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Meiotic Competence and DNA Damage of Porcine Immature Oocytes Following Cryoprotectant Exposure and Vitrification

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ABSTRACT

Effective methodologies for oocyte cryopreservation are currently lacking. Therefore this study aimed to examine the meiotic competence and DNA damage of immature porcine oocytes following cryoprotectant exposure and vitrification. In the first experiment, porcine oocytes were exposed to 4 cryoprotectants for 1 min before *in vitro* Maturation (IVM). The cryopreservatives used were: (1) 40% ethylene glycol [40% EG], (2) 20% EG and 20% glycerol [20% EG/GLY]; (3) 20% EG and 20% propylene glycol (PG) [20% EG/PG]; (4) 20% EG and 20% dimethyl sulphoxide [20% EG/DMSO]) In the second experiment, the porcine oocytes were vitrified with 40% EG, 20% EG/GLY, 20% EG/PG or 20% EG/DMSO before IVM. After cryoprotectant exposure or vitrification, the oocytes were cultured in the maturation medium for 44 h. As a control, oocytes were cultured for 44 h without cryoprotectant exposure and vitrification. In the third experiment, immature oocytes were incubated with 7.5 μg mL^{-1} cytochalasin B for 38.5°C for 30 min, followed by vitrification and culture for 44 h. The meiotic status and DNA fragmentation of the oocytes were examined after IVM. As a cryoprotectant, 20% EG/GLY was superior to 20% EG/PG and 20% EG/DMSO, with respect to the meiotic competence and DNA fragmentation of oocytes. The percentage of DNA fragmentation was significantly lower in oocytes with cryoprotectant exposure and vitrification than in control fresh oocytes, irrespective of the treatment of cytochalasin B. These results indicate that the meiotic competence of porcine oocytes is damaged by cryoprotectant exposure and the competence of vitrified oocytes is not improved by pre-treatment of oocytes with cytochalasin B before vitrification.

Key words: Meiotic competence, DNA damage, vitrification

INTRODUCTION

Mammalian oocytes are one of the most difficult cell types to cryopreserve due to their susceptibility to damage from cooling, freezing and thawing. In particular, oocytes in the Germinal Vesicle (GV) stage have been found to be more susceptible to damage than other nuclear stages. As a result, reproductive cryobiologists face numerous challenges in trying to develop effective cryopreservation methods for both human and animal oocytes. Until recently, attention has been focused on the cryopreservation of mature oocytes. However, at this stage, microtubular spindles are highly sensitive to cooling and cryoprotectant agents fail to prevent chromosomal abnormalities

(Sathananthan *et al*., 1988; Aman and Parks, 1994). When immature oocytes are stored directly after being collected, they are matured and fertilized only when necessary. Oocytes such as these are therefore less susceptible to microtubular and chromosomal destruction (Massip, 2003). However, Cumulus Oocyte Complexes (COCs) have a complex structure and their integrity must be preserved to enable oocyte progression to metaphase II (MII). Treatment of porcine oocytes with cytochalasin B (CB) has been shown to prevent chromosomal damage; however, similar CB concentrations and treatment times may not be applicable to human oocytes (Fujihira *et al*., 2004). To promote advances in cryopreservation technology, the negative effects of this form of cryopreservation on the integrity of several of the unique features of oocytes need to be resolved. Any alterations to these features will have repercussions on normal fertilization, such as premature cortical exocytosis, increased parthenogenetic activation and damage to the cytoskeletal elements of the oocyte, particularly disruption of the meiotic spindle (Dobrinsky *et al*., 2000). CB has been used to reduce injury to oocytes and embryos during vitrification, because it is a cytoskeletal relaxant that is thought to reduce the rigidity and increase the elasticity of the cytoskeletal elements (Isachenko *et al*., 1998; Dobrinsky *et al*., 2000). Cells normally degenerate by 1 of the 2 mechanisms: apoptosis or necrosis. Apoptosis is an endogenous cell degeneration mechanism that is necessary for normal development and tissue homeostasis (Wyllie *et al*., 1980). Previous study has suggested that cryoprotectant and vitrification cause depolymerization and disorganization of the spindle microtubules in porcine oocytes, which in turn results in DNA fragmentation (Isachenko *et al*., 1998). Therefore, the present study aimed to examine the meiotic competence and DNA damage of immature porcine oocytes following cryoprotectant exposure and vitrification.

MATERIAL AND METHODS

Oocyte collection: Ovaries from pre-pubertal crossbred gilts were collected at a local abattoir and transported to the laboratory in physiological saline (0.9% (w/v) NaCl) at 35°C. COCs from follicles (3-6 mm in diameter) were aspirated using an 18-guage needle attached to a 5 mL syringe.

Vitrification and maturation: The holding medium used for handling oocytes during vitrification was Phosphate Buffered Saline (PBS) supplemented with 0.3% Bovine Serum Albumin (BSA) and 50 μg mL^{-1} gentamicin (Sigma, St. Louis, MO, USA). All vitrification solutions were prepared using this holding medium. Oocytes were manipulated and vitrified at room temperature (26°C). In experiment 1, oocytes groups were directly exposed to 4 cryoprotectants (40% ethylene glycol [40% EG], 20% EG and 20% glycerol [20% EG/GLY], 20% EG and 20% propylene glycol [20% EG/PG] and 20% EG and 20% dimethyl sulphoxide [20% EG/DMSO]) for 1 min and then transferred to 0.5 M sucrose in holding medium for 5 min before they were transferred to holding medium for 2 min and *In vitro* Maturation (IVM) medium. In the second experiment, the porcine oocytes were vitrified with 40% EG, 20% EG/GLY, 20% EG/PG or 20% EG/DMSO before IVM. The oocytes were directly exposed to the small drops (40 μL) of each cryoprotectant and loaded into 0.25 mL straws in the middle column of the vitrifying solution separated by air bubbles, with approximately 40 μL of the same medium on each side. Five oocytes were loaded in each straw and immediately but slowly (to avoid cracking) dipped vertically into liquid nitrogen. All the procedures from the exposure of COCs to the final vitrification solution to dipping of the loaded straws into liquid nitrogen were performed within 1 min. Each straw was stored in liquid nitrogen for a week. After warming the straws at 37°C, rehydration was conducted by subsequent washes for 5 min with 0.5 M sucrose in holding medium. After equilibration in the sucrose solution, they were

transferred into maturation medium, consisting of a modified North Carolina State University (NCSU)-37 solution supplemented with 0.6 mM cysteine, 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP; Sigma), 10 IU mL^{-1} equine chorionic gonadotropin (eCG; Teikokuzoki, Tokyo, Japan), 10 IU mL^{-1} human chorionic gonadotropin (hCG; Teikokuzoki), 50 μg mL^{-1} gentamicin (Sigma) and 10% (v/v) porcine follicular fluid. Approximately 50 COCs were cultured in each 500 μL of the maturation medium covered with a layer of mineral oil (Sigma) in a 4-well multi-dish (Nunc A/S, Roskilde, Denmark) for 22 h. Oocytes were then transferred to the maturation medium without hormones or dbcAMP and cultured for an additional 22 h. All cultures were performed at 38.5°C in a humidified incubator containing 5% CO_2. As a control, oocytes were cultured for 44 h without the cryoprotectant exposure or vitrification. In the third experiment, the immature oocytes were incubated with 7.5 μg mL^{-1} cytochalasin B at 38.5°C for 30 min and treated with the same vitrification method as in experiment 2.

Detection of DNA fragmentation in porcine oocytes: After IVM culture the oocytes were denuded of cumulus cells and analysed for DNA damage using a combined technique for simultaneous nuclear staining and the terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL) by a modification of the procedures previously described by Otoi *et al.* (1999). Briefly, the denuded oocytes were washed four times in PBS containing 3 mg mL^{-1} polyvinylalcohol (PBS-PVA) and fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the oocytes were washed four times in PBS-PVA, permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 1 h and incubated in a PBS containing 10 mg mL^{-1} BSA (blocking solution) overnight at 4°C. They were then washed four times in PBS-PVA and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for 1 h at 38.5°C under 5% CO_2 in air. Positive controls (one or two oocytes per TUNEL analysis) were incubated in 1000 IU mL^{-1} deoxyribonuclease I (DNase; Sigma) for 30 min at 38.5°C under 5% CO_2 in air and washed twice in PBS-PVA before TUNEL staining. Negative controls (one or two oocytes per TUNEL analysis) were incubated in fluorescein-dUTP in the absence of TdT. After TUNEL staining, the oocytes were washed three times in PBS-PVA and counterstained with 25 μg mL^{-1} bis-benzimide (Hoechst 33342, Sigma) for 30 min. They were then washed in blocking solution, treated with an anti-bleaching solution (Slow-Fade; Molecular Probes, Eugene, OR, USA), mounted on a glass slide and sealed with clear nail polish. Labeled oocytes were examined using a Nikon Diaphot microscope fitted with epifluorescent illumination. Two standard filter sets were used, a filter with an excitation wave length of 450-490 nm and barrier filter of 520 nm was used to detect fluorescein isothiocyanate (FITC) alone. A filter with an excitation wavelength of 330-380 nm and barrier filter of 420 nm was used to detect the nuclear status of oocytes stained by Hoechst 33342. To assess DNA damage of oocytes from ovaries stored at each time and temperature, the number of nuclei labeled by TUNEL were counted.

Statistical analysis: Data are expressed as Mean±SEMs. The percentages of oocytes reaching each stage of meiosis and oocytes with DNA fragmented nucleus were subjected to arc sin transformation before Analysis of Variance (ANOVA). The transformed data were tested by ANOVA followed by a *post hoc*, Fisher's protected least significant difference test (PLSD test) using the Statview program (Abacus Concepts, Inc, Berkeley, CA). Differences at a probability value (p) of 0.05 or less were considered to be significant.

RESULTS AND DISCUSSION

As shown in Table 1 and 2, the percentage of fresh oocytes reached MII was significantly higher than that of oocytes exposed cryoprotectants and vitrified oocytes. In both exposure and vitrification experiments, moreover, the percentages of oocytes with DNA fragmentation were significantly lower in the fresh group than in the cryoprotectant treatment and vitrification groups. In the exposure experiment, the percentages of oocytes reached MII were significantly higher in 20% EG/GLY than in 20% EG/PG and 20% EG/DMSO. Moreover, the percentages of DNA fragmentation in both exposed and vitrified oocytes were significantly lower in 20% EG/GLY than in 20% EG/PG and 20% EG/DMSO.

As shown in Table 3, the treatment incubation with 7.5 μg mL^{-1} CB before vitrification did not have beneficial effects on the meiotic competence and DNA fragmentation of vitrified-warmed oocytes after IVM culture. However, the percentages of oocytes with DNA fragmentation were significantly lower in the 20% EG/GLY group than in the 20% EG/DMSO group. The present study aimed to examine the meiotic competence and DNA damage of immature porcine oocytes following cryoprotectant exposure and vitrification. The results show that the meiotic competence of oocytes exposed to cryoprotectants and vitrified oocytes was significantly lower than that of fresh oocytes. It has been shown that cryoprotectants disrupt the cortical microfilament network and cause depolymerization and disorganization of the spindle microtubules, which in turn result in chromosomal scattering (Isachenko *et al*., 1998). The results also show that DNA fragmentation of

Table 1: Effects of exposure of cryoprotectants to porcine immature oocytes on meiotic competence and DNA damage of oocytes*

		GVBD oocytes		Total of MII oocytes		Oocytes with DNA-fragmented nucleus	
Cryoprotectants	No. of oocytes examined	No.	%	No.	%	No.	%
Control**	96	80	84.0±3.1[a]	65	68.0±2.6[a]	6	6.5±2.3[a]
40% EG	91	58	64.1±1.9[b]	25	27.8±2.2[b,c]	32	35.1±1.9[b,c]
20% EG/GLY	93	60	64.2±3.5[b]	29	31.2±2.5[b]	28	30.1±2.0[b]
20% EG/PG	94	55	58.7±2.9[b]	20	21.6±1.8[c,d]	38	40.3±2.0[c,d]
20% EG/DMSO	93	55	58.9±4.7[b]	17	17.9±2.7[d]	40	43.1±3.0[d]

*Five replicated trials were carried out. Percentages are presented as Mean±SEM, GVBD: Germinal vesicle breakdown, MII: Metaphase II, EG: Ethylene glycol, GLY: Glycerol, PG: Propylene glycol, DMSO: Dimethyl sulphoxide, **Control oocytes were cultured without cryoprotectant exposure, [a-d]Values with different superscripts in the same column are significantly different at $p<0.05$

Table 2: Meiotic competence and DNA damage of porcine immature oocytes vitrified with various cryoprotectants*

		GVBD oocytes		Total of MII oocytes		Oocytes with DNA-fragmented nucleus	
Cryoprotectants	No. of oocytes examined	No.	%	No.	%	No.	%
Control**	137	117	85.5±2.0[a]	94	68.8±1.8[a]	8	5.6±1.7[a]
40% EG	111	36	33.0±3.8[b]	8	7.2±2.2[b]	51	45.6±3.7[b]
20% EG/GLY	114	36	31.8±2.9[b]	10	9.3±2.4[b]	47	41.4±2.7[b]
20% EG/PG	113	26	23.4±3.9[b]	5	4.4±1.7[b]	65	57.4±3.9[c]
20% EG/DMSO	116	28	24.0±2.5[b]	8	4.7±1.9[b]	63	54.8±2.8[c]

*Seven replicated trials were carried out. Percentages are presented as Mean±SEM, GVBD, Germinal vesicle breakdown, MII: Metaphase II, EG: Ethylene glycol, GLY: Glycerol, PG: Propylene glycol, DMSO: Dimethyl sulphoxide, **Fresh oocytes were cultured as a control, [a-c]Values with different superscripts in the same column are significantly different at $p<0.05$

Table 3: Meiotic competence and DNA damage of porcine immature oocytes vitrified with various cryoprotectants after cytochalasin B treatment*

		GVBD oocytes		Total of MII oocytes		Oocytes with DNA-fragmented nucleus	
Cryoprotectants	No. of oocytes examined	No.	%	No.	%	No.	%
Control**	92	83	90.3±2.5[a]	67	73.2±2.5[a]	5	5.5±1.8[a]
40% EG	83	31	37.4±3.2[b]	8	9.8±1.7[b]	30	36.4±5.2[b,c]
20% EG/GLY	85	31	36.2±3.3[b]	8	9.8±2.7[b]	31	36.3±2.5[b]
20% EG/PG	87	28	32.1±3.1[b]	6	6.8±1.9[b]	37	42.5±5.0[b,c]
20% EG/DMSO	86	26	30.6±2.9[b]	6	7.3±2.6[b]	41	47.9±3.6[c]

*Immature oocytes were vitrified after incubation with 7.5 μg mL^{-1} cytochalasin B for 30 min. Five replicated trials were carried out. Percentages are presented as Mean±SEM, GVBD: Germinal vesicle breakdown, MII: Metaphase II, EG: Ethylene glycol, GLY: Glycerol, PG: Propylene glycol, DMSO: Dimethyl sulphoxide. **Fresh oocytes were cultured as a control, [a-d]Values with different superscripts in the same column are significantly different at $p<0.05$

oocytes increases following cryoprotectant exposure when compared to fresh oocytes, regardless of exposed cryoprotectants. On the other hand, cooling has been identified as a leading cause for the compromised developmental competence of cryopreserved oocytes (Aman and Parks, 1994; Bernard and Fuller, 1996). Several abnormalities in chromosomal DNA have been identified, such as aneuploidy and polyploidy. Such abnormalities are normally associated with the temperature-sensitive nature of microtubules and MII spindles during the processes of cooling and rewarming. Unfortunately, in some species such as bovine and human, MII spindles cannot completely reform after warming owing to the lack of pericentriolar materials in some oocytes (Vincent and Johnson, 1992). In addition, the results also show that DNA fragmentation in cryopreserved oocytes increased significantly compared to that in fresh oocytes, regardless of cryoprotectants used for vitrification. Aslanidi *et al.* (1997) and Ivanov (2000) reported that calcium influx through the membrane may activate a variety of Ca^{2+}-dependent enzymes that induce upregulation or activation of caspases or other proteins that modulate apoptosis. Calcium is a key factor in the control of this evolution (Homa, 1995) and modification of its homeostasis that might occur during cryopreservation protocols could easily result in the abnormal activation of specific events.

The results show that 20% EG combined with 20% glycerol (20% EG/GLY) tended to have a higher cryoprotective effect than did other cryoprotectants. Isachenko *et al.* (2001) obtained 22% MII oocytes after vitrification of porcine GV oocytes by using 40% EG and observed lower cytotoxicity after stepwise exposure than after single-step exposure. In the present study, the MII rates of vitrified-thawed oocyte after IVM culture were less than 10%, regardless of the type of cryoprotectant. The difference in the cryoprotectant effects may result from the procedure of vitrification because oocytes were directly exposed to each cryoprotectant.

During vitrification, porcine oocytes are exposed to a very high concentration of cryoprotectants that trigger an increase in abnormal spindles and cytoskeletal disruption. Exposure of oocytes to cryoprotective additives and/or cooling them from approximately 37 to 20°C or below causes various cytoskeletal and chromosomal alterations (Massip, 2003). In previous studies, a cytochalasin pre-treatment was used to reduce injury to oocytes and embryos during vitrification (Isachenko *et al.*, 1998; Dobrinsky *et al.*, 2000; Fujihira *et al.*, 2004). Some studies have found that cytochalasin increased oocyte survival after vitrification of immature porcine oocytes

(Isachenko *et al.*, 1998; Fujihira *et al.*, 2004); nevertheless, other studies in bovine did not observe any effect in both immature and mature oocytes (Vieira *et al.*, 2002). Rho *et al.* (2002) reported that CB pre-treatment enhanced stabilization of microtubules during vitrification of bovine MII oocyte. Cytochalasin is a cytoskeletal relaxant that is thought to reduce the rigidity of the cytoskeletal elements (Fujihira *et al.*, 2004). CB reduces damage to microtubules and enhances stabilization of spindle microtubules during vitrification of MII oocytes (Rho *et al.*, 2002). It has been suggested that GV oocytes do not have organized meiotic spindles and thus, CB may preserve the functionality of the gap junctions between oocyte and granulosa cells by permitting faster and more uniform penetration of the cryoprotectants (Vieira *et al.*, 2002). However, the results show that CB treatment before vitrification had no significant effects on the meiotic competence and DNA fragmentation of vitrified-warmed oocytes after IVM culture. Differences between the CB effect on GV oocytes in this study and other studies may be as a result of the vitrification procedure, the meiotic stage and/or the species of oocyte.

CONCLUSION

In conclusion, the percentage of DNA fragmentation in oocytes following cryoprotectant exposure and vitrification was significantly lower than that of fresh oocytes. These results indicate that the meiotic competence of porcine oocytes is damaged by cryoprotectant exposure and the competence of vitrified oocytes is not improved by pre-treatment of oocytes with cytochalasin B prior to vitrification, regardless of the type of cryoprotectant.

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