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Glucose in a maturation medium with reduced NaCl improves oocyte maturation and embryonic development after somatic cell nuclear transfer and *in vitro* fertilization in pigs

Yongjin Lee[1,*], Hanna Lee[1,*], Joohyeong Lee[2], Seung Tae Lee[3], Geun-Shik Lee[1] and Eunsong Lee[1,2]

[1]College of Veterinary Medicine, Kangwon National University, Chuncheon 24341, Korea; [2]Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Korea and [3]Division of Applied Animal Science, Kangwon National University, Chuncheon 24341, Korea

Research Article

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Author for correspondence:
Eunsong Lee. College of Veterinary Medicine, Kangwon National University, Chuncheon 24341, Korea. Tel: +82 33 250 8670. Fax: +82 33 259 5625.
E-mail: eslee@kangwon.ac.kr

*These two authors contributed equally to this work.

Summary

This study was conducted to examine whether glucose in maturation medium containing reduced NaCl could improve oocyte maturation and embryonic development in pigs. The base medium was bovine serum albumin-free porcine zygote medium (PZM)-3 containing 10% (v/v) pig follicular fluid (FPZM) or 0.1% (w/v) polyvinyl alcohol (PPZM). Using each medium, the effects of NaCl concentrations (108 and 61.6 mM) and 5.56 mM glucose supplementation (designated as PZM108N, PZM108G, PZM61N, and PZM61G, respectively) were examined using a 2 × 2 factorial arrangement. When oocytes were matured in FPZM, glucose supplementation improved nuclear maturation compared with no supplementation, regardless of the NaCl concentrations. FPZM61G showed a higher blastocyst formation compared with FPZM108N and FPZM108G after parthenogenesis (PA). Blastocyst formations of somatic cell nuclear transfer (SCNT) embryos derived from FPZM61N and FPZM61G were higher compared with those of oocytes from FPZM108N. When oocytes were matured in PPZM, glucose added to PPZM108 and PPZM61 increased nuclear maturation compared with no supplementation. However, glucose added to PPZM108 did not alter embryonic development after PA. Additionally, oocytes matured in PPZM61G showed a higher blastocyst formation compared with those from PPZM61N. In SCNT, blastocyst formation was not influenced by glucose supplementation of PPZM108, but was increased by maturation in glucose-supplemented PPZM61. In embryonic development of *in vitro* fertilization (IVF), oocytes matured in medium with reduced NaCl and glucose showed significantly higher blastocyst formation compared with those matured in PPZM108G. Our results demonstrated that glucose in maturation medium containing 61.6 mM NaCl increased oocyte maturation and embryonic development after PA, SCNT, and IVF.

Introduction

Developmental competence of *in vitro*-produced embryos is influenced by various factors, of which the quality of oocytes is one of the most critical factors determining embryonic viability and successful production of offspring in mammalian species (Lee *et al.*, 2015). Mature oocytes needed for *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) are generally produced by *in vitro* maturation (IVM) of immature oocytes that were obtained from slaughtered gilts ovaries. Therefore, establishment of an optimized IVM system for evaluating the effects of specific components such as energy substrates (Lin *et al.*, 2014), antioxidants (Taweechaipaisankul *et al.*, 2016), and inorganic salts (Lawitts and Biggers, 1992; Lee *et al.*, 2013) is necessary for consistent production of high-quality oocytes.

Previously, it has been reported that IVM of oocytes of pigs in medium with reduced NaCl concentrations inhibits polyspermic fertilization after IVF, by increasing the thickness of the perivitelline space (PVS) and male pronuclear formation (Funahashi *et al.*, 1994). In our study, maturation culture of immature pig oocytes in porcine zygote medium-3 (PZM-3) containing reduced (61.6 mM) NaCl and pig follicular fluid (PFF) was found to increase PVS and improve embryonic development to the blastocyst stage after parthenogenesis (PA) and SCNT compared with that in medium containing 108 mM NaCl (Lee *et al.*, 2013, 2017). In that study, the nuclear maturation of oocytes was significantly decreased although the embryonic development was greatly increased by IVM in medium with reduced NaCl.

Glucose is an important energy substrate commonly included in culture medium for IVM of bovine (Ha *et al.*, 2015) and porcine (Choi *et al.*, 2017) oocytes and known to stimulates meiotic maturation of bovine (Steeves and Gardner, 1999), porcine (Funahashi *et al.*, 2008), and mouse

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(Downs *et al.*, 1996, 1998) oocytes *in vitro*. Previously, Sato *et al.* (2007) reported that glucose contained in the modified North Carolina State University-37 medium stimulated nuclear maturation of pig oocytes and that glucose had an additive effect on nuclear progression. However, they used medium containing PFF to examine the effects of glucose and pyruvate. PFF is similar to serum in its composition and contains glucose, amino acids, and other unknown substances that may be beneficial or detrimental to oocyte maturation (Liu *et al.*, 2002; Hong and Lee, 2007). Therefore, it is desirable to use a chemically defined medium not containing serum components or follicular fluid to clearly evaluate the effect of specific substances added to medium.

PZM-3 is a simple medium with well known components and this medium has been frequently used after replacing its bovine serum albumin (BSA) with polyvinyl alcohol (PVA) to test the precise effect of specific components in medium on pig oocyte maturation. Lactate and pyruvate are included in PZM-3 itself, but glucose is not present (Yoshioka *et al.*, 2002). It has been reported that glucose exists in PFF at 2.1–4.8 mM depending on the storage conditions (Chang *et al.*, 1976). Even though PFF contains various sources of energy substrates such as glucose and amino acids, the concentration of glucose in 10% (v/v) PFF-supplemented PZM-3 is much lower than that (approximately 5.6 mM glucose) in conventional medium such as medium-199 (Wang *et al.*, 1997). Based on this finding, it was suspected that lack of essential nutrients such as glucose and pyruvate might have contributed to the decrease in nuclear maturation of oocytes that were matured in PZM with reduced NaCl in our previous study (Lee *et al.*, 2013, 2017). Therefore, it was hypothesized that glucose supplementation to the glucose-free chemically defined PZM medium would improve nuclear and cytoplasmic maturation of oocytes and resultantly stimulate embryonic development. Therefore, this study was conducted to design and establish an improved IVM system using a medium with reduced NaCl by evaluating the effects of glucose added to IVM medium on oocyte maturation and embryonic development in pigs. To accomplish this, a chemically defined and a conventional medium containing 61.6 or 108 mM NaCl was supplemented with glucose and the effects of glucose supplementation relative to two different NaCl concentrations on nuclear maturation, cumulus cell expansion after IVM, and embryonic development after PA, SCNT, and IVF were compared.

Materials and methods

Culture medium and reagents

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. BSA-free PZM-3 (Lee *et al.*, 2017) containing 108.0 or 61.6 mM NaCl was used as the base medium for IVM of oocytes in this study. The base medium was 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) PVA according to the experimental design (Table 1). PZM-3 containing 0.3% (w/v) BSA, which was modified by adding 0.34 mM trisodium citrate, 2.77 mM myo-inositol, and 10 μM β-mercaptoethanol, was used as the *in vitro* culture (IVC) medium for embryonic development (You *et al.*, 2012).

Collection of immature oocytes and IVM

Ovaries from prepubertal gilts were obtained from a local abattoir. Cumulus–oocyte complexes (COCs) were collected from superficial antral follicles that were 3–8 mm in diameter and those 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 four-well multidish (Nunc, Roskilde, Denmark) containing 500 μl of IVM medium with 80 μg/ml follicle stimulating hormone (Antrin R-10, Kyoritsu Seiyaku, Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (Intervet International BV, Boxmeer, The Netherlands) and then cultured at 39°C under an humidified atmosphere of 5% CO_2 and 95% air. The COCs were washed three times in fresh hormone-free medium after 22 h in maturation culture, then cultured in hormone-free IVM medium for an additional 20 h for SCNT and 22 h for PA and IVF.

Table 1. Abbreviations for media used for *in vitro* maturation in this study

Supplemented to porcine zygote medium-3 (PZM)			Abbreviation	Experiment no.
PVA[a] or PFF[b]	NaCl (mM)	Glucose		
PVA	108.0	No	PPZM108N	2, 3
PVA	108.0	Yes	PPZM108G	2, 3
PVA	61.6	No	PPZM61N	1–3
PVA	61.6	Yes	PPZM61G	1–3
PFF	108.0	No	FPZM108N	4, 5
PFF	108.0	Yes	FPZM108G	4–6
PFF	61.6	No	FPZM61N	4, 5
PFF	61.6	Yes	FPZM61G	4–6

[a]0.1% (w/v) polyvinyl alcohol.
[b]10% (v/v) pig follicular fluid.

Experimental design

In total, eight modified types of IVM medium were used according to the experimental design. Each medium was denoted depending on the addition of PFF (F) or PVA (P), 108.0 (108) or 61.6 mM (61) NaCl, and glucose (G) or no glucose (N) (Table 1). For example, PZM supplemented with PFF, 61.6 mM NaCl, and glucose was abbreviated as FPZM61G and PZM supplemented with PVA, 108 mM NaCl, and no glucose was termed PPZM108N. In Experiment 1, oocytes were matured in a chemically defined PPZM61 supplemented with or without glucose and pyruvate to examine the effects on oocyte maturation and embryonic development after PA. Pyruvate was added to medium as a base energy substrate in subsequent experiments. In Experiments 2 and 3, oocytes were matured in PPZM108 or PPZM61 that was unsupplemented or supplemented further with 5.56 mM glucose. After IVM, nuclear maturation of oocytes, cumulus cell expansion, and embryonic development to the blastocyst stage after PA (Experiment 2) and SCNT (Experiment 3) were examined. The effects of glucose in IVM medium were examined using FPZM instead of PPZM in Experiments 4 and 5 with the same experimental designs as Experiments 2 and 3. Finally, IVM of oocytes in reduced NaCl medium supplemented with glucose (FPZM61G) was evaluated to determine if embryonic development improved after IVF in Experiment 6.

Measurement of cumulus cell expansion after IVM

Following IVM, the degree of cumulus cell expansion was evaluated subjectively as previously described (Vanderhyden *et al.*, 1990).

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Briefly, no response was scored as 0, minimum observable response in which the cells in the outermost layer of the cumulus became round and glistening was scored as 1, the expansion of outer cumulus cell layers was scored as 2, the expansion of all cumulus cell layers except the corona radiata was scored as 3, and the expansion of all cumulus cell layers including the corona radiata was scored as 4.

Preparation of donor cells for SCNT

Porcine fetal fibroblasts were seeded into four-well plates and grown in Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12 (Invitrogen, Grand Island, NY, USA) and 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 single-cell suspension was prepared by trypsinization of the cultured cells, followed by re-suspension in TLH containing 0.4% (w/v) BSA (TLH-BSA) before SCNT (Lee *et al*., 2016).

SCNT and PA

SCNT and PA were performed as previously described (You *et al*., 2012; Lee *et al*., 2016). Briefly, cumulus cells were removed from IVM oocytes by repeated pipetting in 0.1% (w/v) hyaluronidase in TLH-BSA medium. For SCNT, denuded oocytes were stained with 5 μg/ml Hoechst 33342 for 15 min in manipulation medium (calcium-free TLH-BSA). Oocytes were then washed twice with fresh manipulation medium, transferred into a drop of manipulation medium containing 5 μg/ml cytochalasin B (CB), and overlaid with warm mineral oil. Next, oocytes were enucleated by aspirating the first polar body and metaphase II (MII) chromosomes using a 17-μm bevelled glass pipette (Humagen, Charlottesville, VA, USA). Enucleation was confirmed under an epifluorescence microscope (TE300; Nikon, Tokyo, Japan). A single cell was subsequently inserted into the PVS of each oocyte, after which 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 at 1 MHz for 2 s followed by two pulses of 175 V/mm direct current for 30 μs using a cell fusion generator (LF101; NepaGene, Chiba, Japan). Oocytes were subsequently incubated in TLH-BSA for 1 h, after which the fusion rates were evaluated under a stereomicroscope. Two pulses of 120 V/mm direct current were applied for 60 μs in a 280 mM mannitol solution containing 0.1 mM $CaCl_2$ and 0.05 mM $MgCl_2$ to activate reconstructed oocytes. For PA, MII oocytes were activated using a pulse sequence identical to that used to activate SCNT oocytes.

IVF and assessment of fertilization parameters

Fresh semen was purchased from GUMBO GENE (Wonju, Kangwon-do, Korea). For IVF, semen samples were washed two times by centrifugation at 350 *g* for 3 min in Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen) supplemented with 0.1% (w/v) BSA. The sperm pellet was then resuspended in modified Tris-buffered medium (mTBM) supplemented with 0.2% (w/v) BSA. At the end of IVM culture, COCs were placed into 45-μl droplets of mTBM and 5-μl aliquots of the sperm suspension were added to each fertilization droplet to give a final sperm concentration of 1.0×10^6 sperm/ml. Sperm and oocytes were then co-incubated for 6 h, after which inseminated oocytes were pipetted gently to remove cumulus cells and loosely attached spermatozoa. Examination of sperm penetration and pronucleus formation was then performed as previously described (Kwak *et al*., 2012), with brief modifications (solutions 1–4). At 12 h after insemination, the zona pellucida of putative zygotes was dissolved in 0.5% (w/v) pronase (solution 1). The zona-free embryos were then washed in D-PBS containing 0.1% (v/v) formaldehyde (FA) and 0.01% (w/v) PVA (solution 2) for 1 min, after which they were fixed in 1% (v/v) FA and 0.01% (w/v) PVA in D-PBS (solution 3) for 20 min at room temperature. Next, fixed embryos were placed in Hoechst 33342 (solution 4) on a slide. The numbers of spermatozoa penetrated oocytes, and the presence of polyspermy and male pronucleus formation were then examined under an epifluorescence fluorescence microscope.

Post-activation and IVC

After electrical activation, the PA embryos were treated with 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, then 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. The total blastocyst cell count was conducted using Hoechst 33342 staining under an epifluorescence microscope.

Statistical analyses

The independent variables were glucose and/or pyruvate supplementation and NaCl concentration in IVM medium. Cumulus expansion score, proportions of oocytes degenerated, reached MII, fertilization ability, developed to the cleavage and blastocyst stages, and mean number of cells in blastocyst were analyzed as dependent variables. Statistical analyses were conducted using Statistical Analysis System (version 9.4; SAS Institute, Cary, NC, USA). The data were subjected to analysis of variance (ANOVA) using a general linear model procedure. Post-hoc analyses to identify between-group differences were performed using the least significant difference test when treatments differed at a *P*-value less than 0.05. Percentage data were arcsine-transformed prior to analysis to maintain the homogeneity of variance. The results were expressed as the means ± standard error of the mean.

Results

Effect of glucose and/or pyruvate in a chemically defined medium with reduced NaCl (PPZM61) on the development PA embryos

Effects of glucose and pyruvate in a chemically defined maturation with reduced NaCl on oocyte maturation and embryonic development after PA were determined. As shown in Table 2, the absence of glucose and pyruvate in the maturation medium significantly ($P < 0.05$) inhibited nuclear maturation (40.8 ± 6.5%) compared with glucose, pyruvate, and glucose + pyruvate supplementations (59.7 ± 3.7, 62.2 ± 5.2 and 77.2 ± 1.8%, respectively). Conversely, maturation medium without glucose or pyruvate (73.9 ± 9.5%) significantly ($P < 0.01$) showed a higher proportion of degenerated oocytes after IVM than that with glucose (9.8 ± 1.6%), pyruvate (7.2 ± 4.3%) or glucose + pyruvate (4.8 ± 2.9%). Supplementation of

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Table 2. Effect of glucose and/or pyruvate in a chemically defined *in vitro* maturation (IVM) medium containing 61.6 mM NaCl on embryonic development after parthenogenesis (PA)

IVM medium††		No. of oocytes cultured†	% of oocytes degenerated after IVM	% of oocytes reached metaphase II	No. of oocytes activated and cultured†	% of oocytes developed to		No. of cells in blastocyst
Glucose	Pyruvate					≥ 2-cell	Blastocyst	
No	No	218	73.9 ± 9.5^{a}	40.8 ± 6.5^{c}	23	78.5 ± 8.0^{c}	2.1 ± 2.1^{c}	21.0 ± 0.0
Yes	No	123	9.8 ± 1.6^{b}	59.7 ± 3.7^{d}	61	86.6 ± 3.8^{cd}	24.3 ± 3.8^{d}	30.4 ± 1.2
No	Yes	125	7.2 ± 4.3^{b}	62.2 ± 5.2^{d}	69	82.3 ± 2.9^{c}	31.9 ± 2.3^{d}	31.7 ± 2.0
Yes	Yes	124	4.8 ± 2.9^{b}	77.2 ± 1.8^{e}	82	96.1 ± 2.5^{d}	47.8 ± 2.5^{e}	32.5 ± 1.6

†Three replicates.
††Bovine serum albumin-free porcine zygote medium-3 supplemented with 0.1% (w/v) polyvinyl alcohol was used for oocyte maturation.
Values with different superscript letters in the same column are different ($^{a,b}P < 0.01$, $^{c-e}P < 0.05$).

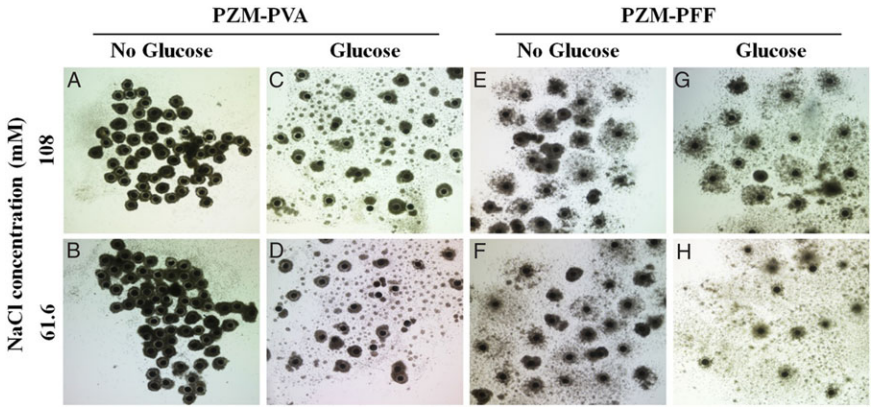

Figure 1. Morphology of pig cumulus–oocyte complexes (COCs) after *in vitro* maturation (IVM). COCs were matured in porcine zygote medium (PZM) containing 108 mM (A, C, E, G) or 61.6 mM NaCl (B, D, F, H) that was further supplemented with polyvinyl alcohol (PVA) (A–D) or pig follicular fluid (PFF) (E–H). COCs were untreated (A, B, E, F) or treated with glucose (C, D, G, H) during IVM culture.

maturation medium with glucose (24.3 ± 3.8%), pyruvate (31.9 ± 2.3%) and glucose + pyruvate (47.8 ± 2.5%) significantly ($P < 0.05$) stimulated blastocyst formation of PA oocytes relative to no supplementation (2.1 ± 2.1%). Simultaneous addition of glucose and pyruvate showed a synergistic effect compared with single addition of glucose or pyruvate.

Effect of glucose supplementation of PPZM108 and PPZM61 on cumulus cell expansion, nuclear maturation and embryonic development after PA

The score of cumulus cell expansion after IVM by glucose treatment significantly ($P < 0.01$) increased in both PPZN108G (1.92 ± 0.14) and PPZM61G (2.12 ± 0.10) compared with PPZM108N (0.68 ± 0.11) and PPZM61N (0.58 ± 0.14) (Fig. 1 and Table 3). Supplementation with glucose significantly ($P < 0.05$) increased nuclear maturation of oocytes compared with no supplementation in both PPZM108 (78.5 ± 4.7% vs. 91.2 ± 1.7%) and PPZM61 (57.7 ± 5.3% vs. 84.3 ± 3.9%). Supplementation of PPZM61 with glucose significantly ($P < 0.05$) improved PA embryonic development to the blastocyst stage relative to no supplementation (36.6 ± 5.2% vs. 51.8 ± 3.4%), but no improving effect was observed when PPZM108 was supplemented with glucose (38.1 ± 1.5% vs. 40.9 ± 2.1%) (Table 3).

Effect of glucose supplementation of PPZM108 and PPZM61 on embryonic development after SCNT

Oocytes matured in PPZM61G (15.2 ± 2.7%) showed a significantly ($P < 0.05$) higher blastocyst formation after SCNT compared with those matured in PPZM61N (7.7 ± 4.5%), while no effect of glucose was observed after supplementation with PPZM108 (14.7 ± 1.8% and 16.2 ± 2.0% for PPZM108G and PPZM108N, respectively) (Table 4). Embryo cleavage (52.8 ± 18.4 to 80.0 ± 3.9%) and mean number of cells in blastocysts (26.3 ± 2.8 to 39.7 ± 3.3 cells per blastocyst) were not influenced by the glucose supplementation.

Effect of glucose supplementation of FPZM108 and FPZM61 on cumulus cell expansion, nuclear maturation, and embryonic development after PA

Effects of glucose supplemented to FPZM108 and FPZM61 on cumulus cell expansion, nuclear maturation, and embryonic development were examined. Similar to PPZM medium, the cumulus cell expansion was significantly ($P < 0.05$) stimulated by glucose addition to FPZM108G and FPZM61G with glucose (2.88 ± 0.09 and 3.06 ± 0.13, respectively) compared with FPZM108N and FPZM61N (2.46 ± 0.10 and 2.56 ± 0.12, respectively) (Fig. 1 and Table 5). Glucose added to FPZM108 (93.7 ± 0.8%) and FPZM61 (92.6 ± 1.7%) significantly improved oocyte maturation when compared with their unsupplemented counterparts (83.4 ± 2.0 and 66.7 ± 3.4%, respectively). However, embryonic development after PA to the blastocyst stage was not altered by glucose supplementation to FPZM108 (37.2 ± 3.0% vs. 36.3 ± 3.6%) and FPZM61 (48.1 ± 4.2%), while FPZM61G (51.2 ± 3.7%) showed a higher blastocyst formation compared with FPZM108G (36.3 ± 3.6%) (Table 5).

Effect of glucose supplementation of FPZM108 and FPZM61 on embryonic development after SCNT

The effect of glucose addition to FPZM on SCNT embryonic development was evaluated. As shown in Table 6, the addition of glucose

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Table 3. Effect of glucose in a chemically defined *in vitro* maturation (IVM) medium on nuclear maturation, cumulus cell expansion after IVM, and embryonic development after parthenogenesis (PA)

IVM medium††						% of oocytes developed to		
NaCl (mM)	Glucose	No. of oocytes cultured†	Cumulus expansion score after IVM*	% of oocytes reached metaphase II	No. of oocytes activated and cultured†	≥ 2-cell	Blastocyst	No. of cells in blastocyst
108.0	No	218	0.68 ± 0.11^{a}	78.5 ± 4.7^{c}	157	85.0 ± 1.9^{c}	38.1 ± 1.5^{c}	37.4 ± 1.7
108.0	Yes	224	1.92 ± 0.14^{b}	91.2 ± 1.7^{d}	201	93.5 ± 1.1^{d}	40.9 ± 2.1^{cd}	36.0 ± 1.6
61.6	No	194	0.58 ± 0.14^{a}	57.7 ± 5.3^{e}	99	82.9 ± 3.3^{c}	36.6 ± 5.2^{c}	31.4 ± 1.7
61.6	Yes	219	2.12 ± 0.10^{b}	84.3 ± 3.9^{cd}	181	97.5 ± 1.4^{e}	51.8 ± 3.4^{d}	36.2 ± 1.4

†Four replicates.
††Bovine serum albumin-free porcine zygote medium-3 supplemented with 0.1% (w/v) polyvinyl alcohol was used for oocyte maturation.*Cumulus cell expansion was scored as 0 (no response), 1 (minimum observable response with the cells in the outermost layer of the cumulus becoming round and glistening), 2 (expansion of outer cumulus cell layers), 3 (expansion of all cumulus cell layers except corona radiate), and 4 (expansion of all cumulus cell layers).
Values with different superscript letters in the same column are different ($^{a,b}P < 0.01$, $^{c\text{-}e}P < 0.05$).

Table 4. Effect of glucose in a chemically defined *in vitro* maturation (IVM) medium containing 61.6 and 108.0 mM NaCl on embryonic development after somatic cell nuclear transfer (SCNT)

IVM medium††						% of oocytes developed to		
NaCl (mM)	Glucose	No. of oocytes cultured†	% of oocytes reached metaphase II	% of SCNT oocytes fused†	No. of SCNT oocytes cultured†	≥ 2-cell	Blastocyst	No. of cells in blastocyst
108.0	No	263	71.8 ± 1.1^{a}	53.4 ± 8.8^{ab}	68	69.9 ± 3.5	16.2 ± 2.0^{a}	32.9 ± 3.6
108.0	Yes	162	83.1 ± 1.4^{b}	63.7 ± 4.1^{b}	80	77.0 ± 4.2	14.7 ± 1.8^{ab}	39.7 ± 3.3
61.6	No	250	48.8 ± 2.1^{c}	38.0 ± 7.7^{a}	37	52.8 ± 18.4	7.7 ± 4.5^{b}	26.3 ± 2.8
61.6	Yes	179	79.1 ± 6.0^{ab}	60.3 ± 5.3^{b}	78	80.0 ± 3.9	15.2 ± 2.7^{a}	38.3 ± 3.9

†Four replicates.
††Bovine serum albumin-free porcine zygote medium-3 supplemented with 0.1% (w/v) polyvinyl alcohol was used for oocyte maturation.
Values with different superscript letters in the same column are different ($^{a\text{-}c}P < 0.05$).

to FPZM108 and FPZM61 had no effect on cleavage, blastocyst development (22.8 ± 1.0% vs. 25.9 ± 0.6% in FPZM108 and 33.8 ± 2.3% vs. 32.2 ± 1.3% in FPZM61) or mean cell number of blastocysts (40.3 ± 2.8 to 43.1 ± 3.6 cells per blastocyst). Regardless of glucose supplementation, oocytes matured in medium with 61.6 mM NaCl (33.8 ± 2.3% and 32.2 ± 1.3%) showed significantly higher blastocyst formation compared with those matured in medium with 108 mM NaCl (22.8 ± 1.0% and 25.9 ± 0.6%).

Effect of glucose added to two maturation media containing 108.0 or 61.6 mM NaCl on fertilization of oocytes and embryonic development after IVF

When oocytes matured in two different media (FPZM108G and FPZM61G) were inseminated *in vitro*, sperm penetration (86.0 ± 4.9% vs. 83.1 ± 6.4%), polyspermic fertilization (49.4 ± 9.0% vs. 47.6 ± 6.6%), and male pronuclear formation (92.1 ± 5.9% vs. 99.1 ± 0.9%) were not influenced by the NaCl concentration of maturation medium (Table 7). However, oocytes matured in FPZM61G showed higher cleavage (62.5 ± 3.6% vs. 77.8 ± 3.1%) and blastocyst formation (16.3 ± 1.9% vs. 21.5 ± 0.3%) than FPZM108G (Table 8).

Discussion

In this study, we examined the effect of glucose supplemented to a chemically defined maturation medium containing low concentrations (61.6 mM) of NaCl on oocyte maturation and embryonic development after PA, SCNT and IVF in pigs. Our results revealed that glucose supplementation of a chemically defined IVM medium containing reduced NaCl dramatically stimulated nuclear maturation and blastocyst formation. Interestingly, the beneficial effect of glucose on embryonic development was greater when glucose was supplemented to PFF-enriched IVM medium with reduced NaCl compared with the supplementation to the medium with the usual concentration (108 mM) of NaCl.

Energy sources are required for maturation and embryonic development of mammalian oocytes *in vitro* (Downs and Utecht, 1999; Hashimoto *et al.*, 2000). Glucose is an essential factor for resumption of meiosis in mouse oocytes (Xie *et al.*, 2016). Glucose is metabolized through the pentose phosphate pathway (PPP), glycolysis, and the tricarboxylic acid cycle and used with ATP during maturation of mammalian oocytes (O'Brien *et al.*, 1996; Krisher and Bavister, 1999; Hashimoto *et al.*, 2000). A recent study reported that metabolites secreted from PPP and glycolysis during IVM of immature pig oocytes improved nuclear and cytoplasmic maturation, whereas nuclear maturation and embryonic development were suppressed by inhibition of glycolysis or PPP (Wen *et al.*, 2020). In addition, pyruvate generated by glucose metabolism has routinely been used for IVM of porcine immature oocytes (Kwak *et al.*, 2012; Taweechaipaisankul *et al.*, 2016). A previous study has shown that pyruvate supplementation during IVM of porcine oocytes contributed to enhancement of nuclear maturation and improves cytoplasmic maturation by increasing intra-oocyte glutathione content (Funahashi *et al.*, 2008). The results of previous and present studies indicated that glucose and pyruvate supplementation to a chemically defined IVM

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Table 5. Effect of glucose in pig follicular fluid (PFF)-enriched *in vitro* maturation (IVM) medium containing 61.6 and 108.0 mM NaCl on nuclear maturation, cumulus cell expansion after IVM, and embryonic development after parthenogenesis (PA)

IVM medium††		No. of oocytes cultured†	Cumulus expansion score after IVM*	% of oocytes reached meta-phase II	No. of oocytes activated and cultured†	% of oocytes developed to		No. of cells in blastocyst
NaCl (mM)	Glucose					≥ 2-cell	Blastocyst	
108.0	No	281	2.46 ± 0.10^{a}	83.4 ± 2.0^{a}	216	86.5 ± 2.6	37.2 ± 3.0^{a}	39.5 ± 1.8
108.0	Yes	273	2.88 ± 0.09^{b}	93.7 ± 0.8^{b}	233	89.5 ± 1.3	36.3 ± 3.6^{a}	35.7 ± 1.4
61.6	No	331	2.56 ± 0.12^{a}	66.7 ± 3.4^{c}	201	93.4 ± 2.4	48.1 ± 4.2^{b}	39.4 ± 1.7
61.6	Yes	288	3.06 ± 0.13^{b}	92.6 ± 1.7^{b}	243	94.4 ± 2.7	51.2 ± 3.7^{b}	36.1 ± 1.2

†Six replicates.
††Bovine serum albumin-free porcine zygote medium-3 supplemented with 10% (v/v) PFF used for oocyte maturation.
*Cumulus cell expansion was scored as 0 (no response), 1 (minimum observable response with the cells in the outermost layer of the cumulus becoming round and glistening), 2 (expansion of outer cumulus cell layers), 3 (expansion of all cumulus cell layers except corona radiate), and 4 (expansion of all cumulus cell layers).
Values with different superscript letters in the same column are different ($^{a-c}P < 0.05$).

Table 6. Effect of glucose in pig follicular fluid (PFF)-enriched *in vitro* maturation (IVM) medium containing 61.6 and 108.0 mM NaCl on embryonic development after somatic cell nuclear transfer (SCNT)

IVM medium††		No. of oocytes cultured†	% of oocytes reached metaphase II	% of SCNT oocytes fused†	No. of SCNT oocytes cultured†	% of oocytes developed to		No. of cells in blastocyst
NaCl (mM)	Glucose					≥ 2-cell	Blastocyst	
108.0	No	308	82.7 ± 1.6^{a}	74.2 ± 2.6	150	85.6 ± 4.8	22.8 ± 1.0^{a}	43.1 ± 3.6
108.0	Yes	299	93.1 ± 0.7^{b}	79.5 ± 2.8	195	85.3 ± 3.9	25.9 ± 0.6^{a}	40.3 ± 2.8
61.6	No	351	68.5 ± 2.9^{c}	77.8 ± 4.1	146	91.7 ± 4.3	33.8 ± 2.3^{b}	43.1 ± 2.3
61.6	Yes	323	92.1 ± 0.5^{b}	76.0 ± 2.3	202	92.7 ± 0.7	32.2 ± 1.3^{b}	42.3 ± 2.1

†Three replicates.
††Bovine serum albumin-free porcine zygote medium-3 supplemented with 10% (v/v) PFF used for oocyte maturation.
Values with different superscript letters in the same column are different ($^{a-c}P < 0.05$).

Table 7. Effect of reduced NaCl in *in vitro* maturation (IVM) medium on *in vitro* fertilization (IVF) of IVM pig oocytes

NaCl (mM) in IVM medium††	No. of IVF oocytes examined†	% of oocytes			Mean no. of spermatozoa in penetrated oocytes
		Penetrated*	Polyspermic**	With male pronucleus**	
108.0	96	86.0 ± 4.9	49.4 ± 9.0	92.1 ± 5.9	2.7 ± 0.1
61.6	97	83.1 ± 6.4	47.6 ± 6.6	99.1 ± 0.9	2.7 ± 0.1

†Three replicates.
††Bovine serum albumin-free porcine zygote medium-3 supplemented with glucose and 10% (v/v) pig follicular fluid was used for oocyte maturation.
*Percentage of the number of examined oocytes.
**Percentage of the number of penetrated oocytes.

Table 8. Effect of reduced NaCl in *in vitro* maturation (IVM) medium on embryonic development after *in vitro* fertilization (IVF)

NaCl (mM) in IVM medium††	No. of IVF oocytes cultured†	% of embryos developed to		No. cells in blastocyst
		≥ 2-cell	Blastocyst	
108.0	159	62.5 ± 3.6^{a}	16.3 ± 1.9^{a}	41.1 ± 2.7
61.6	167	77.8 ± 3.1^{b}	21.5 ± 0.3^{b}	37.4 ± 4.3

†Four replicates.
††Bovine serum albumin-free porcine zygote medium-3 supplemented with glucose and 10% (v/v) pig follicular fluid was used for oocyte maturation.
Values in the same column with different superscript letters are different ($^{a,b}P < 0.05$).

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medium with reduced NaCl stimulated meiosis of porcine oocytes, as well as cytoplasmic maturation and contributed to increased blastocyst formation. Moreover, degradation of IVM oocytes was greatly reduced when oocytes were matured in medium containing glucose and pyruvate. These results further demonstrated that glucose and pyruvate are essential nutrients for oocyte viability and meiosis resumption.

Cumulus cells play an important role in transferring nutrients of culture medium to oocytes and stimulating nuclear maturation (Zhang *et al*., 1995; Erickson and Shimasaki, 2001; Gilchrist and Thompson, 2007). Cumulus cell expansion is known as a key marker for prediction of the quality of IVM oocytes as well as embryonic development potential (Song *et al*., 2018). In this study, glucose supplementation of a chemically defined medium with reduced NaCl stimulated cumulus expansion of oocytes, and these oocytes showed increased nuclear maturation and embryonic development after PA and SCNT. Our results are consistent with those of previous studies that showed mammalian oocytes with great cumulus cell expansion after IVM had increased embryonic development after SCNT (Son *et al*., 2018) and IVF (Zhang *et al*., 1995) and that an increase in cumulus cell expansion is correlated with nuclear maturation acceleration by reducing the penetration of cyclic adenosine monophosphate through gap junctions (Sun *et al*., 2015).

Interestingly, glucose supplementation of a chemically defined medium with 108 mM NaCl increased nuclear maturation and cumulus cell expansion, while embryonic development after PA and SCNT was not affected. Sodium chloride is one of the main components regulating osmolality of a culture medium. In this study, the osmotic pressures of IVM medium containing 61.6 and 108 mM NaCl were approximately 220 and 285 mOsm, respectively. Osmolality of a medium varies with the NaCl concentrations in medium and the changes in the osmolality affect maturation of oocytes and embryonic development (Li and Foote, 1996; Collins and Baltz, 1999). Yamauchi *et al.* (1999) reported that germinal vesicle breakdown was accelerated in porcine oocytes that were cultured under low osmotic conditions (210 mOsm). In another previous study, Kitagawa and Niimura (2006) showed that IVM of porcine oocytes in the medium with low (61.6 mM) NaCl concentration increased the PVS and dramatically reduced the polyspermic fertilization after IVF. Moreover, maturation of oocytes in reduced NaCl (61.6 mM) medium induced enlargement of PVS of oocytes, which led to improved embryonic development after PA and SCNT (Lee *et al*., 2013). In this study, it was not clear if the increased nuclear maturation and blastocyst development after PA was due to reduced NaCl concentration in the medium or to the lower osmotic pressure of the medium. In our previous study (Lee *et al*., 2017) we compared the effects of hypo-osmotic (approximately 220 mOsm) medium containing 61.6 mM NaCl with iso-osmotic (285 mOsm) medium containing 61.6 mM NaCl and 90 mM sucrose. Nuclear maturation of oocytes was increased by IVM culture in iso-osmotic medium, while blastocyst formation after PA was higher in oocytes that were matured in hypo-osmotic medium. The previous and present results indicated that IVM in iso-osmotic medium was desirable for nuclear maturation, whereas hypo-osmotic medium with reduced NaCl was better for embryonic development, and glucose supplementation to IVM medium with reduced NaCl further increased maturation and developmental competence.

PFF is commonly included as a source of macromolecules in IVM medium for porcine oocytes (Chang *et al*., 1976). In our previous study (Lee *et al*., 2017), oocytes matured in medium containing PFF and reduced NaCl showed slightly reduced nuclear maturation and increased embryonic development when compared with those matured in conventional IVM medium containing the usual concentration of NaCl. Therefore, we evaluated the effects of glucose supplementation of IVM medium containing PFF and reduced NaCl on nuclear maturation and embryonic development. Similar to the results obtained using chemically defined medium with reduced NaCl concentrations, glucose supplementation increased cumulus expansion and nuclear maturation and could therefore solve the problem of low nuclear maturation oocytes observed when they were matured in the absence of glucose in the medium.

IVF is a useful method for assessing the developmental potential of embryos because they can be produced without any artificial activation and develop into normal offspring, unlike PA-derived embryos (Kwak *et al*., 2012). Porcine oocytes are more susceptible to polyspermic fertilization than oocytes of other mammalian species when they are fertilized *in vitro* (Kohata *et al*., 2013). Therefore, fertilization parameters such as polyspermic penetration and pronuclear formation with blastocyst development are routinely evaluated when the quality and competence of IVM oocytes are assessed (Koo *et al*., 2005). In our results, oocytes matured in reduced NaCl medium containing PFF and glucose showed significantly higher blastocyst formation without increasing polyspermic fertilization after IVF compared with oocytes matured in a medium containing the usual NaCl concentration. Funahashi *et al.* (1994) reported first that oocytes matured in medium containing a lower NaCl concentration than conventional medium increased the *in vitro* development of IVF embryos. Moreover, in our previous study (Lee *et al*., 2017), oocytes matured in IVM medium containing 61.6 mM NaCl showed improved embryonic development after PA and SCNT. Based on the previous and present results, it is suggested that IVM medium that contains reduced NaCl, PFF, and glucose could be used as a new standard maturation medium to produce oocytes with high developmental competence *in vitro*.

In summary, this study clearly demonstrated that glucose supplementation of IVM medium containing 61.6 mM NaCl stimulated cumulus cell expansion, enhanced nuclear maturation after IVM, and improved embryonic development after PA, SCNT, and IVF. The inhibited nuclear maturation oocytes that were matured in medium with reduced NaCl increased again following glucose supplementation. These findings indicated that the newly designed IVM medium containing reduced (61.6 mM) NaCl and glucose can be utilized for production of more high-quality oocytes with high developmental competence in pigs.

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Conflicts of interest. None of the authors have any conflicts of interest to declare.

Ethical standard. All the experimental procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University, Korea.

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