Effect of Macromolecules in Maturation Medium on Oocyte Maturation and Embryonic Development after Parthenogenesis and Nuclear Transfer in Pigs

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ABSTRACT

The objective of this study was to examine the effect of macromolecule in a maturation medium on nuclear maturation, intracellular glutathione (GSH) level of oocytes, and embryonic development after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT) in pigs. Immature pig oocytes were cultured in maturation medium that was supplemented with each polyvinyl alcohol (PVA), pig follicular fluid (pFF) or newborn calf serum (NBCS) during the first 22 h and the second 22 h. Oocyte maturation was not influenced by the source of macromolecules during *in vitro* maturation (IVM). Embryo cleavage and cell number in blastocyst after PA was altered by the source of macromolecule but no difference was observed in blastocyst formation among treatments. Oocytes matured in PVA-PVA medium showed lower rates of oocyte-cell fusion (70.4% vs. 77~82%) and embryo cleavage (75% vs. 86~90%) after SCNT than those matured in other media but blastocyst formation was not altered (13~27%) by different macromolecules. pFF added to IVM medium significantly increased the intracellular GSH level of oocytes compared to PVA and NBCS, particularly when pFF was supplemented during the first 22 h of IVM. Our results demonstrate that source of macromolecule in IVM medium influences developmental competence of oocytes after PA and SCNT, and that pFF supplementation during the early period (first 22 h) of IVM increases intracellular GSH level of oocytes.

(Key words : follicular fluid, newborn calf serum, somatic cell nuclear transfer, glutathione, pig)

INTRODUCTION

Recent progresses in the field of reproductive biotechnology including *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and somatic cell nuclear transfer (SCNT) make it possible to produce specific animals successfully in several livestock species (Wilmut *et al.*, 1997; Polejaeva *et al.*, 2000; Kawakami *et al.*, 2003). Most of reproductive technologies require the use of a large number of mature oocytes. To obtain plentiful supply of mature oocytes, it is common to collect immature oocytes from ovaries of slaughtered animals and culture them *in vitro*. The quality of *in vitro*-matured oocytes has an utmost importance for deciding developmental competence of embryos after IVF, parthenogenetic activation (PA), and SCNT. Therefore, it is very important to establish a stable *in vitro* maturation (IVM) system to produce mature oocytes of higher quality.

In vivo, oocytes grow in the follicles by being nourished from follicular fluid. The composition of follicular fluid varies and reflects changes in the kinetics of follicular components such as amino acids and steroid hormones during follicular growth (Wiesak *et al.*, 1990; Dostal and Pavlok, 1996; Hong and Lee, 2007). It has been well known that a series of changes occurs during oocyte maturation process including germinal vesicle breakdown, progression to the metaphase Ⅰ and Ⅱ stages. In addition, intercellular coupling between oocytes and cumulus cells decreases 32 h after hCG injection when anaphase Ⅰ or telophase Ⅰ is predominant, and partial uncoupling is observed in oocytes cultured *in vitro* for 16 h (Motlik *et al.*, 1986; Isobe *et al.*, 1998; Mori *et al.*, 2000). These changes during oocyte maturation process may need modifications of culture environments including medium composition (Funahashi and Day, 1993; Schoevers *et al.*, 2003).

Although serum and pFF contain a variety of growth factors, amino acids and steroid hormones that are effective in enhancing developmental competence of oocytes and embryos (Khatir *et al.*, 1997; Seli *et al.*, 1998; Liu *et al.*, 2002; Hong and Lee, 2007), they also contain many unknown substances.

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The variability in the composition of serum and pFF often shows inconsistent outcome in developmental studies. Notwithstanding, pFF or serum still have been widely used due to their beneficial effects on oocytes maturation and embryonic development (Yoshida *et al.*, 1992; Rath *et al.*, 1995; Carolan *et al.*, 1995). In pigs, immature oocytes are routinely cultured for 40～44 h and used for IVF, ICSI or SCNT. During the culture process, oocytes are supplied with various nutrients including carbohydrates, gonadotrophic hormones and macromolecules such as pig follicular fluid (pFF), animal serum, or polyvinyl alcohol (PVA) from the IVM medium (Abeydeera *et al.*, 1998; Kobayashi *et al.*, 2007; García-Mengual *et al.*, 2008). It is common in pigs to culture immature oocytes by two steps; hormones are generally supplemented to medium only for the first half of maturation culture. Many studies examined the effect of gonadotrophic hormones according to different stages of maturation culture (Funahashi and Day, 1993; Schoevers *et al.*, 2003). However, a few studies are available on the effect of macromolecules in IVM medium that was separately supplemented during the first 22 h and the second 22 h of maturation culture.

The objective of this study was to examine the effect of macromolecules (pFF, PVA and newborn calf serum) in IVM medium on nuclear maturation, intracellular glutathione (GSH) level of oocytes after IVM, and *in vitro* development of PA and SCNT embryos in pigs.

MATERIALS AND METHODS

1. Culture Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The base medium for IVM was medium-199 (M-199) (Invitrogen, Grand Island, NY, USA) supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, and 1 μg/ml insulin. This medium was further supplemented with 0.05% (w/v) PVA, 10% (v/v) pFF or 10% (v/v) newborn calf serum (NBCS) according to the experimental design. The *in vitro* culture (IVC) medium used for embryo development was Porcine Zygote Medium-3 (Yoshioka *et al.*, 2002).

2. Oocyte Collection and IVM

Ovaries were obtained from prepubertal gilts at a local abattoir. Follicular contents were aspirated from the superficial follicles (3～8 mm in diameter) of the ovaries with an 18-G needle attached to a 10-mL disposable syringe. Cumulus-oocyte-complexes (COCs) with more than three layers of compact cumulus cells were selected, washed three times in a HEPES-buffered Tyrode's medium containing 0.05% (w/v) PVA (TLH-PVA) (Bavister *et al.*, 1983), and then washed once in IVM medium. A group of 20～25 COCs were placed into each microdrop on a petridish (Becton Dikinson Labware, Lincoln Park, NJ, USA) containing 100 μl of IVM medium with 10 IU/ml eCG (Intervet International BV, Boxmeer, Holland) and 10 IU/ml hCG (Intervet International BV). The COCs were then statically cultured at 39℃ in a humidified atmosphere containing 5% CO_2. After 22 h in the maturation culture, the COCs were washed three times in fresh, hormone-free IVM medium and cultured for an additional 22 h and 18 h for PA and SCNT, respectively.

3. Preparation of Donor Cells

Ear skin fibroblasts from newborn miniature piglets were seeded into four-well plates and were grown in Dulbecco's modified Eagle medium (DMEM) with the nutrient mixture F-12 (Invitrogen), which was supplemented with 15% (v/v) fetal bovine serum from a single batch until a complete monolayer of cells had formed. Donor cells were synchronized at the G0/G1 stage of the cell cycle by contact inhibition for 72～96 h. Cells of the same passage (3～7 passages) were used in each replicate for the various treatments. A suspension of single cells was prepared by trypsinization of the cultured cells, followed by resuspension in TLH containing 0.4% (w/v) BSA (TLH-BSA) prior to the nuclear transfer.

4. SCNT and PA

After 40 h of IVM, cumulus-cell-free oocytes were incubated for 15 min in a manipulation medium (calcium-free TLH-BSA) containing 5 μg/ml Hoechst 33342. Following incubation, the oocytes were washed twice with fresh manipulation medium. The washed oocytes were transferred into a drop of manipulation medium containing 5 μg/ml cytochalasin B (CB) and were overlaid with warm mineral oil. Oocytes were enucleated by aspirating the polar body (PB) and metaphase Ⅱ (MⅡ) chromosomes using a 17-μm beveled glass pipette (Humagen, Charlottesville, VA, USA). Enucleation was confirmed under an epifluorescence microscope (TE300; Nikon, Tokyo, Japan). A single cell was inserted into the perivitelline space of each oocyte. Oocyte-cell couplets were placed on a 1-mm fusion chamber overlaid with 1 ml of 280 mM mannitol solu-

tion containing 0.001 mM $CaCl_2$ and 0.05 mM $MgCl_2$ as previously described (Walker *et al.*, 2002; Song *et al.*, 2009). Membrane fusion was induced by applying an alternating current field of 2 V cycling at 1 MHz for 2 sec, followed by two pulses of 170 V/mm direct current (DC) for 50 μsec using a cell fusion generator (LF101; NepaGene, Chiba, Japan). Oocytes were then incubated for 1 h in TLH-BSA and were evaluated for fusion under a stereomicroscope.

Immediately after incubation, reconstructed oocytes were activated with two pulses of 120 V/mm DC for 60 μsec in a 280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. For PA, the oocytes with the PB at 44 h of IVM were activated using a pulse sequence identical to that used to activate SCNT oocytes.

5. Post-Activation Treatment and Embryo Culture

Following electrical activation, the PA and SCNT embryos were treated respectively with 5 μg/ml CB and 0.4 μg/ml demecolcine in IVC medium for 4 h. The SCNT and PA embryos were washed three times in fresh IVC medium, transferred into 30-μl IVC droplets under mineral oil, and then cultured at 39℃ in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7 days. Cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively, with the day of SCNT or PA designated Day 0. The total blastocyst cell count was performed using Hoechst 33342 staining under an epifluorescence microscope.

6. Measurement of Intracellular GSH Level

Intracellular GSH levels of oocytes were measured by the method previously described (Sakatani *et al.*, 2007). Briefly, CellTracker Blue CMF_2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin; Invitrogen) was used to detect intracellular GSH levels as a blue fluorescence. A group of 20 denuded oocytes from each treatment group was collected 44 h after IVM and incubated for 30 min in TLH-PVA that was supplemented 10 μM CellTracker in the dark. After incubation, oocytes were washed with D-PBS (Invitrogen) containing 0.1% (w/v) PVA, placed into 10-μl droplets and observed for the fluorescence under an epifluorescence microscope (TE300; Nikon, Tokyo, Japan) with a UV filter (370 nm). Fluorescent images were saved as graphic files in tiff format. The fluorescence intensity of oocytes was analyzed by ImageJ software (ver. 1.41o; National Institutes of Health, Bethesda, MD, USA) and normalized to that of oocytes matured in pFF-supplemented medium for entire period of maturation culture.

7. Experimental Design

In Experiment 1, effect of PVA, pFF, and NBCS that were supplemented to maturation medium during the first (0~22 h) and the second (22~44 h) halves of IVM culture on oocyte maturation and embryonic development after PA was examined by a 3 × 3 factorial arrangement (9 experimental groups). Treatment groups were designated as PVA-PVA, pFF-PVA, NBCS-PVA, PVA-pFF, pFF-pFF, NBCS-pFF, PVA-NBCS, pFF-NBCS, and NBCS-NBCS, respectively. Based on the result from Experiment 1, immature oocytes were cultured in IVM medium supplemented with PVA, pFF, and NBCS for the first 22 h and in medium with PVA during the second 22 h of maturation culture in Experiment 2, and then effect of macromolecules on embryonic development after SCNT was examined. The medium supplemented with pFF during the entire period of IVM was served as positive control. In Experiment 3, effect of macromolecules in IVM medium on intracellular GSH levels of oocytes was determined.

8. Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System (version 9.1; SAS Institute, Cary, NC, USA). Data were analyzed using a general linear model followed by the least significant difference mean separation procedure when the treatments differed at $p<0.05$. Percentage data were arcsine-transformed prior to analysis to maintain the homogeneity of variances. The results are expressed as mean ± standard error of the mean (SEM).

RESULTS

Experiment 1: Effect of PVA, pFF and NBCS on Oocyte Maturation and Development of Parthenogenetic Embryos

The proportion of MII oocytes after IVM was not altered (79~93%) by the supplementation with PVA, pFF, or NBCS, but embryo cleavage was influenced by different macromolecules in IVM medium (Table 1). PVA supplementation (48 ± 2%) during the second 22 h of IVM tended to increase (p = 0.0538) embryo development to the blastocyst stage compared to pFF (39 ± 3%) and NBCS supplementation (39 ± 4%) during the second half. Cell number in blastocyst was significantly higher in embryos that had been matured in pFF-supplemented medium (36 ± 1 cells) than in embryos matured in

Table 1. Effect of macromolecule in a maturation medium on oocyte maturation and subsequent development of parthenogenetic pig embryos

Macromolecule in maturation medium†		No. of oocytes matured[a]	% of oocytes reached MII	No. of PA embryos cultured	No. (%) of embryos developed to		No. of cells in blastocyst
0~22 h	22~44 h				≥ 2-cell	Blastocyst	
PVA	PVA	241	89 ± 3	200	86 ± 5^{bc}	50 ± 5	30 ± 2^{b}
pFF	PVA	283	93 ± 2	262	88 ± 3^{bc}	47 ± 2	38 ± 2^{d}
NBCS	PVA	275	88 ± 1	241	90 ± 2^{c}	46 ± 3	34 ± 1^{c}
	Total	799	90 ± 1	703	88 ± 2	48 ± 2	34 ± 1^{AB}
PVA	pFF	280	79 ± 8	222	81 ± 3^{b}	40 ± 2	41 ± 3^{d}
pFF	pFF	280	93 ± 2	260	93 ± 1^{c}	41 ± 7	35 ± 1^{c}
NBCS	pFF	287	79 ± 7	247	94 ± 2^{c}	36 ± 6	35 ± 1^{c}
	Total	847	83 ± 4	729	90 ± 2	39 ± 3	36 ± 1^{A}
PVA	NBCS	279	89 ± 2	242	89 ± 5^{bc}	49 ± 5	34 ± 2^{c}
pFF	NBCS	292	89 ± 3	255	94 ± 2^{c}	41 ± 7	32 ± 1^{b}
NBCS	NBCS	294	88 ± 4	241	96 ± 2^{c}	29 ± 7	35 ± 2^{c}
	Total	865	88 ± 2	738	93 ± 2	39 ± 4	33 ± 1^{B}

† PVA, polyvinyl alcohol; pFF, pig follicular fluid; NBCS, newborn calf serum.
[a] Eleven replicates.
$^{AB, b\sim d}$ Within a column, values with different superscripts are different ($p<0.01$).

PVA- (36 ± 1 cells) and NBCS-supplemented media (33 ± 1 cells) during the second 22 h of IVM.

Experiment 2: Effect of PVA, pFF and NBCS in IVM Medium on Embryonic Development after SCNT

IVM oocytes matured in PVA-PVA medium showed significantly lower rates of oocyte-cell fusion (70 ± 4%) and embryo cleavage (75 ± 4%) than oocytes matured in other media (77~82% of fusion rate and 86~90% of cleavage) (Table 2). However, blastocyst formation after SCNT was not altered (p = 0.1086) by the supplementation of various macromolecules to IVM medium (14~27%), although the oocyte maturation in PVA-PVA group showed the lowest value (13 ± 2%) among treatments.

Experiment 3: Effect of PVA, pFF and NBCS in IVM Medium on Intracellular GSH Level of Oocytes

Intracellular GSH levels of oocytes were significantly influenced by the source of macromolecule in IVM medium (Fig. 1). Oocytes matured in medium that was supplemented with pFF during entire culture period showed the highest level of intracellular GSH among various IVM treatments. PVA supplementation to IVM medium during the first 22 h combined with pFF or NBCS supplementation during the second 22 h of IVM showed the lowest value of intracellular GSH levels. In the retrospective analysis (Fig. 2), GSH level of oocytes was significantly influenced by the source of macromolecule during the first half rather than that during the second half of IVM.

DISCUSSION

Our results demonstrated that intracellular GSH levels and embryonic development of oocytes after PA and SCNT were influenced by the macromolecules that were supplemented to IVM medium. In particular, intracellular GSH level was increased by the pFF supplementation during the early stage of IVM. To the best of our knowledge, this is the first study that reported the effect of macromolecules supplemented to maturation medium at different stages of IVM on oocyte maturation and embryonic development.

In this study, nuclear maturation of oocytes and embryonic

Table 2. Effect of macromolecule in a maturation medium on *in vitro* development of somatic cell nuclear transfer pig embryos

Macromolecule in maturation medium†		No. of oocytes reconstructed[a]	% of oocytes fused	No. of oocytes cultured	% of embryos developed to		No. of cells in blastocyst
0~22 h	22~44 h				≥ 2-cell	Blastocyst	
PVA	PVA	179	70 ± 4[b]	112	75 ± 1[b]	13 ± 2	34 ± 4
pFF	PVA	194	82 ± 3[c]	157	88 ± 3[c]	27 ± 5	36 ± 2
NBCS	PVA	164	77 ± 4[bc]	125	90 ± 2[c]	22 ± 4	34 ± 2
pFF	pFF	199	81 ± 3[c]	160	86 ± 3[c]	26 ± 3	39 ± 3

† PVA, polyvinyl alcohol; pFF, pig follicular fluid; NBCS, newborn calf serum.
[a] Four replicates.
[bc] Within a column, values with different superscripts are different ($p<0.01$).

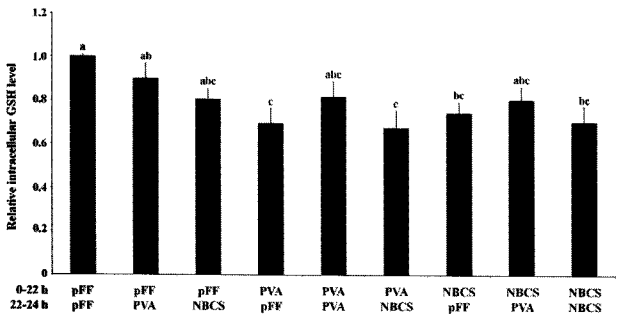

Fig. 1. Effect of different macromolecules in a maturation medium on the intracellular GSH level of *in vitro*-matured oocytes. Intracellular GSH level of oocytes was measured 44 h of IVM. Bars with different letters are significantly different ($p<0.05$).

development to the blastocyst stages after PA and SCNT were not influenced by the source of macromolecules in IVM medium. This result was not consistent with the previous finding that addition of serum to maturation medium inhibited nuclear maturation and embryo cleavage in pigs (García-Mengual *et al.*, 2008), and partially consistent with our previous study (Song and Lee, 2007) in which no difference was found in the oocyte maturation, embryo cleavage and blastocyst formation between IVM media with pFF and PVA. In the SCNT study, the rate of cell-oocyte fusion and developmental competence of reconstructed oocytes to the cleavage stage were decreased when oocytes from PVA-PVA group were used as cytoplasts, but no difference in blastocyst formation was observed among treatments. The result on the embryo development was not consistent with the previous result (Jeong *et al.*, 2008) that blastocyst formation after SCNT was higher in oocytes matured in pFF-supplemented medium than in oocytes matured in PVA-supplemented medium. On the other hand, there have been contradictory results on the effect of macromolecule that embryo cleavage and blastocyst formation of IVF (Abeydeera *et al.*, 1998) and SCNT oocytes (Song and Lee, 2007) was not altered by pFF or PVA supplementation to IVM medium. In this study, oocyte maturation in a defined medium supplemented with PVA showed a comparable developmental competence of PA oocytes to the blastocyst stage to that using pFF- and NBCS-supplemented IVM media. However, SCNT embryos derived from oocytes matured in the defined medium showed decreased blastocyst formation although the difference was not statistically different. Because there are many factors influencing embryo development, it was difficult to know the exact mechanism how the embryo development was influenced by the various macromolecules during IVM. More controlled experiment including *in vivo* developmental study would be needed to know the precise effect of pFF, serum and PVA in a maturation medium on embryonic development in pigs.

GSH is a low molecular thiol compound, protects cells by inhibiting detrimental action of reactive oxygen species (ROS), and improves embryo development. The level of GSH has been examined as a parameter to assess the quality of oocytes because intracellular GSH level is increased by the metabolism of cumulus cells during IVM and closely related with embryonic development (de Matos and Furnus, 2000; Takahashi *et al.*, 2002). In this study, intracellular GSH level of oocytes was increased by the pFF supplementation at the early stage of IVM but not influenced by PVA and NBCS supplementation. It was not clear how the GSH level was influenced by the pFF. pFF contains many substances including carbohydrates, amino acids and growth factors (Orsi *et al.*, 2005; Hong and Lee, 2007).

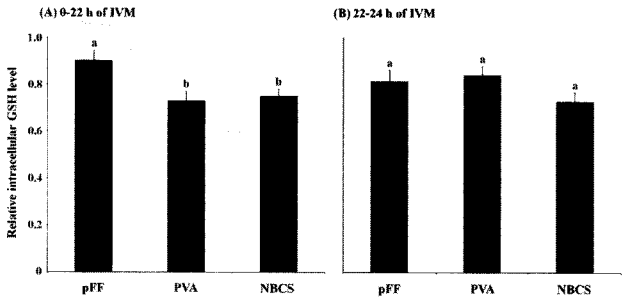

Fig. 2. Intracellular GSH levels of oocytes according to the supplementation with various macromolecules during 0~22 h (A) and 22~24 h of IVM (B). Data presented in Fig. 1 were pooled according to the period of exposure to each macromolecule and re-analyzed. Within the same period of IVM, bars with different letters are significantly different ($p<0.05$).

Cysteamine, β-mercaptoethanol, and several amino acids such as cysteine and cystine stimulate GSH synthesis by cumulus cells and increases intracellular GSH level of oocytes (de Matos and Furnus, 2000; Bing *et al.*, 2002). Therefore, it was possible that unknown substances in pFF might influence GSH synthesis independently or in combination with other substances. Although previous results showed that embryonic development was increased by high intracellular GSH level, the pattern of embryonic development after PA and SCNT in this study did not correspond with the levels of intracellular GSH. We selected and used only oocytes with compact cumulus cells for IVM and added cysteine, a source of GSH for cumulus cells, to the base maturation medium. The base medium for IVM also contains cystine. Moreover, the actual concentration of intracellular GSH was not determined in this study because the GSH level of oocytes was analyzed indirectly by measuring the intensity of fluorescence in each treatment group and normalized to the level of pFF-pFF group. Therefore, our maturation system might support oocytes to maintain the GSH concentration in a certain level and, probably the actual levels of GSH in all the treatment groups might be higher than the minimal level of GSH to support embryonic development. In addition, the influence on the GSH level by macromolecules during the first 22 h of IVM might be related with the period of intercellular uncoupling between oocytes and cumulus cells during the maturation process. It has been reported that intercellular uncoupling is observed in oocytes 16 h after IVM and 32 h after hCG injection (Isobe *et al.*, 1998; Mori *et al.*, 2000).

In summary, our results demonstrate that source of macromolecule in IVM medium influences developmental competence of oocytes after PA and SCNT, and that pFF in IVM medium increases intracellular GSH level of oocytes particularly when supplemented during the first 22 h of maturation rather than at the later stage of IVM.

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