong *et al*., 2004).

The brilliant cresyl blue (BCB) test has been used successfully to select homologous oocytes for IVP. BCB has been used for the selection of competent oocytes of prepubertal pig, goats and cattle (Alm *et al*., 2005; Bhojwani *et al*., 2007; Han *et al*., 2006; Wongsrikeao *et al*., 2006). The test allows the determination of the activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized in growing oocytes but inactive in oocytes that have finished their growth phase (Katska-Ksiazkiewicz *et al*., 2007). Also BCB test is useful test for selecting larger and more competent oocytes for IVP than conventional morphological criteria (Pujol *et al*., 2004). The concentration of 26 μM BCB had been found to be effective for porcine oocytes as it was supportive of a high rate of selected oocytes without apparent loss of viability.

It has been well established that meiosis resumption is facilitated by maturation promoting factor (MPF) in pig oocytes. During oocyte maturation *in vitro*, MPF activation begins at the time of GVBD, sharply rises at the MI stage, declines at anaphase-telophase I transition, and reaches a high level again at the MII stage (Wu *et al*., 1997). It has been well accepted that the high level of MPF and mitogen activated protein (MAP) kinase activity in M II oocytes works as cytostatic factor (CSF) and causes MII arrest in vertebrate oocytes, including mammals. In MII arrested oocytes, MPF activity reaches the highest level and the enhanced ability for oocyte activation (Kikuchi *et al*., 2002; Ye *et al*., 2002).

Therefore, the present study investigated the effect of FBS and pFF on oocyte maturation and in vitro development of porcine oocytes.

MATERIALS AND METHODS

Preparation of Porcine Follicular Fluid (pFF)

All chemicals, unless noted otherwise, were from Sigma, St.Louis, MO. pFF aspirated from the follicles of different size (5~8 mm in diameter) was centrifuged at 2,000 rpm for 30 min at room temperature to remove blood cells and debris. The supernatant was transferred to a sterile centrifuge tube and stored at −20℃ until use. The pFF was passed through 0.22 μm membrane filters before use.

Collection of Oocytes and *In Vitro* Maturation (IVM)

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30~35℃. Cumulus-oocyte complexes (COCs) were collected by the aspiration of ovary antral follicles (3~6 mm diameter) with 18 gauge needle fixed to a 10 ml disposable syringe. The follicular fluid was pooled into 50 ml conical tubes and its sediment washed in Tyrode's lactate (TL)-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA). The COCs with several layers of cumulus cells were selected and washed three times in maturation medium. For maturation culture, approximately 50~100 COCs were transferred into 500 μl maturation medium (TCM-199, Gibco-BRL, GrandIsland, NY, USA) covered with mineral oil in a four-well dish (Nunc, Roskilde, Denmark). Oocytes were matured for 40 to 44 h at 38.5℃ under 5% CO_2 in air. The maturation medium supplemented with 0.1% PVA (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml luteinizing hormone, 0.5 μg/ml follicle stimulating hormone, 10 ng/ml epidermal growth factor, 75 μg/ml penicillin G and 50 μg/ml streptomycin.

Production of Parthenogenetic (PA) Embryos

After maturation, cumulus cells were removed from oocytes by vortexing the COCs in PBS supplemented with 0.1% PVA and 0.1% hyaluronidase for 4 min. Cumulus-free oocytes with the first polar body were placed between 0.2 mm diameter wire electrodes, 1 mm apart in activation medium. The medium used for activation was 0.3 M mannitol supplemented with 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 0.5 mM Hepes. For the fusion, two DC pulses (1 sec interval) of 1.2 kV/cm were applied for 30 μsec using an Electro Cell fusion (NEPA gene, Chiba, JAPAN).

Brilliant Cresyl Blue Test (BCB)

To carry out the BCB test, the compact COCs were washed three times in Dulbecco's PBS modified by the addition of 0.4% BSA (A-7888; mDPBS). Then the COCs were exposed to 26 μM of BCB (B-5388) diluted in mPBS for 90 min at 38.5℃ in humidified air. Following BCB exposure, the COCs were transferred to mDPBS and washed twice. After washing, the COCs were examined under a stereomicroscope and divided into two groups according to their cytoplasm coloration: oocytes with any degree of blue coloration in the cytoplasm (BCB+) and oocytes without blue cytoplasm (BCB-).

Cdc2 Kinase Assay

MPF kinase assay was conducted by measuring the activity of its catalytic subunit, cdc2 kinase, with MESACUP cdc2 kinase assay kit (MBL, Nagoya, Japan). The correlation coefficients between cdc2 kinase activity examined by the MESACUP cdc2 kinase assay kit and histone H1 kinase activity measured by the radioactive method were as high as 0.9961. Briefly, twenty oocytes were denuded from their cumulus cells at 44h of IVM and washed twice with the cdc2 kinase sample buffer containing 50 mM Tris HCl, 0.5 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.01% Brij35, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β-glycerophosphate and 1 mM Na-orthovanadate. Oocytes were then transferred to a microtube containing 5 μl of the buffer and stored at −80℃. At the time of assay, oocytes were lysed by freezing and thawing with liquidnitrogen. Five microliters of oocytes extract were mixed with 45 μl kinase assay buffer containing 25 mM Hepes buffer (MBL), 10 mM $MgCl_2$ (MBL), 10% biotinylated MV peptide (Ser-Lue-Tyr-Ser-Ser-Ser-Pro-Gly-Gly-Ala-Tyr-Cys;MBL) and 0.1 mM ATP, and then incubated for 30 min at 30 ℃.

The phosphorylation reaction was terminated by the addition of 200 μl of stop reagent (PBS containing 50 mM EDTA; MBL), and centrifuged for 15 sec at 14,000 g. For the detection of cdc2 kinase by ELISA, each 100 μl of the reaction mixture were transferred to each microwell strip coated with monoclonal antibody recognizing the phosphorylated form of the biotinylated MV peptide. The microwells were incubated at 25℃ for 60 min, and then washed 5 times with washing solution (PBS). One hundred microliters of horseradish peroxidase conjugated streptavidin solution were added to each well and then incubated at 25℃ for 30 min. After washing, 100 μl of the POD substrate solution were added, and incubated for additional 5 min. Finally 100 μl of stop solution (20% H_3PO_4) was added to each well and the optical density of each well was read at 492 nm with a microplate reader.

Apoptosis Assays

The blastocysts on Days 6 from PA and NT were washed twice in PBS/PVP (PBS supplemented with 0.1 % polyvinylpyrolidone) and fixed in 4% (v/v) paraformaldehyde solution for 24 h at 4℃. Membranes were permeabilized in 0.5% Triton X-100 for 30 min at room temperature.

A TUNEL assay was used to assess the presence of apoptotic cells (*in situ* cell death detection kit, TMR red; Roche, Mannheim), for 1 h at 38.5℃ in the dark. The broken DNA ends of the embryonic cells were labeled with TDT and fluorescein-dUTP. After the reaction stopped, the embryos were washed and transferred into 10 μg/ml Hoechest 33342 for 30 min at room temperature in the dark. The embryos were washed three times and mounted on slides with Prolong antifade Kit (cat. P-748, Molecular Probes, Eugene, OR). The slides were stored at −20℃. The numbers of apoptotic nuclei and total numbers of nuclei were determined from optical images of whole-mount embryos under an epifluorescent microscope (Nikon, Tokyo, Japan).

Preparation of Porcine Fetal Fibroblast Cells

A 35 days porcine fetus was retrieved from the pregnant gilt. After the brain, intestines, and four limbs were removed, tissues were cut into small pieces with fine scissors. The minced cells were incubated for 30 min at 39℃ in PBS supplemented with 0.05% trypsin and 0.02 mM EDTA, and the suspension was centrifuged at 1,200 rpm for 5 min. Cell pellet were resuspended and cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 15% fetal bovine serum and 75 μg/ml antibiotics. The cells were passaged twice, and then frozen using DMEM supplemented with 10% dimethylsulfoxide (DMSO). To be used as donor cells in NT, cells were thawed and cultured until they reached confluence (2 to 8 passages). Before NT, cells were treated with 0.05% trypsin for single-cell isolation.

Production of Nuclear Transfer (NT) Embryos

After maturation, cumulus cells were removed from oocytes by vortexing the COCs in PBS supplemented with 0.1% PVA and 0.1% hyaluronidase for 4 min. Oocytes were enucleated by the aspiration of the first polar body and metaphase-II (MII) plate in a small amount of surrounding cytoplasm with a glass pipette. Enucleation was confirmed by staining the oocytes with 10 μg/ml Hoechst 33342 for 15~20 min at 39℃. All micromanipulation procedures were performed in TCM-199 supplemented with 3 mg/ml BSA and 5 μg/ml cytochalasin B. After enucleation, the oocytes were held in TCM-199 supplemented with 3 mg/ml BSA until injection of donor cells. Cells were trypsinized and held in TCM-199 supplemented with 3 mg/ml BSA. A

single cell with a smooth surface was transferred into the perivitelline space of an enucleated oocyte. Reconstructed oocytes were cultured for 1~2 h in TCM-199 supplemented with 3 mg/ml BSA until fusion. They were then placed between 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber overlaid with 0.3 M mannitol solution supplemented with 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 0.5 mM Hepes. For the fusion, two DC pulses (1 sec interval) of 1.2 kv/cm were applied for 30 μsec using an Electro Cell fusion (NEPA gene, Chiba, JAPAN). After fusion treatment, the reconstructed oocytes were cultured in TCM-199 supplemented with 3 mg/ml BSA for 30 min and the fusion was determined. Embryos were washed and transferred into culture medium (PZM-3, Im *et al*., 2004) covered with mineral oil in a four-well dish.

Statistical Analysis

Data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using the Duncan's multiple range tests. All data were expressed as Least Square (LS) mean±SEM (Standard Error of the sample Mean). A probability of $p<0.05$ was considered statistically significant.

RESULTS

Effect of FBS and pFF on *In Vitro* Maturation and *In Vitro* Development of Porcine PA and NT Embryos

The oocytes matured in the presence of FBS or pFF showed significantly higher ($p<0.05$) maturation rate than control (Table 1). For PA embryos, cleavage and blastocyst rate were significantly higher in FBS or pFF group than that of the control ($p<0.05$). The apoptosis rate was significantly lower in FBS or pFF group than that of the control ($p<0.05$) (Table 2). In NT embryos, there was no difference among treatments in cleavage rate, but developmental rate to the blastocyst stage was significantly higher in pFF or FBS group than the control ($p<0.05$). Also, the apoptosis rate was significantly lower in FBS or pFF group than the control (Table 3) ($p<0.05$).

Table 1. Effect of FBS and pFF on maturation of porcine oocytes

Treatments*	No. of oocytes	No. of (mean±SE) matured oocytes
Control	313	200 (64.0±2.6) ^{c}
FBS	349	259 (73.9±2.3) ^{b}
pFF	355	302 (85.2±1.5) ^{a}
FBS+pFF	350	280 (79.5±2.1) ^{ab}

* Control : TCM199 + 0.1% PVA, FBS : Control + 10% FBS, pFF : Control + 10% pFF, FBS + pFF : Control + 5% FBS + 5% pFF.
^{a,b,c} : Values with different superscripts in the same column differ significantly ($p<0.05$).

BCB Staining and Cdc2 Kinase Activity of the Oocytes Matured in Different Condition

The oocytes matured with FBS and/or pFF showed a significantly higher percentage of BCB+ than the control ($p<0.05$), while the percentage of BCB- was significantly higher in the control (Fig. 1, 2). The cdc2 kinase activity of the oocytes matured with FBS or/and pFF was significantly higher ($p<0.05$) than the control (6.7 to 9.3 vs 3.8) (Fig. 3).

DISCUSSION

This study investigated the effect of FBS and pFF on maturation and *in vitro* development of porcine PA and NT embryos. In this study, pFF supported better maturation and *in vitro* developmental rate.

In this study, the maturation rate with pFF was significantly higher than PVA or FBS treatment (Table 1).

Table 2. Effect of FBS and pFF on *in vitro* development of PA embryos

Treatments*	No. of oocytes	No. of embryos develop to		% TUNEL
		≥2 cell	Blastocycst	
Control	265	179 (77.1±3.3)^{c}	29 (16.2±2.6)^{c}	70/760 (10.8±2.1)^{a}
FBS	249	176 (83.5±3.2)^{bc}	38 (21.6±2.8)^{b}	29/640 (4.9±1.0)^{b}
pFF	240	185 (89.7±2.4)^{a}	56 (30.0±2.5)^{a}	82/1204 (7.4±0.9)^{b}
FBS+pFF	253	202 (86.6±3.9)^{ab}	53 (26.2±3.0)^{ab}	81/1280 (8.2±1.4)^{ab}

*Control : TCM199 + 0.1% PVA, FBS : Control + 10% FBS, pFF : Control + 10% pFF, FBS + pFF : Control + 5% FBS + 5% pFF.
$^{a\sim c}$: Values with different superscripts in the same column differ significantly ($p<0.05$).

Table 3. Effect of FBS and pFF on *in vitro* development of NT embryos

Treatments*	No.of oocytes fused/ manipulated	No. of NT embryos	No. (%±SE) of NT embryos develop to		% TUNEL
			≥2cell	Blastocyst	
Control	123/163	123	96 (81.0±8.3)	20 (16.5±2.5)^{b}	14/452 (3.1±0.7)^{a}
FBS	136/167	136	106 (80.2±7.2)	32 (24.6±3.8)^{ab}	8/657 (1.3±0.5)^{b}
pFF	136/177	136	118 (87.3±3.5)	38 (28.5±3.8)^{a}	3/525 (0.5±0.3)^{b}
FBS+pFF	117/165	117	98 (84.1±4.6)	18 (15.5±2.0)^{b}	4/319 (1.3±0.6)^{b}

* Control : TCM199 + 0.1% PVA, FBS : Control + 10% FBS, pFF : Control + 10% pFF, FBS + pFF : Control + 5% FBS + 5% pFF.
$^{a-c}$: Values with different superscripts in the same column differ significantly (p<0.05).

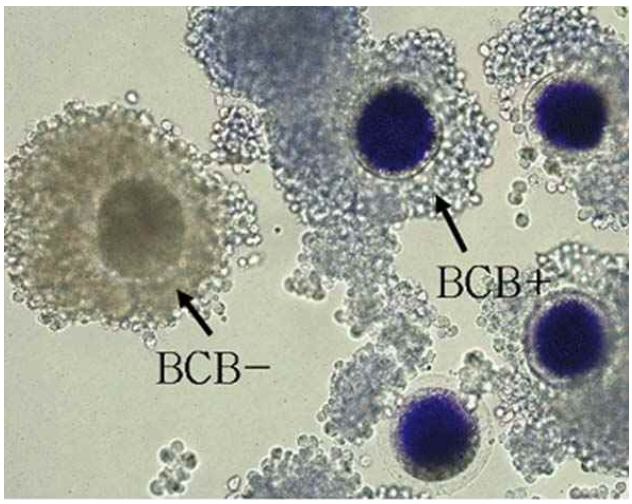

Fig. 1. Porcine COCs after exposure to BCB. BCB + : blue colored and BCB : unstained. (×30)

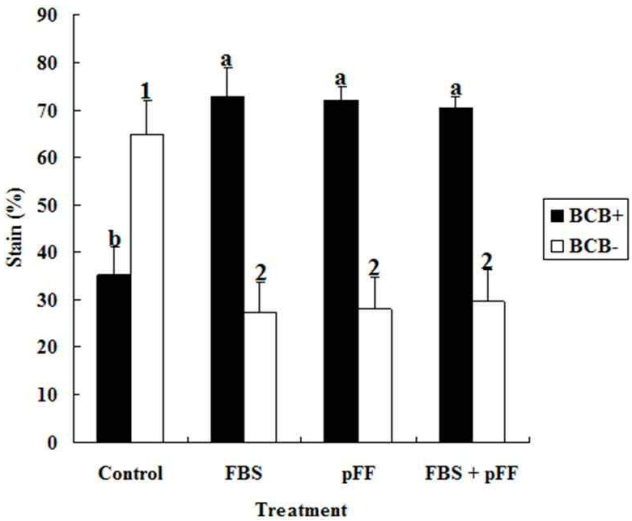

Fig. 2. BCB staining of porcine oocytes after *in vitro* maturation. Control : TCM-199 + 0.1% PVA, FBS : Control + 10% FBS, pFF : Control + 10% pFF, FBS + pFF : Control + 5% FBS + 5% pFF. ^{a,b,1,2} : Bars with different letters are significantly different (p<0.05).

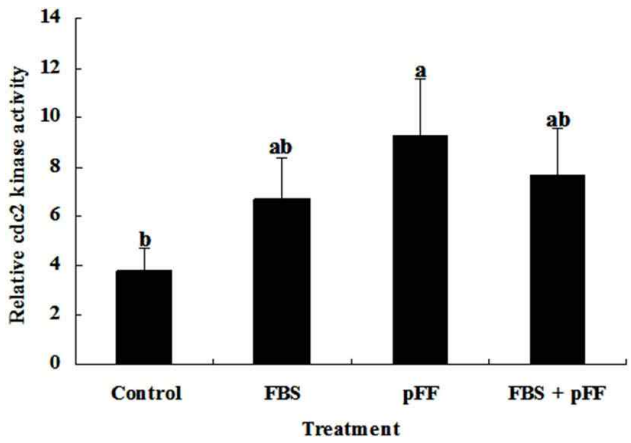

Fig. 3. The activity of cdc2 kinase in porcine oocytes matured in various maturation system. Control : TCM199 + 0.1% PVA, FBS : Control + 10% FBS, pFF : Control + 10% pFF, FBS + pFF : Control + 5% FBS + 5% pFF. Data are expressed as relative percentage of the level of p34cdc2 activity in porcine oocytes. ^{a,b} : Bars with different letters are significantly different (p<0.05).

Some researchers have confirmed that the developmental ability of *in vitro* produced blastocysts from oocytes matured in TCM 199 with 10% pFF was higher (Funahashi *et al*., 1994; Marques *et al*., 2007). Qian *et al*. (2003) reported that supplementaion of pFF supported consistent over 80% of maturation rate. Recently, Suzuki *et al*. (2006) also reported that when oocytes were matured in NCSU 37 supplemented with 10% pFF showed a higher maturation rate than 10% FBS (74 vs. 15 to 41%). They suggested the possibility that one or two mechanisms are operating; a promotion factor from pFF that increases the maturation rate, and an inhibitory factor from FBS that suppresses the rate. Different media, hormone or modification of the culture system might explain the difference in maturation rate. In many previous reports, exposure of early embryos to FBS was detrimental to blastocysts development *in vitro* and significantly reduced the formation of hatched blastocysts (Dobrinsky *et al*., 1996; Bavister *et al*., 1995).

In order to select the comparable oocytes to *in vivo* oocytes, BCB staining was conducted. It has been demonstrated that brilliant cresyl blue (BCB) can be used for the selection of competent oocytes of prepubertal pigs, goats and cattle (Rodriguez*et al*., 2002; Pujol *et al*., 2004). BCB is a vital blue dye, which determines the intracellular activity of glucose phosphate dehydrogenase (G6PD), an enzyme synthesized during the oocyte growth phase but with decreased activity in oocytes that have finished their growth phase (Tian *et al*., 1998). Oocytes that have finished their growth have a blue colored cytoplasm, because G6PD activity decreased (Rodriguez-Gonzalez *et al*., 2003). In the present study, the percentage of MII oocytes stained with BCB after IVM was higher in FBS and pFF group than those of PVA group (Fig. 1, 2).

In all animals, oocyte maturation involves the activation of various signal pathways that converge to activate MPF. During the final stage of maturation, immature oocytes develop from germinal vesicle (GV) to metaphase II (MII). Based on these morphological changes, the maturation process is divided into the meiotic stages germinal vesicle (GV), characterized by a visible prominent nucleus, diakinese (D), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) (Motlik *et al*., 1978). At this stage, meiosis is again arrested and the level of MPF in the cytoplasm reaches the highest level. One of the mechanisms that regulate MPF activity is a modification of the phosphorylation status of $p34^{cdc2}$ kinase by Myt1/Wee1 and cdc25. In the present study the oocytes matured in the medium supplemented with FBS or pFF showed a significantly higher activity of cdc2 kinase (Fig. 3). Funahashi *et al*. (1996) showed that the MPF activity of pig oocytes matured *in vitro* was influenced by the culture medium, and a correlation between MPF activity in metaphase II oocytes and their ability to develop to pronucleus stage (Naito *et al*., 1992; 1995; Wechrend *et al*., 2001).

In conclusion, the oocytes matured in TCM 199 supplemented with FBS or pFF showed significantly higher maturation rates. However, the developmental rate to blastocyst stage was higher in the oocytes matured in the presence of pFF. These results indicate that FBS or pFF can support the increment of cdc2 kinase activity level in the cytoplasm of oocytes resulting in the *in vitro* developmental ability.

REFERENCES

1. Algriany O, Bevers M, Schoevers E, Colenbrander B, Dieleman S (2004): Follicle sizedependent effects of sow follicular fluid on *in vitro* cumulus expansion, nuclear maturation and blastocyst formation of sow cumulus oocytes complexes. Theriogenology 62:1483-1497.
2. Alm H, Torner H, Lohrke B, Viergutz T, Ghoneim IM, Kanitz W (2005): Bovine blastocyst development rate *in vitro* is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose6phosphate dehydrogenase activity. Theriogenology 63:2194-2205.
3. Bavister BD (1995): Culture of preimplantation embryos: facts and artifacts. Hum Reprod 1:91-148.
4. Bhojwani S, Alm H, Torner H, Kanitz W, Poehland R. (2007): Selection of developmentally competent oocytes through brilliant cresyl blue stain enhances blastocyst development rate after bovine nuclear transfer. Theriogenology 67:341-345.
5. Dobrinsky JR, Johnson LA, Rath D (1996): Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development. Biol Reprod 55:1069-1074.
6. Funahashi H, Stumpf TT, Terlouw SL, Cantley TC, Rieke A, Day BN (1994): Developmental ability of porcine oocytes matured and fertilized *in vitro*. Theriogenology 41:1425-1433.
7. Funahashi H, Day BN (1993): Effects of different serum supplements in maturation medium on meiotic and cytoplasmic maturation of pig oocytes. Theriogenology 39:965-973.
8. Han ZB, Lan GC, Wu YG, Han D, Feng WG, Wang JZ, Tan JH (2006): Interactive effects of granulosa cell apoptosis, follicle size, cumulus-oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reproduction 132:749-758.
9. Hong JY, Yong HY, Lee BC, Hwang WS, Lim JM, Lee ES (2004): Effects of amino acids on maturation, fertilization and embryo development of pig follicular oocytes in two IVM media. Theriogenology 62:1473-1482.
10. Hunter MG (2000): Oocyte maturation and ovum quality in pigs. Rev Reprod 5:122-130.
11. Im GS, Lai L, Liu Z, Hao Y, Wax D, Bonk A, PratherRS (2004): *In vitro* development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology 61:1125-1135.
12. Katska-Ksiazkiewicz L, Opiela J, Ryńska B (2007): Effects of oocyte quality, semen donor and embryo co-culture system on the efficiency of blastocyst production in goats. Theriogenology 68:736-744.
13. Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H (2002): Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. Biol Reprod 66:1033-1041.
14. Lonergan P, Rizos D, GutierrezAdan A, Fair T, Bo-

land MP (2003): Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. Reprod Domest Anim 38:259-267.
15. Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P (2001): Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 56:17-29.
16. Marques MG, Nicacio AC, de Oliveira VP, Nascimento AB, Caetano HV, Mendes CM, Mello MR, Milazzotto MP, Assumpção ME, Visintin JA (2007): *In vitro* maturation of pig oocytes with different media, hormone and meiosis inhibitors. Anim Reprod Sci 97:375-381.
17. Men Hongsheng, Agca Yuksel, Critser ES, Critser JK (2005): Beneficial effects of serum supplementation during *in vitro* production of porcine embryos on their ability to survive cryopreservation by open pulled straw vitrification. Theriogenology 64:1340-1349.
18. Motlik J, KoefoedJohnson HH, Fulka J (1978): Breakdown of the germinal vesicle in bovine oocytes cultivated *in vitro*. J Exp Zool 205:377-384.
19. Naito K, Daen FP, Toyoda Y (1992): Comparison of histone H1 kinase activity during meiotic maturation between two types of porcine oocytes matured in different media *in vitro*. Biol Reprod 47:43-47.
20. Naito K, Hawkins C, Yamashita M, Nagahama Y, Aoki F, Kohmoto K, Toyoda Y, Moor RM (1995): Association of p34 cdc2 and cyclin B1 during meiotic maturation in porcine oocytes. Dev Biol 168:627-634.
21. Prather RS, Hawley RJ, Carter DB, Lai L, Greenstein JL (2003): Transgenic swine for biomedicine and agriculture. Theriogenology 59:115-123.
22. Pujol M, LópezBéjar M, Paramio MT (2004): Developmental competence of heifer oocytes selected using the brilliant cresyl blue (BCB) test. Theriogenology 61:735-744.
23. Qian Y, Shi WQ, Ding JT, Sha JH, Fan BQ (2003): Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilized *in vitro*. J Reprod Dev 49:167-174.
24. RodriguezGonzalez E, LopezBejar M, Izquierdo D, Paramio MT (2003): Developmental competence of prepubertal goat oocytes selected with brilliant cresyl blue and matured with cysteamine supplementation. Reprod Nutr Dev 43:179-187.
25. Suzuki M, Misumi K, Ozawa M, Noguchi J, Kaneko H, Ohnuma K, Fuchimoto D, Onishi A, Iwamoto M, Saito N, Nagai T, Kikuchi K (2006): Successful piglet production by IVF of oocytes matured *in vitro* using NCSU-37 supplemented with fetal bovine serum. Theriogenology 65:374-386.
26. 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.
27. Tian WN, Braunstein LD, Pang J, Stuhlmeier KM, Xi QC, Tian X, and Stanton RC (1998): Importance of glucose6phosphate dehydrogenase activity for cell growth. J Biol Chem 273:10609-10617.
28. Wehrend A, Meinecke B (2001): Kinetics of meiotic progression, M phase promoting factor (MPF) and mitogen-activated protein kinase (MAP kinase) activities during *in vitro* maturation of porcine and bovine oocytes: species-specific differences in the length of the meiotic stages. Anim Reprod Sci 66:175-184.
29. Wongsrikeao P, Otoi T, Yamasaki H, Agung B, Taniguchi M, Naoi H, Shimizu R, Nagai T (2006): Effects of single and double exposure to brilliant cresyl blue on the selection of porcine oocytes for *in vitro* production of embryos. Theriogenology 66:366-372.
30. Wu B, Ignotz G, Currie WB, Yang X (1997): Dynamics of maturation-promoting factor and its constituent proteins during *in vitro* maturation of bovine oocytes. Biol Reprod 56:253-259.
31. Ye J, Flint APF, Campbell KH, Luck MR (2002): Synchronization of porcine oocyte meiosis using cycloheximide and its application to the study of regulation by cumulus cells. Reprod Fertil Dev 14: 433-442.
32. Yoon KW, Shin TY, Park JI, Roh SH, Lim JM, Lee BC, Hwang WS, Lee ES (2000): Development of porcine oocytes from preovulatory follicles of different size after maturation in media supplemented with follicular fluids. Reprod Fertil Dev 12:133-139.
33. Yoshida M, Ishizaki Y, Kawagishi H, Kojima Y (1992): Effects of pig fluid on maturation of pig oocytes *in vitro* and on their subsequent fertilizing and development capacity *in vitro*. J Reprod Fertil 95:481-488.

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