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In vitro maturation using αMEM with reduced NaCl enhances maturation and developmental competence of pig oocytes after somatic cell nuclear transfer

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ABSTRACT

Background: Compared to medium containing 108 mM sodium chloride (NaCl), *in vitro* maturation (IVM) using a simple medium with reduced (61.6 mM) NaCl increases the cytoplasmic maturation and embryonic development of pig oocytes.
Objectives: This study determines the effect of a complex medium containing reduced NaCl on the IVM and embryonic development of pig oocytes.
Methods: Pig oocytes were matured in Minimum Essential Medium Eagle-alpha modification (αMEM) supplemented with 61.6 (61αMEM) or 108 (108αMEM) mM NaCl, and containing polyvinyl alcohol (PVA) (αMEMP) or pig follicular fluid (PFF) (αMEMF). Medium-199 (M199) served as the control for conventional IVM. Cumulus cell expansion, nuclear maturation, intra-oocyte glutathione (GSH) contents, size of perivitelline space (PVS), and embryonic development after parthenogenesis (PA) and somatic cell nuclear transfer (SCNT) were evaluated after IVM.
Results: Regardless of PVA or PFF supplementation, oocytes matured in 61αMEM showed increased intra-oocyte GSH contents and width of PVS ($p < 0.05$), as well as increased blastocyst formation ($p < 0.05$) after PA and SCNT, as compared to oocytes matured in 108αMEMP and M199. Under conditions of PFF-enriched αMEM, SCNT oocytes matured in 61αMEMF showed higher blastocyst formation ($p < 0.05$), compared to maturation in 108αMEMF and M199, whereas PA cultured oocytes showed no significant difference.
Conclusions: IVM in αMEM supplemented with reduced NaCl (61.6 mM) enhances the embryonic developmental competence subsequent to PA and SCNT, which attributes toward improved oocyte maturation.

Keywords: Pigs; embryonic development; *in vitro* oocyte maturation; sodium chloride; somatic cell nuclear transfer technique

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Conflict of Interest
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INTRODUCTION

Pigs are an advantageous animal model for biomedical research due to their similarities to humans in physiological and anatomical characteristics. Combined with gene editing technology, it became possible to produce various transgenic cloned pigs [1,2]. Till date, pig animal models are being applied in the medical field for the purpose of treating human diseases [3] and development of livestock animals [4]. However, in generating recombinant embryos by the somatic cell nuclear transfer (SCNT) technology, the production efficiency of cloned pigs is extremely low [5] and the risk of miscarriage and malformations is high [6]. The most fundamental factors for implantation (viz., maintenance of pregnancy and continuous development of the transplanted embryos) are the desired qualities of recombinant embryos produced *in vitro* [5]. To obtain embryos with potentially high developmental capacity, the essential important factors are production of good quality recipient oocytes, with a simultaneous optimization of the *in vitro* maturation (IVM) system that continuously maintains the quality of oocytes.

In our previous study, IVM was achieved using a bovine serum albumin (BSA)-free porcine zygote medium (PZM)-3 medium supplemented with 10% (v/v) pig follicular fluid (PFF). The embryonic development after parthenogenesis (PA) and SCNT was increased in oocytes matured in medium containing reduced sodium chloride (NaCl) (61.6 mM), compared to oocytes matured in PZM-3 medium containing the usual concentration of NaCl (108 mM) [7]. Funahashi et al. [8] were the first to report that a PFF-enriched Whitten's medium containing reduced NaCl concentrations (44.5 to 68.5 mM) for pig IVM conspicuously enhanced the embryonic development of porcine oocytes. They reported that the proportion of embryos forming a male pronucleus and blastocyst formation after *in vitro* fertilization (IVF) was increased in oocytes matured in medium containing lower concentration of NaCl. In addition, Kitagawa and Niimura [9] reported that IVM of porcine oocytes cultured in the North Carolina State University (NCSU)-37 medium with reduced (61.6 mM) NaCl concentration resulted in increased width of the perivitelline space (PVS) and dramatically lowered polyspermic fertilization after IVF. Interestingly, although the types of basal media used in our and other previous reports were different, the results of embryonic development and embryo quality in terms of blastocyst cell number were similar. During the IVM and development of mammalian oocytes, the different effects of simple and complex media have been reported, by considering the origin of oocytes [10], the presence or absence of PFF [11], and the type of *in vitro* produced (IVP) embryos [10,11]. It is considered that subtle changes in the ratio and concentration of various factors may exert positive or negative effects on maturation and embryonic development of oocytes by altering the inherent endogenous ingredients and morphology of oocytes. Therefore, we assumed the suitability of evaluating the effect of reduced NaCl in medium during IVM on oocyte maturation, and on the later development of pig oocytes, using a complex medium instead of a simple medium.

Minimum Essential Medium Eagle-alpha modification (αMEM) [12] and Medium-199 (M199) [13] are classified as complex media, being differentiated from a simple medium such as PZM-3 or NCSU-37 [14]. M199 is widely used as a standard medium for IVM of most mammalian oocytes including sheep [15], bovine [16], and pigs [17]. However, αMEM is only used as a base medium for maturation of mouse oocytes [18]. In pigs, αMEM has been mainly used for the *in vitro* development of immature oocytes derived from small antral follicles (1–2 mm in diameter) [19]; however, application of αMEM for IVM of pig oocytes from medium size follicles (3–8 mm in diameter) has not yet been reported. In addition, although the

effects of various simple media containing reduced NaCl on IVM of pig oocytes have been reported, there are no reports on studies using a complex medium containing low NaCl concentration as an IVM medium. This study therefore aimed to investigate the effects of reduced NaCl using a complex medium rather than a simple medium, on oocyte maturation and embryonic development after PA or SCNT. To investigate the effect of reduced NaCl concentration in a complex medium, customized αMEM media containing 61.6 mM or 108 mM NaCl were prepared and used.

MATERIALS AND METHODS

Culture media and reagents

All chemicals used in this study were purchased from Sigma-Aldrich (USA), unless otherwise noted. M199 (Cat# 12340-030; Invitrogen, USA) was used as the standard medium for IVM of oocytes. To evaluate the effect of reducing NaCl concentration in medium, the base medium used for IVM was αMEM (Cat# LM 008-01; WELGENE, Korea), modified by adding 61.6 or 108 mM NaCl. Both media were equally supplemented with 0.91 mM pyruvate, 0.6 mM cysteine, 10 ng/mL epidermal growth factor, 1 μg/mL insulin, 75 μg/mL kanamycin, and 10% (v/v) PFF or 0.1% (w/v) polyvinyl alcohol (PVA), according to the experimental design. PZM-3 containing 0.3% (w/v) BSA was modified by adding 0.34 mM trisodium citrate, 2.77 mM myo-inositol, and 10 μM β-mercaptoethanol, and used as the *in vitro* culture (IVC) medium for embryonic development [17].

Collection of cumulus-oocyte complexes (COCs) and IVM

Ovaries from pre-pubertal gilts were obtained from a local abattoir. Follicular contents were aspirated from superficial follicles measuring 3–8 mm in diameter. COCs with multiple layers of compact cumulus cells were selected and washed several times in HEPES-buffered Tyrode's medium containing 0.05% (w/v) PVA (TLH-PVA). A group of 50–80 COCs was placed into each well of a 4-well multi-dish (Nunc, Denmark) containing 500 μL of IVM medium supplemented with 80 μg/mL follicle stimulating hormone (Antrin R-10; Kyoritsu Seiyaku, Japan) and 10 IU/mL human chorionic gonadotropin (Intervet International BV, The Netherlands), and subsequently cultured at 39°C under humidified atmosphere of 5% CO_2 and 95% air. After 22 h in maturation culture, the COCs were washed three times in fresh hormone-free medium and cultured in hormone-free IVM medium for an additional 20 h for SCNT and PA.

Experimental design

M199 medium is commonly used for maturation of various mammalian oocytes as well as pigs; however, it was not possible to obtain a customized M199 with reduced NaCl. We therefore used αMEM and modified its NaCl concentrations to 61.6 or 108 mM, in accordance with our experimental design. The osmotic pressures of αMEM containing 108 and 61.6 mM NaCl were 285 ± 2 and 220 ± 2 mOsm, respectively.

A group of oocytes matured in the M199 medium was used as the standard control in all experiments. Depending on the addition of PVA (P) or PFF (F) and 61.6 (61) or 108.0 (108) mM NaCl, the αMEM was denoted accordingly. For example, αMEM supplemented with PVA and 108.0 mM NaCl was abbreviated as 108αMEMP and αMEM supplemented with PFF and 61.6 mM NaCl was termed 61αMEMF. Experiment 1 evaluated the cumulus cell expansion, size of PVS, and intra-oocyte glutathione (GSH) contents of oocytes matured in a chemically

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defined αMEMP medium containing different concentrations (61.6 and 108 mM) of NaCl. In experiments 2 and 3, the cleavage ability of early embryos and the proportion of embryos developing into blastocysts after PA and SCNT of oocytes matured in αMEMP containing low NaCl were analyzed. In experiment 4, the quality and developmental competence of oocytes matured in αMEMF medium containing low NaCl were evaluated in the same design as experiments 1, 2, and 3, using a maturation medium containing PFF instead of a chemically defined medium.

Determination of cumulus cell expansion score and nuclear maturation of IVM oocytes

Following IVM, the score of cumulus cell expansion was evaluated subjectively, as previously described [20]: no response, 0; minimum observable response in which the cells in the outermost layer of the cumulus became round and glistening, 1; expansion of outer cumulus cell layers, 2; expansion of all the cumulus cell layers except the corona radiata, 3; and expansion of all the cumulus cell layers including the corona radiata, 4. After analyzing the cumulus cell expansion, COCs were denuded by repeated pipetting in TLH containing 0.4% (w/v) BSA (TLH-BSA) and 0.1% (w/v) hyaluronidase, followed by staining with 5 μg/mL Hoechst 33342 in TLH-BSA for 15 min. The nuclear maturation of IVM oocytes was evaluated under fluorescence microscope (TE300; Nikon, Japan), according to the method described in a previous study [21].

Determination of PVS size and diameter of IVM oocytes

Following IVM, images of denuded metaphase II (MII) oocytes in each experimental group were recorded using a digital camera (DS-L3; Nikon) attached to an inverted microscope (TE300; Nikon). The size of each part of the matured oocyte was measured using ImageJ software (version 1.49q; National Institutes of Health, USA), and sizes of the PVS and cytoplasm were calculated as described previously [22]. Briefly, mean diameters of oocytes were calculated by averaging the longest and the shortest diameters of each oocyte. The PVS size was calculated by subtracting the diameter of ooplasm from the inner diameter of zona pellucida.

Measurement of intra-oocyte GSH contents

The intra-oocyte GSH contents of IVM oocytes were measured by applying previously described methods [17,23]. Briefly, CellTracker Blue 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (Invitrogen) was used to detect intra-oocyte GSH based on blue fluorescence. From each treated group, denuded oocytes having visually extruded first polar body were incubated in the dark for 30 min in TLH-PVA supplemented with 10 μM CellTracker, followed by incubation for an additional 30 min in IVC medium. The oocytes were subsequently washed with Dulbecco's phosphate-buffered saline (Invitrogen) containing 0.1% (w/v) PVA, and placed in 2-μL micro-droplets. Fluorescence was observed under an epifluorescence microscope (TE300; Nikon) with ultraviolet ray filters at 370 nm. The fluorescence intensity of each oocyte was analyzed using the ImageJ software (version 1.49q; National Institutes of Health), and normalized to that of oocytes matured in standard M199.

Preparation of donor cell for SCNT

Porcine fetal fibroblasts were seeded into a 4-well plate and cultured in Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12 (Invitrogen) and 15% (v/v) fetal bovine serum (Invitrogen), until a complete monolayer of cells was obtained. 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 (3–5 passages) were used in each replicate for the various treatments. A

single-cell suspension was prepared by trypsinization of the cultured cells, followed by re-suspension in TLH-BSA [17].

SCNT and PA

SCNT and PA were performed as previously described [20]. Briefly, cumulus cells were removed from IVM oocytes by repeated pipetting in TLH-BSA containing 0.1% (w/v) hyaluronidase. For SCNT, denuded oocytes were stained with 5 μg/mL Hoechst 33342 for 15 min in calcium-free TLH-BSA. Oocytes were then washed twice with fresh calcium-free TLH-BSA, and transferred into a drop of manipulation medium containing 5 μg/mL cytochalasin B (CB) under warm mineral oil. Next, oocytes were enucleated by aspirating the first polar body and MII chromosomes using a 17-μm beveled glass pipette (Humagen, USA). Enucleation was confirmed under an epifluorescence microscope (TE300; Nikon). A single cell was subsequently inserted into the PVS of each oocyte, after which oocyte-cell couplets were placed in a 1-mm fusion chamber overlaid with 1 mL of 280 mM mannitol containing 1 μM $CaCl_2$ and 50 μM $MgCl_2$. Using a cell fusion generator (LF101; NepaGene, Japan), membrane fusion was induced by applying an alternating current field of 2 V at 1 MHz for 2 sec, followed by two pulses of 170–175 V/mm direct current for 30 μsec. Oocytes were subsequently incubated in TLH-BSA for 30 min, after which the fusion rates were evaluated under a stereomicroscope. Two pulses of 120 V/mm direct current were applied for 60 μsec in a 280 mM mannitol solution containing 100 μM $CaCl_2$ and 50 μM $MgCl_2$ to activate the reconstructed oocytes. For PA, MII oocytes were activated using a pulse sequence identical to that used to activate SCNT embryos.

Post-activation treatment and IVC of embryos

After electrical activation, the PA embryos were exposed to 5 μg/mL CB and SCNT embryos with 0.4 μg/mL demecolcine combined with 1.9 mM 6-dimethylaminopurine in IVC medium for 4 h. The SCNT and PA embryos were subsequently washed three 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 7 days. Cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively, with the day of SCNT or PA designated as Day 0. Count of total cell number per blastocyst was conducted using Hoechst 33342 staining under an epifluorescence microscope (TE300; Nikon).

Statistical analysis

The independent variables were standard IVM medium and αMEM medium containing different NaCl concentrations in IVM medium. Score of cumulus cell expansion, nuclear maturation, size of PVS, intra-oocyte GSH contents, development to the cleavage and blastocyst stages, and mean cell number of blastocysts after PA and SCNT, were analyzed as dependent variables. Statistical analyses were conducted using the Statistical Analysis System (version 9.4; SAS Institute, USA). Data were subjected to analysis of variance using a general linear model procedure. *Post hoc* analyses to identify between-group differences were performed using the least-significant-difference test when treatments were different at p value less than 0.05. Percentage data were arcsine-transformed prior to analysis to maintain the homogeneity of variance. The results are expressed as the means ± standard error of the mean.

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RESULTS

Effect of chemically defined αMEMP with reduced NaCl on cumulus cell expansion, nuclear maturation, size of PVS, and intra-oocyte GSH contents

Considering the NaCl concentrations, cumulus cell expansion after IVM showed no difference between groups (**Fig. 1**). The proportion of oocytes attaining the MII stage was significantly ($p < 0.05$) decreased in oocytes matured in 61αMEMP (71.6% ± 3.6%), as compared to M199 (84.1% ± 1.9%) and 108αMEMP (87.6% ± 3.5%). However, the size of PVS (11.5 ± 0.9 µm vs. 6.2 ± 0.4 µm and 5.9 ± 0.5 µm, respectively) and GSH (1.32 ± 0.11 pixels/oocyte vs. 1.00 ± 0.05 pixels/oocyte and 1.09 ± 0.09 pixels/oocyte, respectively) contents were significantly ($p < 0.05$) increased (**Table 1**).

Effect of chemically defined αMEMP medium with reduced NaCl on embryonic development after PA and SCNT

After PA and SCNT, we evaluated the effect of chemically defined αMEMP medium with reduced NaCl, on embryonic development. As presented in **Table 2**, exposure to PA showed no difference in the cleavage rate (92.7% ± 3.3% to 97.4% ± 2.6%) and average of total cell number (33.4 ± 1.7 cells to 38.0 ± 2.2 cells) per blastocyst among the experimental groups.

Table 1. Effect of IVM in chemically defined αMEMP with reduced NaCl, on cumulus cell expansion, nuclear maturation, intra-oocyte GSH contents, and PVS size of pig oocytes

Medium for IVM	No. of oocytes cultured*	Cumulus expansion score after IVM	% of oocytes that reached MII	No. of MII oocytes examined*	Diameter of cytoplasm (µm)	Width of the PVS (µm)	Relative level (pixels/oocyte) of GSH (n = 40)
M199	159	2.01 ± 0.12	84.1 ± 1.9^{b}	45	111.4 ± 0.5^{b}	6.2 ± 0.4^{a}	1.00 ± 0.05^{a}
61αMEMP	158	1.95 ± 0.12	71.6 ± 3.6^{a}	45	106.1 ± 0.5^{a}	11.5 ± 0.9^{b}	1.32 ± 0.11^{b}
108αMEMP	155	1.99 ± 0.09	87.6 ± 3.5^{b}	45	112.8 ± 0.3^{b}	5.9 ± 0.5^{a}	1.09 ± 0.09^{a}

IVM, *in vitro* maturation; αMEMP, Minimum Essential Medium Eagle-alpha modification supplemented with 0.1% (w/v) polyvinyl alcohol; NaCl, sodium chloride; GSH, glutathione; PVS, perivitelline space; M199, medium-199 supplemented with 0.1% (w/v) polyvinyl alcohol; 61αMEMP, Minimum Essential Medium Eagle-alpha modification containing 61.6 mM sodium chloride and 0.1% (w/v) polyvinyl alcohol; 108αMEMP, Minimum Essential Medium Eagle-alpha modification containing 108 mM sodium chloride and 0.1% (w/v) polyvinyl alcohol; MII, metaphase II.
*Four replicates.
[a,b]Values with different superscript letters in the same column are different ($p < 0.05$).

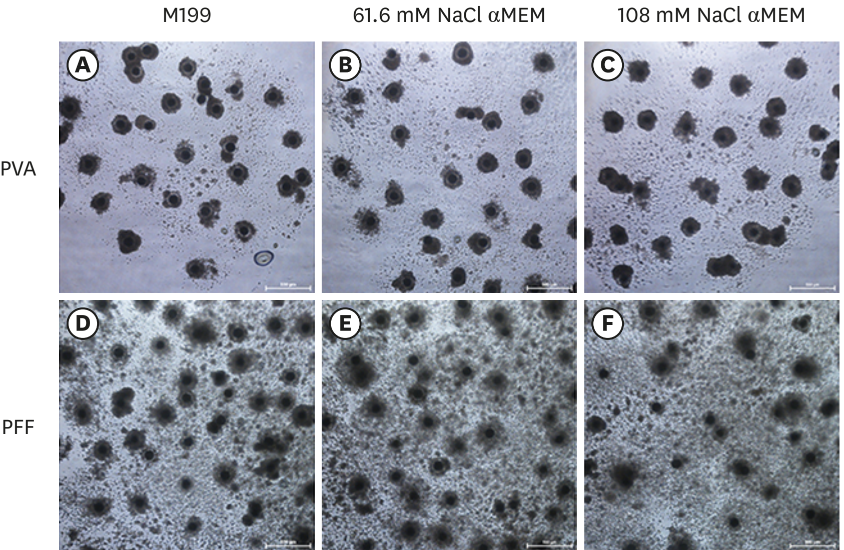

Fig. 1. Morphology of pig COCs after *in vitro* maturation. COCs were matured in M199 (A, D) and αMEM containing 61.6 (B, E) or 108 mM NaCl (C, F) that were further supplemented with 0.1% (w/v) PVA (A, B, C) or 10% (v/v) PFF (D, E, F). Scale bar is 500 µm.
COCs, cumulus-oocyte complexes; M199, medium-199; αMEM, Minimum Essential Medium Eagle-alpha modification; NaCl, sodium chloride; PVA, polyvinyl alcohol; PFF, pig follicular fluid.

However, the rate of blastocysts formation was significantly ($p < 0.05$) increased in the 61.6 mM NaCl αMEMP medium (52.6% ± 3.6%) group, as compared to 108 mM NaCl αMEMP medium (38.1% ± 5.4%) and M199 (39.7% ± 2.6%) medium. Consistent with the results of embryonic development competence of PA embryos, no difference was observed in the cleavage rate (90.8% ± 2.0% to 94.7% ± 1.4%) of SCNT embryo development. However, significant differences ($p < 0.05$) were observed in the rate of blastocyst formation; oocyte maturation showed the highest blastocyst formation rate (44.7% ± 3.6%) in the 61.6 mM NaCl αMEMP medium, as compared to oocytes cultured in 108 mM NaCl αMEMP medium (30.7% ± 2.2%) and M199 (35.9% ± 3.2%) medium (**Table 3**). No significant differences were obtained between M199 and 108 mM NaCl αMEMP medium for embryonic development after PA and SCNT.

Effect of PFF-enriched αMEMF medium with reduced NaCl on cumulus cell expansion, nuclear maturation, size of PVS, and intra-oocyte GSH contents

We further evaluated the effects of PFF-enriched αMEMF medium with reduced NaCl on oocyte quality and maturation of oocytes after IVM. In IVM, cumulus cell expansion was not influenced by the NaCl concentration and base medium (**Fig. 1**). In contrast to chemically defined medium conditions, the nuclear maturation of matured oocytes in the 61αMEMF medium did not differ from oocytes matured in M199 and the conventional concentration 108 mM NaCl αMEMF medium. However, compared to oocytes matured in 108αMEMF and M199 medium, the size of PVS was increased in oocytes cultured in the 61αMEMF medium (4.9 ± 0.4 μm and 5.5 ± 0.4 μm vs. 10.1 ± 0.5 μm, respectively), whereas diameter of the cytoplasm was observed to decrease (113.8 ± 0.6 μm and 113.5 ± 0.6 μm vs. 110.1 ± 0.6 μm, respectively). Moreover, GSH contents (1.03 ± 0.13 pixels/oocyte and 1.00 ± 0.14 pixels/oocyte vs. 1.53 ± 0.19 pixels/oocyte, respectively) were significantly ($p < 0.05$) improved in oocytes matured in αMEMF with reduced NaCl, as compared to other groups (**Table 4**).

Table 2. Effect of IVM in a chemically defined αMEMP with reduced NaCl on embryonic development of pig oocytes after PA

Medium for IVM	No. of PA embryos cultured*	% of embryos developed to		No. of cells in blastocyst
		≥ 2-cell	Blastocyst	
M199	156	92.7 ± 3.3	39.7 ± 2.6[a]	38.0 ± 2.2
61αMEMP	138	97.4 ± 2.6	52.6 ± 3.6[b]	33.4 ± 1.7
108αMEMP	164	95.2 ± 1.9	38.1 ± 5.4[a]	34.8 ± 1.9

IVM, *in vitro* maturation; αMEMP, Minimum Essential Medium Eagle-alpha modification supplemented with 0.1% (w/v) polyvinyl alcohol; NaCl, sodium chloride; PA, parthenogenesis; PVA, polyvinyl alcohol; M199, medium-199 supplemented with 0.1% (w/v) polyvinyl alcohol; 61αMEMP, Minimum Essential Medium Eagle-alpha modification containing 61.6 mM sodium chloride and 0.1% (w/v) polyvinyl alcohol; 108αMEMP, Minimum Essential Medium Eagle-alpha modification containing 108 mM sodium chloride and 0.1% (w/v) polyvinyl alcohol.
*Four replicates.
[a,b]Values with different superscript letters in the same column are different ($p < 0.05$).

Table 3. Effect of IVM in a chemically defined αMEMP with reduced NaCl on embryonic development of pig oocytes after SCNT

Medium for IVM	No. of SCNT embryos cultured*	% of embryos developed to		No. of cells in blastocyst
		≥ 2-cell	Blastocyst	
M199	174	93.3 ± 2.3	35.9 ± 3.2[a]	39.8 ± 2.1
61αMEMP	154	94.7 ± 1.4	44.7 ± 3.6[b]	38.5 ± 1.8
108αMEMP	172	90.8 ± 2.0	30.7 ± 2.2[a]	38.3 ± 2.3

IVM, *in vitro* maturation; αMEMP, Minimum Essential Medium Eagle-alpha modification supplemented with 0.1% (w/v) polyvinyl alcohol; SCNT, somatic cell nuclear transfer; NaCl, sodium chloride; M199, medium-199 supplemented with 0.1% (w/v) polyvinyl alcohol; 61αMEMP, Minimum Essential Medium Eagle-alpha modification containing 61.6 mM sodium chloride and 0.1% (w/v) polyvinyl alcohol; 108αMEMP, Minimum Essential Medium Eagle-alpha modification containing 108 mM sodium chloride and 0.1% (w/v) polyvinyl alcohol.
*Four replicates.
[a,b]Values with different superscript letters in the same column are different ($p < 0.05$).

Table 4. Effect of IVM in a PFF-enriched αMEMF with reduced NaCl, on cumulus cell expansion, nuclear maturation, intra-oocyte GSH contents, and PVS space size of pig oocytes

Medium for IVM	No. of oocytes cultured*	Cumulus expansion score after IVM	% of oocytes that reached MII	No. of MII oocytes examined*	Diameter of cytoplasm (mm)	Size of the PVS (mm)	Relative level (pixels/oocyte) of GSH (n = 40)
M199	168	3.11 ± 0.15	88.1 ± 2.5	40	113.5 ± 0.6[b]	5.5 ± 0.4[a]	1.00 ± 0.14[a]
61αMEMF	169	3.05 ± 0.07	81.6 ± 4.0	40	110.1 ± 0.6[a]	10.1 ± 0.5[b]	1.53 ± 0.19[b]
108αMEMF	164	3.25 ± 0.13	88.9 ± 0.8	40	113.8 ± 0.6[b]	4.9 ± 0.4[a]	1.03 ± 0.13[a]

IVM, *in vitro* maturation; PFF, pig follicular fluid; αMEMF, Minimum Essential Medium Eagle-alpha modification supplemented with 10% (v/v) pig follicular fluid; NaCl, sodium chloride; GSH, glutathione; MII, metaphase II; PVS, perivitelline space; M199, medium-199 supplemented with 10% (v/v) pig follicular fluid; 61αMEMF, Minimum Essential Medium Eagle-alpha modification containing 61.6 mM sodium chloride and 10% (v/v) pig follicular fluid; 108αMEMF, Minimum Essential Medium Eagle-alpha modification containing 108 mM sodium chloride and 10% (v/v) pig follicular fluid.
*Four replicates.
[a,b]Values with different superscript letters in the same column are different ($p < 0.05$).

Effect of PFF-enriched αMEMF medium with reduced NaCl on embryonic development after PA and SCNT

Finally, we analyzed the competence of embryonic development of oocytes matured in 61.6 mM NaCl αMEMF medium containing 10% (v/v) PFF, which is generally used for the *in vitro* pig oocyte maturation. As shown in **Table 5**, the cleavage rate of oocytes matured in 61αMEMF (98.9% ± 1.1%) medium after exposure to PA was significantly ($p < 0.05$) increased, as compared to PA embryos matured in M199 (92.3% ± 1.1%) and 108αMEMF (94.4% ± 1.3%) medium. However, the blastocyst formation rate (48.1% ± 3.4% to 52.4% ± 4.3%) did not differ significantly in all IVM media groups (**Table 5**). In contrast, unlike the development of PA embryos, embryonic development after SCNT was significantly ($p < 0.05$) higher in the 61.6αMEMF medium group (52.8% ± 5.0%) than the 108αMEMF (35.6% ± 21.0%) and M199 (28.0% ± 6.0%) groups. Cleavage rate (88.0% ± 3.1% to 95.4% ± 2.0%) and mean cell number (38.8 ± 2.0 cells per blastocyst to 41.2 ± 1.9 cells per blastocyst) of blastocyst were not significantly different between all three media groups (**Table 6**).

Table 5. Effect of IVM in a PFF-enriched αMEMF with reduced NaCl on embryonic development of pig oocytes after PA

Medium for IVM	No. of PA embryos cultured*	% of embryos developed to		No. of cells in blastocyst
		≥ 2-cell	Blastocyst	
M199	148	92.3 ± 1.1[a]	49.9 ± 3.8	36.5 ± 1.7
61αMEMF	137	98.9 ± 1.1[b]	52.4 ± 4.3	37.8 ± 1.6
108αMEMF	145	94.4 ± 1.3[a]	48.1 ± 3.4	38.1 ± 2.0

IVM, *in vitro* maturation; PFF, pig follicular fluid; αMEMF, Minimum Essential Medium Eagle-alpha modification supplemented with 10% (v/v) pig follicular fluid; NaCl, sodium chloride; PA, parthenogenesis; M199, medium-199 supplemented with 10% (v/v) pig follicular fluid; 61αMEMF, Minimum Essential Medium Eagle-alpha modification containing 61.6 mM sodium chloride and 10% (v/v) pig follicular fluid; 108αMEMF, Minimum Essential Medium Eagle-alpha modification containing 108 mM sodium chloride and 10% (v/v) pig follicular fluid.
*Four replicates.
[a,b]Values with different superscript letters in the same column are different ($p < 0.05$).

Table 6. Effect of IVM in a PFF-enriched αMEMF with reduced NaCl on embryonic development of pig oocytes after SCNT

Medium for IVM	No. of SCNT embryos cultured*	% of embryos developed to		No. of cells in blastocyst
		≥ 2-cell	Blastocyst	
M199	190	88.0 ± 3.1	28.0 ± 6.0[a]	41.2 ± 1.9
61αMEMF	193	95.4 ± 2.0	52.8 ± 5.0[b]	38.8 ± 2.0
108αMEMF	172	91.8 ± 2.7	35.6 ± 2.1[a]	39.4 ± 2.0

IVM, *in vitro* maturation; PFF, pig follicular fluid; αMEMF, Minimum Essential Medium Eagle-alpha modification supplemented with 10% (v/v) pig follicular fluid; NaCl, sodium chloride; SCNT, somatic cell nuclear transfer; M199, medium-199 supplemented with 10% (v/v) pig follicular fluid; 61αMEMF, Minimum Essential Medium Eagle-alpha modification containing 61.6 mM sodium chloride and 10% (v/v) pig follicular fluid; 108αMEMF, Minimum Essential Medium Eagle-alpha modification containing 108 mM sodium chloride and 10% (v/v) pig follicular fluid.
*Four replicates.
[a,b]Values with different superscript letters in the same column are different ($p < 0.05$).

DISCUSSION

In mammals, IVM of immature oocytes is an essential step for producing a large number of mature oocytes for further use in IVF and SCNT. The current study aimed to improve an IVM system for consistent production of high-quality pig oocytes. To achieve this, we examined the effect of αMEM with reduced NaCl (61.6 mM) on the maturation and quality of oocytes, and the subsequent embryonic development after PA and SCNT. Our results reveal that reducing the NaCl concentration from 108 mM to 61.6 mM in αMEM stimulates enlargement of the PVS and increases the intra-oocyte GSH contents, irrespective of PFF or PVA supplementation. Especially, αMEM supplemented with 61.6 mM NaCl during IVM resulted in enhanced embryonic development after SCNT, as compared to αMEM and M199 supplemented with 108 mM NaCl.

Good quality oocytes with high developmental capacity are ascertained by various assessments such as GSH contents [17], enlargement of PVS [7], and regulation of maturation promoting factors in IVM oocytes [24]. During IVM, physical shocks, alterations in the pH and temperature, and exposure to ambient light are suggested to trigger or induce reactive oxygen species (ROS) [25]. ROS are generated in the process of oxidative phosphorylation, which is known to be involved in production of mitochondrial adenosine triphosphate [26]. ROS in oocytes not only induce apoptosis, but also causes abnormal chromosome spindles, leading to the failure of meiosis and subsequent embryonic development [27]. GSH is one of the powerful antioxidants known to effectively scavenge ROS. Previous studies have reported that high GSH content in pig oocytes enhances the cytoplasmic maturation and promotes blastocyst formation after PA and SCNT [7,17]. Moreover, enlargement of PVS in matured oocytes is a factor that evaluates the quality of oocytes with high developmental potential, and is also closely related to GSH synthesis [28]. It has been known that enlargement of PVS stimulates the influx of glycine into oocytes, and glycine accumulated in oocytes promotes GSH synthesis [29]. A previous study suggested that *in vivo* matured pig oocytes with high developmental ability have a wider PVS, as compared to IVM oocytes [30]. Additionally, oocytes matured in Whitten's medium containing lower NaCl concentration showed a gradual increase in the intra-oocyte GSH contents and also in the size of PVS after IVM, which probably contributes to the enhancement of subsequent embryonic development after IVF [8].

In this study, intra-oocyte GSH contents and PVS size of oocytes matured in αMEM medium supplemented with 61.6 mM NaCl were higher than values obtained in oocytes matured in αMEM with 108.0 mM NaCl and the standard M199. Moreover, embryonic development to the blastocyst stage after PA and SCNT of oocytes matured in αMEM with 61.6 mM NaCl was improved in a similar pattern. Although the base medium was different from previous studies [7,22], it was confirmed that the intra-oocyte GSH contents and the PVS size of oocytes matured in αMEM with 61.6 mM NaCl during IVM were increased, as compared to oocytes matured in a medium containing the usual concentration of NaCl. The osmotic pressure of a medium is altered by various components. Of the components, NaCl acts as the main osmolyte in a medium. From this point, a question was raised about whether the enhanced embryonic development of oocytes that were matured in a medium with 61.6 mM NaCl was attributed to the lowered NaCl concentration or the decreased osmotic pressure of a medium. When we compared in our previous study [7] the effects of 61.6 mM NaCl in iso-osmotic (285 mOsm) and hypo-osmotic (220 mOsm) medium, oocytes matured in a hypo-osmotic medium having 61.6 mM NaCl showed a better embryonic development to the

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blastocyst stage than those matured in iso-osmotic medium with 61.6 mM NaCl while IVM in iso-osmotic medium with 61.6 mM NaCl showed a higher blastocyst formation than that in the same medium with 108 mM NaCl. From our previous and present observations, it was considered that the enhanced embryonic development of oocytes in this study was due to both the reduced NaCl concentration in the IVM medium and the decreased osmotic pressure of the medium.

Interestingly, although the nuclear maturation was lower in oocytes matured in a chemically defined αMEMP containing 61.6 mM NaCl than in oocytes matured in a medium with the normal NaCl concentration, the embryonic development to the blastocyst stage after PA and SCNT was higher in oocytes matured in medium with reduced NaCl. In our previous study, we determined that oocytes cultured for IVM in a chemically defined PZM-3 containing 5.56 mM glucose and supplemented with either 61.6 or 108 mM NaCl showed no difference in nuclear maturation as well as embryonic development after PA and SCNT [20]. M199 and αMEM media used in the current study also contain 5.56 mM glucose. Thus, although the only differentiating factor between the previous and present study was the different base medium for IVM, we obtained dissimilar results for nuclear maturation and embryonic development. A clear reason for the decreased nuclear maturation of oocytes matured in a chemically IVM medium could not be elucidated in this study; however, we hypothesize that the contrary results could be attributed to a complex interaction by the diverse components present in the maturation medium. Further investigations on the effects of the components present in αMEM are necessary, and additional research is required to improve nuclear maturation while maintaining high embryonic developmental capacity.

PFF contains various substances including amino acids, energy substrates, and hormones, that are beneficial to pig oocyte maturation [31]. In addition, PFF is known to provide an optimal environment for the *in vivo* development of oocytes before ovulation [31]. Therefore, in most laboratories, PFF is routinely added to IVM medium for successful induction of maturation of immature pig oocytes. Previous reports indicate that addition of PFF to IVM medium effectively inhibits polyspermy after IVF [32] and contributes to improving the normal monospermic fertilization [33]. In another study, oocytes matured in a medium containing PFF showed enhanced embryonic development [34]. Generally, PFF is aspirated from the antral follicles, and the composition of PFF differs depending on the size of follicles [35]. A previous study reported that cumulus cell viability, monospermic fertilization of IVF embryos, and subsequent embryonic developmental competence are reduced in oocytes matured in medium containing PFF derived from small antral follicles (3–4 mm in diameter), compared to oocytes matured in medium containing larger (5–6 mm in diameter) follicle-derived PFF [36]. In general, PA and SCNT embryos have different patterns for remodeling of donor nuclei and nuclear ploidy formation [37]. In addition, depending on the method in which IVP embryos are produced, reprogramming [38] for successful embryonic development in pre-implantation embryos and the expression patterns [39] of specific genes have been reported differently. In the current study, embryonic development of oocytes matured in a chemically defined αMEM with 61.6 mM NaCl was equally promoted after PA and SCNT. However, under PFF-enriched IVM conditions, improved development of SCNT embryos was observed in oocytes matured in αMEM containing 61.6 mM NaCl, as compared to oocytes matured in αMEM and M199 containing 108 mM NaCl. However, no significant difference was observed in the embryonic development after PA. These results differ from a previous study which reported that oocytes matured in PFF-enriched PZM-3 medium containing 61.6 mM NaCl show increased embryonic development after both PA and

SCNT [7]. Although not fully verified, based on the results obtained in previous studies, we speculate that different developmental capacity of PA and SCNT-derived embryos might be influenced by various factors constituting the base medium, and unidentified factors present in the PFF.

It is generally known that the IVM period of pig oocytes is relatively longer than mouse and bovine oocytes. Therefore, pig oocytes are more likely to be exposed longer to unfavorable factors during IVM, resulting in deteriorated quality of IVM oocytes. One study used αMEM to induce meiotic arrest of pig oocytes [40]. However, to our knowledge, ours is the first study that examines the effects of αMEM as an IVM medium on pig oocyte maturation and embryonic development. In the current study, maturation of pig oocytes in αMEM and M199 containing the normal NaCl concentration showed no difference in oocyte maturation and embryonic development after PA and SCNT. Although additional analysis of molecular events such as gene expression patterns, chromosome spindles, and specific protein contents is required, we propose that αMEM medium could be a complex medium that can replace M199 for IVM of pig oocytes.

In summary, significant increases in the PVS width and intra-oocyte GSH contents after IVM were induced in oocytes matured in αMEM containing 61.6 mM NaCl, than in oocytes matured in αMEM with 108 mM NaCl and standard M199. This indicates that cytoplasmic maturation is improved by IVM culture under lowered NaCl condition, and might contribute to the enhancement of embryonic development after PA and SCNT. These findings further signify that αMEM with reduced NaCl can be used as an IVM medium for obtaining pig oocytes with high developmental potential, and may contribute to increasing the production efficiency of SCNT pig embryos.

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