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—Original Article—

Comparison of Ethylene Glycol and Propylene Glycol for the Vitrification of Immature Porcine Oocytes

Tamás SOMFAI[1)], Michiko NAKAI[2)], Fuminori TANIHARA[2,3)], Junko NOGUCHI[2)], Hiroyuki KANEKO[2)], Naomi KASHIWAZAKI[4)], István EGERSZEGI[5)], Takashi NAGAI[1,6)] and Kazuhiro KIKUCHI[2)]

[1)]*NARO Institute of Livestock and Grassland Science, Ibaraki 305-0901, Japan*
[2)]*Division of Animal Sciences, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan*
[3)]*The United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi 753-8515, Japan*
[4)]*Graduate School, Azabu University, Kanagawa 229-8501, Japan*
[5)]*Research Institute for Animal Breeding and Nutrition, Herceghalom, H-2053, Hungary*
[6)]*WCU Biomodulation Major, Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Republic of Korea*

Abstract. Our aim was to optimize a cryoprotectant treatment for vitrification of immature porcine cumulus-oocyte complexes (COCs). Immature COCs were vitrified either in 35% ethylene glycol (EG), 35% propylene glycol (PG) or a combination of 17.5% EG and 17.5% PG. After warming, the COCs were *in vitro* matured (IVM), and surviving oocytes were *in vitro* fertilized (IVF) and cultured. The mean survival rate of vitrified oocytes in 35% PG (73.9%) was higher ($P<0.05$) than that in 35% EG (27.8%). Oocyte maturation rates did not differ among vitrified and non-vitrified control groups. Blastocyst formation in the vitrified EG group (10.8%) was higher ($P<0.05$) than that in the vitrified PG group (2.0%) but was lower than that in the control group (25.0%). Treatment of oocytes with 35% of each cryoprotectant without vitrification revealed a higher toxicity of PG on subsequent blastocyst development compared with EG. The combination of EG and PG resulted in 42.6% survival after vitrification. The maturation and fertilization rates of the surviving oocytes were similar in the vitrified, control and toxicity control (TC; treated with EG+PG combination without cooling) groups. Blastocyst development in the vitrified group was lower ($P<0.05$) than that in the control and TC groups, which in turn had similar development rates (10.7%, 18.1% and 23.3%, respectively). In conclusion, 35% PG enabled a higher oocyte survival rate after vitrification compared with 35% EG. However, PG was greatly toxic to oocytes. The combination of 17.5% EG and 17.5% PG yielded reasonable survival rates without toxic effects on embryo development.

Key words: Cryoprotectant, Embryo development, Immature oocyte, *In vitro* fertilization, Pig, Vitrification

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Oocyte cryopreservation combined with assisted reproductive techniques such as *in vitro* fertilization (IVF) is a basic strategic technology for gene banking of female germplasm [1]. In recent years, there have been several attempts to cryopreserve porcine oocytes with varying results [2]. However, to our knowledge, viable piglets have not been obtained so far from cryopreserved unfertilized oocytes, underlining the difficulty of gamete cryopreservation in porcine species. Previously, we applied solid surface vitrification to cryopreserve *in vitro* matured and immature porcine oocytes using 35% (v/v) ethylene glycol (EG) as a permeating cryoprotectant [3]. Although survival rates were relatively high for *in vitro* matured oocytes (71.4%), their fertilization and developmental competences after IVF were greatly impaired [3]. On the other hand, oocytes vitrified as cumulus cell-oocyte complexes (COCs) at the immature germinal vesicle (GV) stage could maintain/regain their ability to undergo normal fertilization and development, resulting in the production of high-quality blastocysts [4]. However, unlike matured oocytes, the survival rate for immature COCs was very low (27.7%) [4]. In gene banks, low oocyte survival rates limit the number of transferable embryos; therefore, improvement of the survival rates in immature oocytes is essential.

Some previous studies have demonstrated that immature mammalian oocytes have lower permeability to cryoprotective agents (CPAs) and higher permeability to water compared with matured oocytes [5, 6]. Therefore, a plausible reason for the low survival rates of immature porcine oocytes in the vitrification system we reported previously [3, 4] could be insufficient permeation of the permeable CPA. This suggests that changing EG to a more permeable CPA may be a possible means of improving survival of immature oocytes. In a recent study comparing the permeating speed of several CPAs through the mouse oocyte membrane, propylene glycol (PG; also labeled 1-propanediol) and dimethyl sulfoxide (DMSO) exhibited significantly better permeability than EG [7]. When used as the CPA for vitrification, DMSO has been found to be detrimental to the meiotic competence of immature porcine oocytes [8], whereas EG treatment of oocytes did not affect their maturation, fertilization

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Correspondence: T Somfai (e-mail: somfai@affrc.go.jp)

and subsequent embryo development [3, 4]. On the other hand, the feasibility of PG for the cryopreservation of porcine oocytes remains unknown.

The aim of the present study was to improve the survival of immature porcine oocytes in our vitrification system without reducing their meiotic and developmental competences and ultimately to improve the yield of IVF-derived embryos available for embryo transfer. In the present study, we compared the feasibilities of EG and PG, either alone or in combination, for the vitrification of immature porcine oocytes.

Materials and Methods

Collection and vitrification of COCs

Ovaries from prepubertal cross-bred gilts (Landrace × Large White) were collected at a local slaughterhouse and brought to the laboratory within 1 h in Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical, Tokyo, Japan) at 35−37 C. COCs were collected by scraping follicles 3 to 6 mm in diameter into a collection medium consisting of medium 199 (with Hank's salts; Sigma Chemical, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (Gibco; Invitrogen, Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), and antibiotics [100 units/ml penicillin G potassium (Sigma) and 0.1 mg/ml streptomycin sulfate (Sigma)]. The COCs were cryopreserved by the solid surface vitrification (SSV) method of Dinnyes *et al.* [9] with some modification. Briefly, COCs were treated for 20 min in a basic medium (BM) consisting of modified North Carolina State University (NCSU)-37 [10] medium without glucose, 20 mM HEPES, 4 mg/ml bovine serum albumin (BSA), 50 μM β-mercaptoethanol, 0.17 mM sodium pyruvate and 2.73 mM sodium in the presence of 5 μg/mL cytochalasin B (Sigma, C-6762). The COCs were then treated with equilibration medium comprised of BM supplemented with 5 μg/ml cytochalasin B and, in total, 4% (v/v) of permeable CPA consisting of either EG (Sigma, E-9129), PG (Nacalai Tesque, Kyoto, Japan, 29218-35) or a mixture of the two (2% [v/v] EG + 2% [v/v] PG). They were treated with equilibration medium for 13−15 min at 38.5 C, washed 3 times in 20-μl droplets of vitrification solution at 38.5 C, pipetted into a glass capillary tube in groups of 25 to 30 and finally dropped with approximately 2−3 μl vitrification solution onto the cold surface of aluminum foil floating on liquid nitrogen (LN_2). The vitrification solution was BM supplemented with 50 mg/ml polyvinylpyrrolidone (Sigma, P-0930), 0.3 M trehalose (Sigma, T-0167) and, in total, 35% (v/v) permeable CPA comprised of either EG, PG or a mixture of the two (17.5% [v/v] EG + 17.5% [v/v] PG). Washing in vitrification medium and placing microdrops with COCs on the cold surface were performed in approximately 30 sec in total. The vitrified droplets were either warmed after keeping them on the cold surface for approximately 5 min or placed in 2-ml cryotubes (Iwaki 2732-002; AGC Techno Glass, Tokyo, Japan) partly immersed in LN_2, and then were stored in LN_2 for 14−30 days until use. Vitrified droplets were warmed by transfer into a warming solution (0.4 M trehalose in BM) at 38.5 C. One to two minutes later, oocytes were consecutively transferred for 1-min periods into 500-μl droplets of BM supplemented with 0.2, 0.1 and 0.05 M trehalose. They were then washed in BM without trehalose at 38.5 C and subjected to *in vitro* maturation (IVM).

IVM

The maturation culture medium was a modified NCSU-37 solution [10] containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 μM β-mercaptoethanol, 1 mM dibutyryl cAMP (dbcAMP; Sigma), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan), and 10 IU/ml hCG (500 units; Puberogen, Novartis Animal Health, Tokyo, Japan). Maturation was performed in 4-well dishes (Nunclon MultiDishes, Nalge Nunc International, Roskilde, Denmark) in 500-μl droplets of IVM medium without oil coverage for 22 h in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39 C. The COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for an additional 22 h under the same atmosphere. Forty to fifty COCs were cultured in each droplet.

Evaluation of live/dead status and nuclear maturation after IVM

At the end of IVM, COCs were transferred for 30 sec to 1 ml collection medium supplemented with 0.1% (w/v) hyaluronidase and gently pipetted to partially remove the cumulus cells. The oocytes were washed twice in a hyaluronidase-free collection medium and observed under a stereomicroscope. Oocytes with clear signs of membrane damage (brownish, faded cytoplasm) were removed, and only oocytes with a normal spherical shape, smooth surface and dark and evenly granulated cytoplasm were considered live oocytes. Live oocytes with a visible first polar body (1PB; the indicator of the metaphase II [MII] stage) were considered matured.

IVF and in vitro embryo culture

The IVF and *in vitro* embryo culture (IVC) procedures were performed by the method of Kikuchi *et al.* [11]. The medium used for IVF was a modified Pig-FM [12] medium containing 10 mM HEPES, 2 mM caffeine and 5 mg/ml BSA (Fraction V, Sigma). After being washed 3 times in IVF medium, oocytes were transferred into 90-μl IVF droplets (approximately 20 oocytes in each droplet) covered by paraffin oil (Paraffin Liquid; Nacalai Tesque). Frozen-thawed epididymal spermatozoa from a Landrace boar were preincubated at 37 C in medium 199 (with Earle's salts, pH adjusted to 7.8) for 15 min [13]. To obtain the final sperm concentration (1×10^5 cells/ml), 10 μl of the sperm suspension was introduced into the IVF medium containing oocytes and co-incubated for 3 h at 39 C under 5% CO_2, 5% O_2 and 90% N_2. The day of IVF was defined as day 0. After removal of spermatozoa from the surface of the zona pellucida by gentle pipetting with a fine glass pipette, IVC was performed in 500-μl drops of IVC-PyrLac on days 0 to 2 and of IVC-Glu on days 2 to 7 [11] in 4-well dishes in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39 C. The IVC-PyrLac was BM medium without HEPES supplementation. The IVC-Glu was the original NCSU-37 medium containing 5.55 mM glucose and supplemented with 4 mg/mL BSA and 50 μM β-mercaptoethanol.

Evaluation of fertilization

The fertilization status of oocytes was assessed 10 h after IVF. Oocytes were mounted on glass slides and fixed with acetic alcohol (acetic acid 1:ethanol 3) for at least 3 days, stained with 1% (w/v) orcein (Sigma) in acetic acid, rinsed in glycerol:acetic acid:water (1:1:3) and then examined under a phase-contrast microscope with × 40 or × 100 objectives. The presence and numbers of female and

male pronuclei and/or sperm heads and extrusion of the second polar body (PB) were investigated in the oocytes. Oocytes were considered penetrated when a sperm head or heads, male pronucleus with the corresponding sperm tail or both were detected in the cytoplasm. Oocytes were considered activated when a female pronucleus was detected in the cytoplasm. Oocytes with a female pronucleus without penetrating sperm were considered parthenogenetic. Normal fertilization was defined by the presence of 1 female pronucleus and 1 male pronucleus and the extrusion of both the 1st and the 2nd PBs. Oocytes with one penetrating sperm in the cytoplasm were defined as monospermic.

Evaluation of embryo development and blastocyst cell numbers

Cleavage rates on day 2, blastocyst rates on days 6 and 7 and total cell numbers in blastocysts on day 7 were recorded. On day 2, embryos with 2−4 blastomeres were considered cleaved [14]. On days 6 and 7, expanded embryos without a visible perivitelline space containing more than 10 blastomeres and a blastocoel were considered blastocysts. To assess total cell numbers in embryos, blastocysts were incubated overnight with 25 μg/ml Hoechst 33342 (H33342, Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol. After washing in 99.5% ethanol, they were mounted on glass slides in glycerol droplets, flattened by cover slips and examined under UV light with an excitation wavelength of 330–385 nm by using an epifluorescence microscope (IX-71, Olympus, Tokyo, Japan). A digital image of each embryo was taken, and the total numbers of nuclei labeled by H33342 were counted using the NIH ImageJ (v. 1.40) software [15].

Experimental design

Experiment 1: This experiment was performed to compare the feasibilities of EG and PG for oocyte vitrification. Immature COCs were vitrified using either EG (EG-vitrified group) or PG (PG-vitrified group) as the permeable CPA. After vitrification, microdrops were warmed without storing, and COCs were subjected to IVM. After 44 h of IVM, oocytes were denuded to assess their live/dead and maturation status. Live oocytes were then subjected to IVF and IVC. Survival and maturation rates (% live oocytes/total and % MII-stage oocytes/live oocytes, respectively) after IVM and cleavage, blastocyst development and blastocyst cell numbers were compared with those of COCs not subjected to vitrification (control). Four replications were performed.

Experiment 2: This experiment was performed to compare the toxic effects on survival, maturation, fertilization and developmental abilities of immature oocytes after treatment with EG or PG. Immature COCs were subjected to equilibration, vitrification and warming solutions according to the SSV vitrification protocol without cooling. The equilibration and vitrification solutions contained either EG (EG-treated group) or PG (PG-treated group) as the permeable CPA. After treatment, COCs were subjected to IVM. After 44 h of IVM, oocytes were denuded to assess membrane integrity and maturation. Live oocytes were then subjected to IVF and IVC. At 10 h after IVF in each group, 10−20 oocytes were randomly selected to analyze fertilization results. The remaining oocytes were subsequently cultured. Survival and maturation rates after IVM, fertilization rates after IVF and cleavage, blastocyst development and blastocyst cell numbers were compared with those of COCs that were not subjected to CPA treatment (control). The experiment was replicated 5 times.

Experiment 3: This experiment was performed to assess the toxicity and feasibility of the EG and PG combination for oocyte vitrification. Immature COCs were vitrified using a mixture of EG and PG as the permeable CPA during equilibration (2% EG [v/v] + 2% PG [v/v] and vitrification (17.5% EG [v/v] + 17.5% PG [v/v]). After treatment, COCs were subjected to IVM. After 44 h of IVM, oocytes were denuded to assess live/dead status and maturation. Live oocytes were then subjected to IVF and IVC. Fertilized oocytes were either fixed at 10 h after IVF to analyze fertilization results or subsequently cultured. Survival and maturation rates after IVM, fertilization rates after IVF and cleavage, blastocyst development and blastocyst cell numbers were compared with those of COCs subjected to CPA treatment without cooling (toxicity control) and those of COCs without CPA treatment or cooling (control). The experiment was replicated 9 times in total; 5 replications were performed to assess survival, maturation and embryo development, and the other 4 replications were performed to assess IVF results.

Statistical analysis

All data were expressed as mean ± SEM values. Percentage data were arcsine transformed before analysis. Data on survival, maturation, fertilization status, *in vitro* development and embryo cell numbers were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. All data were analyzed using the KyPlot package (Ver. 4.0, KyensLab, Tokyo, Japan); $P<0.05$ was defined as the significance level.

Results

Feasibility of 35% EG and 35% PG for oocyte vitrification

The proportion of surviving oocytes in the PG-vitrified group (73.9%) was significantly higher than that in the EG-vitrified group (27.8%); however, these values were significantly lower than that of the control group (95.2%) (Table 1). The percentages of live oocytes reaching the MII stage were similar among the treatment groups (ranging from 62.4% to 71.1%). After IVF of live oocytes, the cleavage rates in both the EG-vitrified and PG-vitrified groups (29.9%, and 32.1%, respectively) were similar to one another, but significantly lower than that in the control group (52.0%) (Table 2). Nevertheless, a significantly lower rate of cultured oocytes developed by day 7 to the blastocyst stage in the PG-vitrified group (2.0%) than in the EG-vitrified group (10.7%), and these values were both significantly lower than that of the control group (24.1%) (Table 2). Likewise, blastocyst formation rates calculated from the numbers of cleaved embryos and the original numbers of vitrified oocytes in the PG-vitrified group were lower than those in the EG-vitrified group. There was no significant difference in the total number of cells in day 7 blastocysts among the control, EG-vitrified and PG-vitrified groups (48.7 ± 1.5, 50.5 ± 3.8 and 47.7 ± 5.9, respectively) (Table 2).

Toxicity of 35% EG- or 35% PG-based CPA solution treatment

After CPA treatment and IVM, the proportions of live oocytes in the control and PG-treated groups were similar to one another and were significantly higher than that in the EG-treated group (97.5%

Table 1. Survival and maturation of immature porcine oocytes after vitrification in different permeable cryoprotectants

Treatment groups	Total no. of oocytes examined	No. of live oocytes (% total)	No. of oocytes matured to MII (% live oocytes)
Control	160	152 (95.2 ± 2.2)[a]	71 (64.0 ± 3.2)
EG-vitrified	310	87 (27.8 ± 3.0)[c]	53 (62.4 ± 3.9)
PG-vitrified	365	197 (73.9 ± 3.8)[b]	106 (71.1 ± 4.7)

Four replicates were performed. Percentage data are presented as mean ± SEM values. [a,b,c] Percentages with different letters in the same column differ significantly ($P<0.05$). MII = metaphase II, EG = ethylene glycol, PG = propylene glycol.

Table 2. *In vitro* embryo development of live vitrified oocytes following IVM and IVF

Treatment groups	Total cultured	Cleaved embryos (%)	Blastocysts (day 6) (% cultured)	Blastocysts (day 7) (% cultured)	Blastocysts (day 7) (% cleaved)	Blastocysts (day 7) (% oocytes examined*)	Total cells in blastocysts
Control	152	80 (52.0 ± 2.6)[a]	31 (20.2 ± 4.6)[a]	(24.1 ± 3.8)[a]	37 (46.6 ± 7.9)[a]	(23.1 ± 4.0)[a]	48.7 ± 1.5
EG-vitrified	87	25 (29.9 ± 5.2)[b]	6 (7.0 ± 1.0)[b]	(10.7 ± 1.2)[b]	9 (37.5 ± 4.1)[a]	(2.8 ± 0.2)[b]	50.5 ± 3.8
PG-vitrified	197	64 (32.1 ± 3.5)[b]	2 (1.0 ± 0.6)[c]	(2.0 ± 0.2)[c]	4 (6.7± 1.0)[b]	(1.5 ± 0.09)[c]	47.7 ± 5.9

Four replications were performed. Data are presented as mean ± SEM values. [a,b,c] Percentages with different letters in the same column differ significantly ($P<0.05$) (one-way ANOVA). *Numbers of examined oocytes are shown in Table 1.

Table 3. Survival and maturation of immature porcine oocytes after treatment with different permeable cryoprotectants

Treatment groups	Total no. of oocytes examined	No. of live oocytes (% total)	No. of oocytes matured to MII (% live oocytes)
Control	302	293 (97.5 ±1.6)[a]	230 (78.2 ± 4.8)
EG-treated	389	302 (78.7 ± 2.5)[b]	238 (78.6 ± 3.3)
PG-treated	326	308 (94.7 ± 2.6)[a]	213 (68.0 ± 3.8)

Five replicates were performed. Percentage data are presented as mean ± SEM values. [a,b] Percentages with different letters in the same column differ significantly ($P<0.05$). MII = metaphase II, EG = ethylene glycol, PG = propylene glycol.

and 94.7% *vs.* 78.7%, respectively) (Table 3). The percentages of live oocytes reaching the MII stage were similar among the treatment groups (ranging from 68.0% to 78.6%). After IVF of live oocytes, the cleavage rates were not statistically different among groups (ranging from 29.6% to 41.6%); however, blastocyst formation rates for cultured embryos appeared to be significantly lower in the PG-treated group compared with the EG-treated group and the control on day 6 (3.3% *vs.* 13.4% and 10.6%, respectively) and on day 7 (4.1% *vs.* 18.4% and 15.2%, respectively) (Table 4). Likewise, the blastocyst formation rate calculated from the number of cleaved embryos in the PG-treated group (16.3%) was lower than those in the EG-treated group and the control (47.8% and 36.4%, respectively). There was no significant difference in any aspects of embryo development between the control and EG-treated groups. There was no significant difference in the total number of cells in day 7 blastocysts among the control, EG-treated and PG-treated groups (53.9 ± 6.1, 58.4 ± 5.6 and 41.1 ± 5.3, respectively) (Table 4). Additionally, there was no significant difference among groups in the fertilization status of oocytes after IVF (Table 5).

The feasibility of the EG–PG combination for oocyte vitrification

The proportions of live oocytes in the control (96.3%) and toxicity control (97.3%) groups were similar and significantly higher than that

Table 4. *In vitro* embryo development of live CPA-treated oocytes following IVM and IVF

Treatment groups	Total cultured	Cleaved embryos (%)	Blastocysts (day 6)	Blastocysts (day 7)			Total cells in blastocysts
			(% cultured)	(% cultured)	(% cleaved)	(% oocytes examined*)	
Control	222	94	24		34		53.9 ± 6.1
		(41.6 ± 4.2)	(10.6 ± 2.9)[a]	(15.2 ± 3.4)[a]	(36.4± 6.8)[a]	(11.8 ± 3.0) [a]	
EG-treated	229	86	29		40		58.4 ± 5.6
		(37.9 ± 3.7)	(13.4 ± 2.1)[a]	(18.4 ± 3.5)[a]	(47.8± 7.5)[a]	(11.6 ± 2.9) [a]	
PG-treated	224	67	7		9		41.1 ± 5.3
		(29.6 ± 4.1)	(3.3 ± 1.1)[b]	(4.1 ± 1.0)[b]	(16.3± 6.6)[b]	(2.9 ± 0.7) [b]	

Five replications were performed. Data are presented as mean ± SEM values. [a,b] Percentages with different letters in the same column differ significantly (P<0.05) (One-way ANOVA). *Numbers of examined oocytes are shown in Table 3.

Table 5. Fertilization status of live CPA-treated oocytes following IVM and IVF

Treatment groups	No. of oocytes						
	Total	Activated (% total)	Penetrated (% total)	Parthenogenetic (% total)	Normal (% total)	MPN (% penetrated)	Monospermy (% penetrated)
Control	60	15 (25.1 ± 7.2)	12 (20.4 ± 4.8)	3 (4.6 ± 3.1)	10 (16.7 ± 4.3)	11 (87.5 ± 12.5)	11 (95.0 ± 5.0)
EG-treated	60	15 (24.7 ± 3.9)	16 (26.3 ± 3.6)	0 (0 ± 0)	10 (16.3 ± 2.7)	15 (93.7 ± 6.2)	13 (77.5 ± 10.3)
PG-treated	60	13 (20.7 ± 3.6)	9 (15.3 ± 3.6)	5 (7.0 ± 4.7)	6 (10.5 ± 2.3)	8 (91.6 ± 8.3)	9 (100 ± 0)

Four replicates were performed. Percentage data are presented as means ± SEM. Percentage data are presented as mean values. No significant differences were detected among treatment groups (P<0.05). MPN = male pronucleus.

Table 6. Survival and maturation of immature porcine oocytes after cryoprotectant treatment or vitrification using a combination of EG and PG as permeable cryoprotectants

Treatment groups	Total no. of oocytes examined	No. of live oocytes (% total)	No. of oocytes matured to MII (% live oocytes)
Control	245	236 (96.3 ± 1.8)[a]	166 (69.9 ± 2.9)
Toxicity control	245	238 (97.3 ± 0.8)[a]	170 (70.8 ± 3.8)
Vitrified	397	173 (42.6 ± 5.3)[b]	119 (66.4 ± 4.4)

Five replicates were performed. Percentage data are presented as mean ± SEM values. Toxicity control was carried out by subjecting COCs to CPA treatment without cooling. [a,b] Percentages with different letters in the same column differ significantly (P<0.05). MII = metaphase II, EG = ethylene glycol, PG = propylene glycol, COCs = cumulus-oocyte complexes, CPA = cryoprotective agent.

in the vitrified group (42.6%) (Table 6). After IVF of live oocytes, the cleavage rates did not differ statistically among the groups (ranging from 35.9% to 44.6%) (Table 7). The rate of blastocyst development from cultured oocytes in the vitrified group was significantly lower than those in the control and toxicity control groups on day 6 (6.9% *vs.* 15.1% and 17.2%, respectively) and day 7 (10.7% *vs.* 18.1% and 23.3%, respectively). Blastocyst formation rates calculated from cleaved embryos and the original numbers of oocytes in the vitrified group (26.1% and 4.7%, respectively) were significantly lower than those in the control (52.0% and 17.4%, respectively) and toxicity control (51.6% and 22.8%, respectively) groups. There was no significant difference in any aspects of embryo development between the control and toxicity control groups. There was no significant difference in the total number of cells in day 7 blastocysts among the control, toxicity control and vitrified groups (59.3 ± 6.5, 53.3 ± 8.2 and 61.6 ± 6.7, respectively) (Table 7). Moreover, there was no significant difference in the fertilization status of oocytes after IVF among groups (Table 8).

Discussion

In the present study, we compared the feasibility of EG and PG for vitrification of immature porcine oocytes for the first time and improved the efficacy of our vitrification protocol by combining EG and PG as a permeable CPA. This approach resulted in a 42.6% survival rate without affecting meiotic competence and with only a slight reduction in blastocyst development (10.7%). Furthermore, the quality, as measured by the total cell numbers of blastocysts produced from vitrified immature oocytes, was similar to that of non-vitrified control oocytes. To our knowledge, this demonstrates

Table 7. *In vitro* embryo development after IVM and IVF of live immature oocytes treated or vitrified with a combination of EG and PG as permeable cryoprotectants

Treatment groups	Total cultured	Cleaved embryos (%)	Blastocysts (day 6)	Blastocysts (day 7)			Total cells in blastocysts
			(% cultured)	(% cultured)	(% cleaved)	(% oocytes examined)*	
Control	236	88	37		44		59.3 ± 6.5
		(35.9 ± 6.4)	(15.1 ± 2.5)[a]	(18.1 ± 2.5)[a]	(52.0 ± 7.2)[a]	(17.4 ± 2.2)[a]	
Toxicity control	237	107	43		56		53.3 ± 8.2
		(44.6 ± 3.0)	(17.2 ± 2.8)[a]	(23.3 ± 2.7)[a]	(51.6 ± 4.1)[a]	(22.8 ± 2.6)[a]	
Vitrified	173	70	12		20		61.6 ± 6.7
		(41.2 ± 2.0)	(6.9 ± 1.3)[b]	(10.7 ± 2.3)[b]	(26.1 ± 5.7)[b]	(4.7 ± 1.2)[b]	

Five replications were performed. Data are presented as mean ± SEM values. *Numbers of examined oocytes are shown in Table 6. Toxicity control was carried out by subjecting COCs to CPA treatment without cooling. [a,b,c] Percentages with different letters in the same column differ significantly ($P<0.05$) (One-way ANOVA). EG = ethylene glycol, PG = propylene glycol, COCs = cumulus-oocyte complexes, CPA = cryoprotective agent.

Table 8. Fertilization status after IVM and IVF of live immature oocytes treated or vitrified with a combination of EG and PG as permeable cryoprotectants

Treatment groups	No. of oocytes						
	Total	Activated (% total)	Penetrated (% total)	Parthenogenetic (% total)	Normal (% total)	MPN (% penetrated)	Monospermy (% penetrated)
Control	94	26 (31.2 ± 6.8)	22 (25.6 ± 6.6)	4 (5.6 ± 1.6)	12 (15.0 ± 2.3)	22 (100 ± 0)	13 (69.1 ± 12.0)
Toxicity control	95	21 (25.9 ± 7.6)	18 (22.2 ± 5.4)	3 (3.7 ± 2.3)	10 (13.6 ± 3.2)	18 (100 ± 0)	10 (53.3 ± 3.3)
Vitrified	76	15 (20.3 ± 2.2)	16(21.6 ± 3.2)	0 (0 ± 0)	68 (12.0 ± 2.7)	15 (94.4 ± 5.5)	9 (62.5 ± 12.5)

Four replicates were performed. Percentage data are presented as means ± SEM. Toxicity control was carried out by subjecting COCs to CPA treatment without cooling. No significant differences were detected among treatment groups ($P<0.05$). MPN = male pronucleus, EG = ethylene glycol, PG = propylene glycol, COCs = cumulus-oocyte complexes, CPA = cryoprotective agent.

the highest efficacy in *in vitro* embryo production obtained by IVF of vitrified porcine oocytes reported to date.

In *Experiment 1*, replacing EG with the highly permeable PG dramatically improved the survival rates of oocytes (from 27.8% to 73.9%) without affecting their meiotic competence, suggesting that the permeation speed of a CPA basically determines survival outcomes in immature porcine oocytes. On the other hand, blastocyst development after IVF of oocytes vitrified in the PG-based medium was significantly lower than that of oocytes vitrified in EG. Accordingly, the overall efficacy of blastocyst production (calculated from the original number of oocytes subjected to vitrification) was significantly higher when EG was used as a CPA (2.8%) as compared with that obtained using PG (1.5%). The developmental competence of oocytes preserved in PG was severely reduced; most notably, the blastocyst development of cleaved embryos was significantly reduced compared with oocytes in the non-vitrified control and those vitrified in EG. These results suggest a specific toxic effect of PG on oocytes that causes subsequent detrimental effects during embryo development.

Based on the results of *Experiment 1*, *Experiment 2* was designed to compare the toxicity of EG and PG. Exposure of immature oocytes to EG containing equilibration and vitrification media followed by the rehydration procedure caused a smooth but significant (21.3%) reduction in the rate of live oocytes; however, the ability of oocytes to mature and subsequently develop remained similar to that of the control oocytes. These results are in accordance with our previous results [4]. On the other hand, when oocytes were exposed to the medium containing PG, the maturation and development rates of live oocytes did not decrease; however, embryo development after IVF was impaired compared with those in the control and EG-treated oocytes. In addition, fertilization results did not differ between the PG-treated and control oocytes. Thus, we assume that before the cooling procedure, treatment with a PG containing vitrification solution has already exerted a detrimental effect on immature oocytes, which manifests after fertilization during embryo development. These results are in accordance with previous reports describing the lower toxicity of EG compared with PG in matured porcine and murine oocytes [16, 17]. Therefore, in terms of toxicity, 35% (v/v) EG appears to be more feasible than 35% (v/v) PG for the cryopreservation of immature porcine oocytes. Nevertheless, membrane damage induced by the EG treatment in more than 20% of oocytes suggests that 35% EG itself is not an appropriate solution either. The most plausible cause of such CPA-induced membrane damage is excessive osmotic stress in the oocyte membrane. In the present study, the osmolarity of vitrification solutions containing 35% EG was 5.47 Osm/l which was significantly higher than that that of the vitrification solution containing 35% PG (4.10 Osm/l). Again, these results suggest that inefficient permeation of 35% EG in immature oocytes can cause high osmotic stress and membrane damage in oocytes even without cooling.

Previous reports suggested that the combination of different permeable cryoprotectants may be a possible means of improving oocyte cryopreservation protocols by reducing their specific toxicity in mice [17, 18]. Indeed, in several previous studies, combinations of permeable CPAs were successfully used to reduce the toxicity

of the individual agents during cryopreservation of embryos and oocytes in several mammalian species including the mouse, cattle, rabbit, human and buffalo [19–25]. In *Experiment 3* of the present study, the combination of 17.5% EG and 17.5% PG (in total, 35% [v/v] CPA) for vitrification resulted in 42.6% survival of immature pig oocytes, which was significantly higher than that observed with 35% EG (27.8%) but lower than that obtained with 35% PG (73.9%) in *Experiment 1*. Nevertheless, the similar viability, fertilization and embryo development between the control and toxicity control groups revealed that equilibration and vitrification solutions employing a combination of EG and PG did not exert a toxic effect on oocytes, which was otherwise observed with 35% PG in *Experiment 2*. Furthermore, the vitrification solution containing a mixture of EG and PG (4.97 Osm/l) did not cause membrane damage in oocytes, indicating that the osmolarity of this solution is better suited to the vitrification of immature porcine oocytes compared with 35% EG. Thus the combination of EG and PG allowed more than 10% of vitrified immature oocytes to develop to the blastocyst stage after IVF, and their quality (as measured by total cell numbers) was similar to that of the control blastocysts. Furthermore, the overall efficacy for blastocyst production calculated from the original number of oocytes subjected to vitrification was 4.7%, which is significantly higher than that obtained by the use of only EG or PG in *Experiment 1* (2.8% and 1.5%, respectively). Nevertheless, the developmental competence of oocytes vitrified in the mixture of EG and PG was still significantly reduced compared with that of the non-vitrified control and toxicity control groups, which indicates the need for further improvement of our vitrification protocol.

In conclusion, the present study revealed that replacing EG with the more permeable PG significantly improved the survival of vitrified immature porcine oocytes; however, PG exerted a severe toxic effect on oocytes, reducing blastocyst development after IVF. On the other hand, combining EG with PG resulted in improved oocyte survival compared with EG only and had no toxic effects on embryo development. This approach resulted in improved blastocyst yield after IVF of vitrified immature oocytes.

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References

1. **Ledda S, Bogliolo L, Succu S, Ariu F, Bebbere D, Leoni GG, Naitana S.** Oocyte cryopreservation: oocyte assessment and strategies for improving survival. *Reprod Fertil Dev* 2007; **19**: 13–23. [Medline]
2. **Zhou GB, Li N.** Cryopreservation of porcine oocytes: recent advances. *Mol Hum Reprod* 2009; **15**: 279–285. [Medline]
3. **Somfai T, Kikuchi K, Nagai T.** Factors affecting cryopreservation of porcine oocytes. *J Reprod Dev* 2012; **58**: 17–24. [Medline]
4. **Somfai T, Noguchi J, Kaneko H, Nakai M, Ozawa M, Kashiwazaki N, Egerszegi I, Rátky J, Nagai T, Kikuchi K.** Production of good-quality porcine blastocysts by *in vitro* fertilization of follicular oocytes vitrified at the germinal vesicle stage. *Theriogenology* 2010; **73**: 147–156. [Medline]
5. **Le Gal F, Gasqui P, Renard JP.** Differential osmotic behavior of mammalian oocytes before and after maturation: a quantitative analysis using goat oocytes as a model. *Cryobiology* 1994; **31**: 154–170. [Medline]
6. **Agca Y, Liu J, Peter AT, Critser ES, Critser JK.** Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability characteristics. *Mol Reprod Dev* 1998; **49**: 408–415. [Medline]
7. **Pedro PB, Yokoyama E, Zhu SE, Yoshida N, Valdez DM Jr, Tanaka M, Edashige K, Kasai M.** Permeability of mouse oocytes and embryos at various developmental stages to five cryoprotectants. *J Reprod Dev* 2005; **51**: 235–246. [Medline]
8. **Gupta MK, Uhm SJ, Lee HT.** Cryopreservation of immature and *in vitro* matured porcine oocytes by solid surface vitrification. *Theriogenology* 2007; **67**: 238–248. [Medline]
9. **Dinnyés A, Dai Y, Jiang S, Yang X.** High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. *Biol Reprod* 2000; **63**: 513–518. [Medline]
10. **Petters RM, Wells KD.** Culture of pig embryos. *J Reprod Fertil Suppl* 1993; **48**: 61–73. [Medline]
11. **Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T.** Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. *Biol Reprod* 2002; **66**: 1033–1041. [Medline]
12. **Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodriguez-Martinez H.** Capacitation status and *in vitro* fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int J Androl* 2002; **25**: 84–93. [Medline]
13. **Kikuchi K, Nagai T, Ikeda H, Noguchi J, Shimada A, Soloy E, Kaneko H.** Cryopreservation and ensuing *in vitro* fertilization ability of boar spermatozoa from epididymides stored at 4°C. *Theriogenology* 1998; **50**: 615–623. [Medline]
14. **Dang-Nguyen TQ, Kikuchi K, Somfai T, Ozawa M, Nakai M, Maedomari N, Viet-Linh N, Kanai Y, Nguyen BX, Nagai T.** Evaluation of developmental competence of *in vitro*-produced porcine embryos based on the timing, pattern and evenness of the first cleavage and onset of the second cleavage. *J Reprod Dev* 2010; **56**: 593–600. [Medline]
15. **Abramoff MD, Magelhaes PJ, Ram SJ.** Image processing with ImageJ. *Biophoton Int* 2004; **11**: 36–42.
16. **Taniguchi M, Arikawa R, Kaedei Y, Tanihara F, Namula Z, Viet VL, Sato Y, Otoi T.** Effects of cryoprotectant agents and equilibration methods on developmental competence of porcine oocytes. *Cryo Letters* 2011; **32**: 410–414. [Medline]
17. **Szurek EA, Eroglu A.** Comparison and avoidance of toxicity of penetrating cryoprotectants. *PLoS One* 2011; **6**: e27604. [Medline]
18. **Fahy GM, Wowk B, Wu J, Paynter S.** Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology* 2004; **48**: 22–35. [Medline]
19. **Ishimori H, Takahashi Y, Kanagawa H.** Viability of vitrified mouse embryos using various cryoprotectant mixture. *Theriogenology* 1992; **37**: 481–487.
20. **Ishimori H, Saeki K, Inai M, Nagao Y, Itasaka J, Miki Y, Seike N, Kainuma H.** Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. *Theriogenology* 1993; **40**: 427–433. [Medline]
21. **Vicente JS, Garcia-Ximenez F.** Osmotic and cryoprotective effects of a mixture of DMSO and ethylene glycol on rabbit morulae. *Theriogenology* 1994; **42**: 1205–1215. [Medline]
22. **Cai XY, Chen GA, Lian Y, Zheng XY, Peng HM.** Cryoloop vitrification of rabbit oocytes. *Hum Reprod* 2005; **20**: 1969–1974. [Medline]
23. **Selman H, Angelini A, Barnocchi N, Brusco GF, Pacchiarotti A, Aragona C.** Ongoing pregnancies after vitrification of human oocytes using a combined solution of ethylene glycol and dimethyl sulfoxide. *Fertil Steril* 2006; **86**: 997–1000. [Medline]
24. **Mahmoud KG, Scholkamy TH, Ahmed YF, Seidel GE Jr, Nawito MF.** Effect of different combinations of cryoprotectants on *in vitro* maturation of immature buffalo (*Bubalus bubalis*) oocytes vitrified by straw and open-pulled straw methods. *Reprod Domest Anim* 2010; **45**: 565–571. [Medline]
25. **Liang YY, Srirattana K, Phermthai T, Somfai T, Nagai T, Parnpai R.** Effects of vitrification cryoprotectant treatment and cooling method on the viability and development of buffalo oocytes after intracytoplasmic sperm injection. *Cryobiology* 2012; **65**: 151–156. [Medline]