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Modification of maturation condition improves oocyte maturation and *in vitro* development of somatic cell nuclear transfer pig embryos

Kilyoung Song[1], Eunsong Lee[2,*]

[1]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
[2]*School of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chunchon 200-701, Korea*

This study examined effects on the developmental competence of pig oocytes after somatic cell nuclear transfer (SCNT) or parthenogenetic activation (PA) of : 1) co-culturing of oocytes with follicular shell pieces (FSP) during *in vitro* maturation (IVM); 2) different durations of maturation; and 3) defined maturation medium supplemented with polyvinyl alcohol (PVA; control), pig follicular fluid (pFF), cysteamine (CYS), or β-mercapto-ethanol (β-ME). The proportion of metaphase II oocytes was increased ($p < 0.05$) by co-culturing with FSP compared to control oocytes (98% vs. 94%). However, blastocyst formation after SCNT was not improved by FSP co-culture (9% vs. 12%). Nuclear maturation of oocytes matured for 39 or 42 h was higher ($p < 0.05$) than that of oocytes matured for 36 h (95-96% vs. 79%). Cleavage (83%) and blastocyst formation (26%) were significantly higher ($p < 0.05$) in oocytes matured for 42 h than in other groups. Supplementation of a defined maturation medium with 100 μM CYS or 100 μM β-ME showed no stimulatory effect on oocyte maturation, embryo cleavage, or blastocyst formation after PA. β-ME treatment during IVM decreased embryo cleavage after SCNT compared to pFF or PVA treatments, but no significant difference was found in blastocyst formation (7-16%) among the four treatment groups. The results indicated that maturation of oocytes for 42 h was beneficial for the development of SCNT embryos. Furthermore, the defined maturation system used in this study could support *in vitro* development of PA or SCNT embryos.

Key words: embryo development, oocyte maturation, parthenogenesis, pig, somatic cell nuclear transfer

*Corresponding author
Tel: +82-33-250-8670; Fax: +82-33-244-2367
E-mail: eslee@kangwon.ac.kr

Introduction

Somatic cell nuclear transfer (SCNT) has been successfully applied to produce clone animals in a wide range of species, including sheep [36], cattle [8], mice [34], goats [4] and pigs [31]. SCNT is now a routine method that is employed for the production of transgenic pig or bio-organs for xenotransplantation, but in spite of the success of this technique, embryo viability and efficiency of piglet production have remained low. Therefore, truly practical application of SCNT will require an increase in its efficiency through modifications in oocyte maturation and embryo manipulation methods.

Preparation of oocytes is one of the critical factors that determine the developmental competence of embryos produced by *in vitro* fertilization (IVF), SCNT, or parthenogenetic activation (PA). In pigs, oocytes that were matured both *in vivo* and *in vitro* have been used for SCNT. *In vitro*-matured (IVM) oocytes are known to have a lower developmental competence after IVF or SCNT compared to *in vivo*-derived oocytes [27]. Still, IVM oocytes have been used in most laboratories because their use makes it feasible to obtain a large number of oocytes from slaughtered ovaries at relatively low cost. Many factors influence the karyoplasmic and cytoplasmic maturation of oocytes *in vitro*, including co-culturing with follicular cells such as cumulus cells or granulosa cells, duration of maturation, and type of IVM media [3,13,14]. It has been reported that co-culturing of pig oocytes with follicle shells during IVM increases cleavage of PA embryos and blastocyst formation of IVF pig embryos [3,23]. Oocytes are matured *in vivo* through mutual interaction of oocytes and their surrounding follicular cells, which include granulosa and cumulus cells. Follicular cells are known to secrete various factors such as growth factors and hormones [7,25], and to influence oocyte maturation and embryo development after IVF [3] or SCNT [15]. The beneficial role of follicular cells during oocyte maturation has been extensively studied in other domestic species [24,33], but there are a few reports available on the effect of follicular cells on SCNT embryo development in pigs [15].

Most pig oocytes reach the metaphase II (MII) stage 36 h after the start of IVM, but some oocytes expel their first polar bodies after 24 h of IVM [1,26]. It has been reported that the age of IVM oocytes affects the developmental potential of the oocytes after IVF, PA, or SCNT [13,16,26]. Different durations (24 to 42 h) of IVM have been employed to produce MII pig oocytes for SCNT, but the optimal duration of IVM remains controversial.

In order to understand the factors that play roles in oocyte maturation in IVM medium, a defined maturation system without porcine follicular fluid (pFF) or serum has been introduced in several studies [6,28]. However, the developmental ability of oocytes matured in defined media still tends to be lower than that of oocytes matured in media supplemented with pFF [14,32]. Cysteamine (CYS) is a thiol compound that is known to be a scavenger of hydroxyl radical, and may contribute to maintaining the redox status in oocytes [12,37]. Addition of CYS to a maturation medium increased glutathione (GSH) synthesis in bovine oocytes [9] and enhanced *in vitro* development of porcine embryos derived from intracytoplasmic sperm injection [22]. Beta-mercaptoethanol (β-ME), another thiol compound, has been shown to function as an antioxidative agent by increasing the intracellular GSH content of oocytes matured *in vitro* and to facilitate pig embryo development to blastocysts after IVF [2].

In this study, to improve the developmental capacity of SCNT pig embryos, we examined the effects of: 1) co-culturing of oocytes with follicular shell pieces (FSPs) during IVM; 2) different durations of maturation; and 3) defined medium supplemented with CYS or β-ME on oocyte maturation and subsequent developmental competence of pig embryos produced by SCNT and PA.

Materials and Methods

Culture media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (USA). The basic medium for IVM was TCM199 (Invitrogen, USA) supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, 1 μg/ml insulin, and 10% (v/v) pFF (TCM-pFF). This medium was modified by deleting cysteine and substituting pFF with 0.05% (w/v) polyvinyl alcohol (TCM-PVA) and used as a defined medium in Experiments 3 and 4. Porcine follicular fluid was collected from follicles 3-8 mm in diameter, centrifuged at 1,900 × *g* for 15 min, filtered, and stored at –20°C until use. Porcine follicular fluid from the same batch was used in all experiments. The *in vitro* culture (IVC) medium for embryo development was North Carolina State University (NCSU)-23 medium containing 0.4% (w/v) bovine serum albumin (BSA) [30], which was modified by replacing glucose with 0.5 mM pyruvate and 5.0 mM lactate [29].

Oocyte collection and IVM

Porcine ovaries from pre-pubertal gilts were collected at a local abattoir and transported to the laboratory in sterile saline at 37°C. Follicles 3-8 mm in diameter were aspirated using an 18-gauge needle fixed to a 10-ml disposable syringe, and follicular contents were pooled into 15-ml conical tubes and allowed to settle as sediment. The sediment was observed in HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) PVA (TLH-PVA) [5] under a stereomicroscope, and only cumulus-oocyte complexes (COCs) with more than 3 layers of compact cumulus cells were selected. After washing twice in TLH-PVA and once in IVM medium, 50-90 COCs in a group were placed into each well of a 4-well multi-dish (Nunc, Denmark) containing 500 μl of IVM medium with 5 IU/ml eCG (Intervet International BV, Holland) and 5 IU/ml hCG (Intervet International BV, Holland). COCs were cultured at 39°C in a humidified atmosphere of 5% CO_2 in air. After 22 h of maturation culture, the COCs were washed 3 times in a fresh hormone-free IVM medium and then cultured in IVM medium without hormones for an additional 14, 17, 20, or 22 h according to the experimental design. Oocytes with clearly extruded polar bodies were considered to be mature MII oocytes.

Preparation of donor cells

Porcine ear skin fibroblasts bearing the human decay accelerating factor gene were seeded in a 4-well plate at 35% confluency and grown until contact-inhibited. A single cell suspension was prepared by trypsinization of cultured cells and resuspension in TLH containing 0.4% (w/v) BSA (TLH-BSA) prior to nuclear transfer.

Nuclear transfer (NT)

After 40 h of maturation culture in Experiments 1 and 4 and 36-42 h in Experiment 2, cumulus cells were removed from oocytes by gently pipetting the oocytes in IVM medium containing 0.1% (w/v) hyaluronidase. Denuded oocytes were incubated for 15 min in manipulation medium (calcium-free TLH-BSA containing 5 μg/ml Hoechst 33342), washed twice in fresh manipulation medium, and transferred into a manipulation medium drop containing 5 μg/ml cytochalasin B overlaid with mineral oil. MII oocytes were enucleated by aspirating the first polar body and MII chromosomes using 17-μm bevelled glass pipettes (Humagen, USA), and enucleation was confirmed under an epifluorescent microscope (TE300; Nikon, Japan).

After enucleation, a single cell was placed 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 containing 0.001 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Membrane fusion was induced by applying an alternating current field of 2 V, 1 MHz for 2 sec followed by two pulses of 170 V/cm direct current (DC) for 50 μsec

using a cell fusion generator (LF101; NepaGene, Japan). Oocytes were subsequently incubated for 1 h in TLH-BSA and evaluated for fusion rates under a stereomicroscope prior to activation. Preliminary results showed that the fusion method used in this study was not sufficient for oocyte activation because less than 1% of MII oocytes were cleaved after electro-stimulation and none of those developed to the blastocyst stage.

Activation and embryo culture

Reconstructed oocytes were activated by two pulses of 120 V/cm DC for 60 μsec in 280 mM mannitol containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Oocytes were thoroughly washed in IVC medium, transferred into 30-μl IVC droplets under mineral oil, and cultured at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 6 days. Cleavage and blastocyst formation were evaluated on Days 2 and 6, respectively (the day of SCNT was designated Day 0). Total cell number in blastocysts was assessed using Hoechst 33342 staining under ultraviolet light.

Parthenogenetic activation of oocytes

After 44 h of IVM, oocytes were denuded and those with the first polar body were placed in a 1-mm fusion chamber and activated by applying two DC pulses of 120 V for 60 μsec separated by 1 sec in 280 mM mannitol containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Electro-stimulated oocytes were incubated in IVC medium containing 10 μg/ml cycloheximide for 5 h, washed 3 times in fresh IVC medium, transferred into 30-μl IVC droplets under mineral oil, and cultured at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 6 days.

Experimental design

All oocytes used in respective experiments were randomly allocated to each treatment group, and a minimum of four replications were performed. In Experiment 1, oocytes were matured with FSPs to determine the effect of the co-culture system on IVM. Transparent and clear FSPs were selected from follicular sediments at the time of oocyte selection, and were then washed twice in TLH-PVA and once in IVM medium. Finally, 20-25 FSPs were transferred to each well of a culture dish containing 40-50 COCs. In Experiment 2, the effect of a duration of maturation of 36 h (group I), 39 h (group II), and 42 h (group III) on oocyte maturation and developmental competence of SCNT embryos was examined. TCM-PVA was supplemented with 100 μM CYS or 100 μM β-ME, and the effect of CYS and β-ME on oocyte maturation and subsequent developmental capacity after PA (Experiment 3) and SCNT (Experiment 4) was assessed. TCM-pFF was used as a positive control.

Statistical analysis

Data were analyzed by a general linear model procedure using the Statistical Analysis System (version 8.2; SAS Institute, USA), followed by the least significant difference mean separation procedure when treatments differed at $p < 0.05$. Percentage data were subjected to arcsine transformation prior to analysis to maintain homogeneity of variance. Results are expressed as mean ± SE.

Results

Effect of co-culture with FSPs (Experiment 1)

COCs matured by co-culturing with FSPs exhibited a significantly higher maturation rate than those matured without co-culturing (98% vs. 94%, $p < 0.05$). Fusion and embryo cleavage with SCNT were not different between the two groups. Blastocyst formation was slightly increased in oocytes with co-culturing, but the increase was not statistically significant. The mean cell number in blastocysts was significantly lower for oocytes matured by co-culturing than for oocytes without co-culturing (33 vs. 42 cells, $p < 0.05$) (Table 1).

Effect of maturation period on development of NT embryos (Experiment 2)

The proportions of MII oocytes that were found to be in the MII stage in group II (95%) and group III (96%) were significantly ($p < 0.05$) higher than the proportion in group I (79%), but the fusion rate was significantly ($p < 0.05$) lower in group II (77%) and group III (75%) compared to group I (87%). The cleavage rate of SCNT embryos increased significantly ($p < 0.05$) as the oocyte maturation duration increased. In SCNT, the blastocyst development rate of group III (26%) was significantly ($p < 0.05$) higher than that of group I (14%) and group II (16%), but there was no

Table 1. Effect of follicular shell pieces co-culturing during *in vitro* maturation on oocyte maturation, cell fusion, and *in vitro* development of somatic cell nuclear transfer pig embryos*

Co-culture during IVM	Maturation		Reconstruction		Embryo development (%)			Cell number/ Blastocyst
	N	MII (%)	N	Fused (%)	N	≥2-cell	Blastocyst	
No	461	94 ± 1[a]	418	74 ± 5	272	76 ± 3	9 ± 3	42 ± 3[a]
Yes	478	98 ± 1[b]	414	74 ± 6	314	76 ± 3	12 ± 2	33 ± 2[b]

*Four replicates.

[a-b]Values in the same column with different superscript letters are significantly different ($p < 0.05$).

Table 2. Effect of different durations of maturation on oocyte maturation, cell fusion, and *in vitro* development of somatic cell nuclear transfer pig embryos*

Maturation period (h)	Maturation		Reconstruction		Embryo development (%)			Cell number/ Blastocyst
	N	MII (%)	N	Fused (%)	N	≥2-cell	Blastocyst	
36	404	79 ± 7[a]	295	87 ± 3[a]	244	58 ± 4[a]	14 ± 2[a]	36 ± 2
39	407	95 ± 2[b]	368	77 ± 4[ab]	254	71 ± 3[b]	16 ± 2[a]	39 ± 3
42	443	96 ± 2[b]	347	75 ± 5[b]	246	83 ± 2[c]	26 ± 2[b]	36 ± 2

*Five replicates.
[a-c]Values in the same column with different superscript letters are significantly different ($p < 0.05$).

Table 3. Effect of cysteamine or β-mercaptoethanol in a defined maturation medium on oocyte maturation, cell fusion, and *in vitro* development of somatic cell nuclear transfer derived pig embryos*

Treatment†	Maturation		Reconstruction		Embryo development (%)			Cell number/ Blastocyst
	N	MII (%)	N	Fused (%)	N	≥2-cell	Blastocyst	
TCM-pFF	617	88 ± 2	509	69 ± 6	366	72 ± 2[a]	16 ± 6	33 ± 2
TCM-PVA	622	90 ± 3	550	69 ± 6	393	77 ± 4[a]	9 ± 2	37 ± 3
CYS	631	88 ± 3	541	68 ± 6	379	69 ± 2[ab]	10 ± 3	34 ± 2
β-ME	611	86 ± 3	460	68 ± 8	318	60 ± 3[b]	7 ± 1	30 ± 3

*Six replicates.
†pFF: 10% (v/v) porcine follicular fluid; PVA: 0.05% (w/v) polyvinyl alcohol; CYS: 100 μM cysteamine; β-ME: 100 μM β-mercaptoethanol.
[a-b]Values in the same column with different superscript letters are significantly different ($p < 0.05$).

significant difference in embryo cell number among the groups (Table 2).

Parthenogenetic development of oocytes matured in a defined medium (Experiment 3)

The addition of CYS or β-ME to IVM medium (n = 386 to 389 oocytes per treatment, 4 replicates) did not improve the oocyte maturation rate over that of the control (93%, 93% vs. 94%, respectively). The embryo cleavage (68%, 68% vs. 67%, respectively), blastocyst formation (25%, 26% vs. 23%, respectively), and embryo cell number (46, 49 vs. 44 cells, respectively) of PA embryos were not influenced by the presence of CYS or β-ME during IVM. Irrespective of the CYS or β-ME supplementation, defined maturation medium could support oocyte maturation and *in vitro* development of PA embryos comparable to those achieved with TCM-pFF (89%, 63%, 28%, and 47 cells for MII rate, cleavage, BL formation, and embryo cell number, respectively).

Development of NT oocytes matured in a defined medium (Experiment 4)

In vitro development of SCNT embryos using oocytes matured in a defined medium is summarized in Table 3. Compared with the control (TCM-PVA), supplementation with CYS or β-ME did not influence the oocyte maturation (86-88% vs. 90%), fusion (68-68% vs. 69%), blastocyst formation (7-10% vs. 9%), or embryo cell number (30-34 cells vs. 37 cells) after SCNT. Presence of β-ME in IVM medium significantly ($p < 0.05$) decreased the cleavage rate of SCNT embryos compared to the control (60% vs. 72%). Oocytes matured in defined medium showed similar developmental capacity after SCNT to those matured in TCM-pFF.

Discussion

In the production of SCNT embryos, maturation of recipient oocytes is considered to one of the primary factors influencing the developmental competence of embryos. It was investigated whether changes in IVM medium and duration of maturation influence the oocyte maturation and *in vitro* development of porcine embryos after SCNT or PA through a series of experiments. The results of this study demonstrated that co-culturing of immature oocytes with FSPs increased oocyte maturation, and that the rates of cell fusion, cleavage, and blastocyst formation in SCNT embryos were greatly influenced by the IVM period of recipient oocytes. In addition, we found that a defined maturation medium could support oocyte maturation and subsequent development of SCNT embryos comparable to undefined medium containing pFF.

Follicular cells surrounding oocytes secrete specific proteins that are required for cytoplasmic maturation [23]. Previous studies have demonstrated that co-culturing of pig oocytes with follicular cells during IVM is beneficial to oocyte maturation [15,23]. In the present study, co-culturing of oocytes with FSPs improved the nuclear maturation rate, but did not enhance *in vitro* development of SCNT embryos. This result is inconsistent with the previous findings that

IVM of pig oocytes with FSPs improved blastocyst formation of IVF [3] and SCNT pig embryos [15]. In this study, 40-50 oocytes were co-cultured with 20-25 FSPs, compared to 8-12 FSPs used for 40-50 oocytes in NCSU-23 containing 10% pFF in a previous study [3] or two inverted follicular shells used for 20-25 oocytes in 2 ml of M199 with 10% FBS in another [15]. These differences in FSP number and IVM medium might be attributed to the differences in the results obtained. Despite the higher nuclear maturation observed in co-cultured oocytes in this study, SCNT embryos derived from those oocytes showed decreased embryo cell number. The reason that the embryo cell number was decreased was not known. It has been thought that unknown factors secreted from FSPs or suboptimal culture conditions might influence cytoplasmic maturation, resulting in lower embryo cell number. Although the embryo cell number was decreased in co-cultured oocytes, it (33 cells) was still comparable to that of IVF (29-30 cells) [3] or SCNT blastocysts (30-34 cells) [17] in other studies.

Generally, MII oocytes are used as recipient oocytes for the production of SCNT embryos. In pigs, immature oocytes can be fully matured *in vitro* after 38 h, but oocytes expel their first polar bodies over a wide range of maturation times [16,26]. Oocyte age influences the activity of maturation/M-phase promoting factor (MPF) [19] and *in vitro* fertilizability [11]. MPF, which induces the M-phase in eukaryotic cells including oocytes [21], increased during the process of oocyte maturation and remained at a high level during meiotic arrest. MPF activity in aged oocytes gradually decreased, while the activation ability or fragmentation frequency gradually increased [18,20]. In this study, oocytes matured for 42 h were superior to those matured for 36 or 39 h in terms of cleavage and blastocyst formation after SCNT. Our result contrasted the previous findings that 40 h maturation of oocytes was more beneficial for SCNT embryo development than 38 h or 42 h [13], and that pig oocytes matured for 33 h showed higher cleavage and blastocyst formation after SCNT than those matured for 44 h [16]. Hölker *et al.* [13] suggested that decreased developmental competence of SCNT oocytes matured for 42 h might be attributable to inactivation of MPF and premature activation of oocytes. However, in the report [19] that examined changes of MPF in pig oocytes, histone H1 kinase activity gradually decreased from 36 h to 72 h of maturation, and the activity at 48 h was not significantly different from that at 36 h of maturation. From this study, it was not clear whether the differences in the developmental competence of SCNT embryos were due to altered MPF activity as a consequence of different durations of maturation. Interestingly, the fusion rate after cell injection was significantly higher in oocytes matured for 36 h than in oocytes matured for 42 h, although blastocyst formation was lower. It may be good to consider the possibilities that ooplasmic membranes of young MII oocytes may be vulnerable to electric fusion pulses, and that their cytoplasmic maturation is not enough to support the development of SCNT embryos.

Porcine follicular fluid is known to contain unknown factors such as hormones and growth factors. However, the developmental competence of oocytes could be affected by different batches. It is useful to establish a defined maturation system for understanding the role of a specific substance present in the medium. In this study, except for the decreased cleavage of SCNT embryos observed in the presence of β-ME, nuclear maturation of oocytes, cleavage, and blastocyst formation after PA or SCNT were not affected by the maturation of oocytes in medium containing PVA or pFF, even with the addition of CYS or β-ME to the maturation medium. In SCNT experiments, β-ME added to the maturation medium significantly decreased embryo cleavage, which mirrored the finding that oocytes matured in a defined medium containing β-ME showed significantly lower cleavage of pig embryos after intracytoplasmic sperm injection [22]. Many studies [2,10,22,35] have demonstrated the beneficial effect of CYS or β-ME added to a maturation medium on IVM, IVF, or embryo development in bovine or porcine systems, but we did not observe such a stimulatory effect in this study. In general, the developmental capacity of SCNT embryos is known to be lower than that of fertilized embryos. This impaired developmental capacity of SCNT embryos might exist in too low a range to be improved by the beneficial effects of CYS or β-ME.

It was possible to improve oocyte maturation and SCNT embryo development through modifications of maturation conditions, which indicated that IVM of oocytes for 42 h was beneficial for oocyte maturation and *in vitro* development of SCNT pig embryos. In addition, a defined maturation system developed in this study could support *in vitro* development of PA or SCNT pig embryos. This system could be a good tool for understanding the roles of specific factors during oocyte maturation. Further research is needed to evaluate the effects of our modifications on post-transfer viability and implantation of SCNT embryos.

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