# The Effect of the Addition and Removal of Various Cryoprotectants on the Nuclear Maturation and ATP Content of Immature Porcine Oocytes

Y. Tsuzuki[1, *], K. Nozawa[1, 2] and K. Ashizawa[1]

[1] Laboratory of Animal Reproduction, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan

**ABSTRACT :** This study was undertaken to investigate the influence of exposure and removal of four different cryoprotectants (CPAs) on the ATP content of cumulus cell-enclosed (COs) and cumulus cell-denuded (DOs) immature porcine oocytes. The *in vitro* nuclear maturation of the COs, exposed to and removed from the CPAs was also assessed. Both COs and DOs were exposed to 1.5 M concentrations of each CPA (ethylene glycol (EG); propylene glycol (PG); dimethyl-sulfoxide (DMSO); and glycerol (G)) for durations of 5, 15, and 30 minutes at room temperature ($23.5\pm1.5°C$), and immersed in physiological saline supplemented with 20% (v/v) fetal bovine serum for 5 minutes ($39°C$) to remove each CPA. Before, during and after exposure to each CPA, the ATP content of both the COs and the DOs was measured. After removal from each CPA an aliquot of the COs was matured for $44\pm2$ h, and their nuclear maturation rates were measured up to the metaphase stage of the second meiotic division (the M-II stage). The maturation rates up to the M-II stage were not significantly different between all the groups that were exposed to each CPA for 5 minutes. For 15 and 30 minute exposures, the maturation rates of the COs exposed to PG, DMSO and EG were almost the same as those of the control groups; however, the rates of G group exposed for 15 and 30 minutes were significantly lower ($p<0.05$) than the control group. These groups were also found to have a decrease in the ATP content of COs and DOs during and after exposure for the same periods ($p<0.05$). In addition, although the ATP contents of the COs after exposure to EG for any period were the same as the controls, the ATP content of the DOs exposed to EG for any period were significantly lower ($p<0.05$) than those of the controls. When the ATP content of the COs and DOs of each CPA were compared, the DOs exposed to PG were found to have a significantly greater level ($p<0.05$) than DOs exposed to G for any duration. In addition, the ATP content of DOs exposed to PG for 30 min and removal was also higher ($p<0.05$) than when exposed to DMSO for the same period. These findings indicate that PG may be a useful CPA for the cryopreservation of immature porcine oocytes. (**Key Words :** Cryoprotectant, Porcine Oocytes, ATP Content)

## INTRODUCTION

Oocyte cryopreservation provides greater flexibility in breeding programs than embryo cryopreservation (Payner and Fuller, 2007). However, mammalian oocytes are much more readily damaged by cryopreservation than mammalian embryos (Coticchio et al., 2007; Gardner et al., 2007). Differences in plasma membrane permeability to water and cryoprotectants (CPAs) could account for this damage, as could the idiosyncratic physiology of the oocyte itself (Gardner et al., 2007). It is common knowledge that, compared with immature oocytes, the *in vivo* or *in vitro* matured oocytes are the preferred stage because of their superior cryotolerance (Otoi et al., 1995; Rojas et al., 2004). However, the meiotic spindles of the matured oocytes are markedly affected by the exposure to CPAs, cooling, and freezing (Van der Elst et al., 1992; Tharasanit et al., 2006; Gardner et al., 2007; Ledda et al., 2007; Mugnusson et al., 2007); exposure to the aforementioned conditions results in an increase in spindle abnormalities and aneuploidy (Pickering et al., 1990; Stachecki et al., 2006; Wu et al., 2006). To avoid this problem, and until suitable protocols for *in vitro* maturation are available, cryopreservation of immature oocytes is more appropriate (Van der Elst et al., 1992; Agca et al., 1998); immature stage (GV stage) oocytes have a nuclear envelope to protect the nuclear DNA (Hunter and Polge, 1966).

CPAs are divided into two groups: permeable to and non-permeable to the cell membrane (Friedler et al., 1988; Edashige and Kasai, 2007). The permeable CPAs are low molecular weight non-electrolytes that are completely

miscible with water (Leibo, 2008). These CPAs can lower the freezing point and replace some of the bound water molecules in and around proteins (Shaw and Jones, 2003); alternatively, they can stabilize the proteins by enhancing disulphide bridge formation under conditions where other stresses, such as dehydration during freezing, can occur (Fahy et al., 1990; Fuller, 2004). The permeable CPAs commonly used worldwide are ethylene glycol (EG; MW = 62.1), propylene glycol (PG; MW = 76.1), dimethyl-sulfoxide (DMSO; MW = 78.1), and glycerol (G; MW = 92.1) (Friedler et al., 1988; Pedro et al., 2005; Leibo, 2008). Different CPAs can have different effects on the viability and developmental potential of cryopreseved oocytes and embryos (Ashwood-Smith, 1987; Friedler et al., 1988; Bağis and Odaman Mercan, 2005; Yamada et al., 2007).

Most CPAs are relatively innocuous when compared to, for example, equivalent concentrations of salts; in most cases, there is an appreciable time- and temperature-dependent effect (Fuller, 2004). However, CPAs have some cellular toxicity, and the nature of the chemical toxicities from CPAs are complex, given the ranges of different molecular structures of the commonly used agents (Friedler et al., 1988; Fahy et al., 1990; 2004; Fuller, 2004). This toxicity as well as chilling injuries are the major adverse consequences following cryoprocedures (Ledda et al., 2007). For developing an efficient cryopreservation protocol, a balance between the toxicity and cryoprotective ability of a CPA is critical (Fuller, 2004; Gupta et al., 2007).

For mammalian oocytes, CPAs have a direct toxic effect on structures such as microtubules (a main component of the meiotic spindle) and microfilaments; this toxicity causes disassembly, which can be reversed if the CPA exposure is of a relatively brief duration or at lower temperatures (Fuller, 2004; Gardner et al., 2007). In addition, the denaturation of proteins in the cytoplasm, degeneration of the cell membrane induced by the high osmolarity, relocation of cell organelles and zona hardening of the oocytes can also be induced by CPAs (Friedler et al., 1988; Fabbri et al., 2001; Fuller, 2004; Coticchio et al., 2007).

With vitrification, a widely-used method for cryopreservation of oocytes and embryos, CPAs are used in markedly higher concentrations (30% or more) than those of conventional methods (slow freezing method: approximately 10%); the use of CPAs at high concentrations decreases the survivability of mammalian oocytes and embryos before freezing (Rall, 1987; Fahy et al., 1990; Shaw and Jones, 2003; Edashige et al., 2007; Yavin and Arav, 2007).

Slow freezing combined with a 1 to 2 M CPA concentration is also widely used for mammalian oocytes and embryos (Shaw and Jones, 2003; Mukaida and Kasai, 2004). In this method, the immature and mature oocytes of some species (mice, cattle, and humans) can be preserved

without a significant decrease in survivability (Kubota et al., 1998; Stachecki and Willadsen, 2000; Jain and Paulson, 2006; Stachecki et al., 2006). However, immature porcine oocytes can not be cryopreserved by this method because they cannot survive below 15°C (Didion et al., 1990; Paynter and Fuller, 2007).

ATP could be a useful marker for the cell viability of human (Van Blerkom et al., 1995), bovine (Stojkovic et al., 2001; Nagano et al., 2006) and porcine oocytes (Bravinii et al., 2007). Rittmeyer and Nydegger (1992) reported that hydroxyethyl starch, a non-permeable CPA for the extracellular environment of the cells, reduced the ATP content of human blood cells. In view of this phenomenon, there may be some potent influences of permeable CPAs upon the intracellular ATP. The literature contains a number of reports describing the effects of type, exposure period or temperature of the CPA on the survivability or developmental potential of oocytes (Gardner et al., 2007); however, to the best of our knowledge, our study (Tsuzuki et al., 2001) was the first to describe the relationship between the CPA and the ATP content of the oocytes.

Cumulus cells attached to the oocytes can support oocyte maturation by supplying nutrients as well as hormonal stimulation through the cell membrane or gap junctions formed between cumulus cell projections and the oocytes (Downs, 1993; Ledda et al., 2007). The cumulus cells are also one of the important factors for the permeability of the CPAs to the oocytes (Fujihira et al., 2005; Ledda et al., 2007). However, aside from our study, no reports describe the relationship between CPAs, ATP content, or attachment of the cumulus cells to the oocytes; we reported that the use of 1.5 M DMSO+0.25 M sucrose as a cryoprotectant decreased the ATP content of matured bovine oocytes *in vitro* (under the presence or absence of cumulus cells) (Tsuzuki et al., 2001). After presenting this report, we inferred that further study was necessary to elucidate the relationship between CPAs, attached cumulus cells and ATP of porcine oocytes.

The present study was undertaken to investigate the influence of four kinds of CPAs (EG, PG, DMSO and G; each at 1.5 M concentration) on the nuclear maturation and ATP content of immature porcine oocytes with or without cumulus cells.

## MATERIALS AND METHODS

### Oocyte collection

The ovaries of gilts with unknown estrus cycle (approximately 16 to 18 months of age) were collected after slaughter from a local abattoir and transported to our laboratory immersed in physiological saline supplemented with 400 U/ml penicillin (No. 26239-42, Nacalai Tesque, Kyoto, Japan) and 500 μg/ml streptomycin sulfate (No.

32237-72, Nacalai Tesque) within three hours. Oocytes attached with cumulus cells (COs) were aspirated from superficial follicles (2 to 6 mm in diameter) and washed five times with 2 ml of pre-warmed (39°C) Hank's salted TCM-199 (HTCM-199, No. M-0393, Sigma-Aldrich, St Louis, MO, USA) that contained 2% (v/v) heat-inactivated (56°C, 30 min) calf serum (No. 16170-086, Gibco BRL Products, Grand Island, NY, USA), 98 U/penicillin G potassium and 98 µg/ml streptomycin sulfate. Subsequently, the COs which had regulatory granular cytoplasm in black and more than one layer of cumulus cells were selected and washed four times with 2 ml of pre-warmed (39°C) HTCM-199; these cells were used for nuclear maturation and ATP assay as described below.

**Nuclear maturation assay**

Selected COs were matured in drops (culture volume, 6.25 µl medium/CO) comprised of modified NCSU37 (Petters and Wells, 1993) supplemented with 1.2 mM cystine (No. 10309-12, Nacalai Tesque), 100 µM β-mercaptoethanol (No. M-7522, Sigma-Aldrich), 51.5 mM taurin (No. T-7146, Sigma-Aldrich), 2% (v/v) essential amino acid (No. 11130-041, Gibco BRL Products), 1% (v/v) non-essential amino acids (No. 11140-050, Gibco BRL Products), 5% (v/v) heat-inactivated fetal bovine serum (FBS, No.16140-063, Gibco BRL Products), 0.60 µg/ml follicle stimulating hormone (No.F-2293, Sigma-Aldrich), 0.23 µg/ml luteinizing hormone (No. L-9773, Sigma-Aldrich), 5 µg/ml Insulin (No. I-6634, Sigma-Aldrich), 15 ng/ml epidermal growth factor (No. E-7127, Sigma-Aldrich), 5 µg/ml phenol red (No. P-3532, Sigma-Aldrich) and antibiotics (100 U/ml penicillin G potassium, No. P-4687, Sigma-Aldrich, 100 µg/ml streptomycin sulfate, No. S-1277, Sigma-Aldrich, and 100 µg/ml dibekacin sulfate, No. DBK, Meiji Seika Co., Japan) for 42 to 46 h in 5% $CO_2$, 95% room air at 39°C under 100% humidity under paraffin oil (No. 26137-85, Nacalai Tesque, Tsuzuki et al., 2008).

**Exposure of the oocytes to four different CPAs**

The exposure and removal of each CPA was done using the method reported by Kubota et al. (1998) with some modification. The concentration of each CPA was set at the same osmolarity (1.5 M). The COs were exposed to ethylene glycol (EG, No. 15209-85, Nacalai Tesque), propylene glycol (PG, No. 292-18, Nacalai Tesque), dimethyl-sulfoxide (DMSO, No. 134-07, Nacalai Tesque) or glycerol (G, No.170-18, Nacalai Tesque) in isotonic (0.15 M) NaCl (physiological saline, Agca et al., 1998; Kleinhans and Mazur, 2007) supplemented with 20% (v/v) FBS (FBS-PS, Tsuzuki et al., 2001) for 5, 15, and 30 minutes at room temperature (23.5±1.5°C). Since exposure periods of the

CPAs were set from 5 to more than 20 minutes in other reports (Otoi et al., 1994; Kubota et al., 1998; Paynter and Fuller, 2007), we used 5, 15, and 30 minute exposure periods for this study. After exposure, the COs with 20 to 40 µl FBS-PS were immersed in 2 ml of pre-warmed (39°C) FBS-PS to remove each CPA from each CO. Subsequently, these COs without further selection were washed with modified NCSU37 as previously described; then matured. In addition, we produced cumulus cell-denuded oocytes (DOs) following aspiration from the ovaries; these DOs were exposed to and removed from each CPA to assess the ATP content of the oocytes (a process similar to that of the COs groups). After removal of each CPA and IVM, the COs without further selection had their cumulus cells removed by a vortex mixture for several seconds and were mounted on the slide glass; they were then fixed with acetic-alcohol (ethanol:acetic acid = 3:1, v/v) for at least six hours at room temperature. Subsequently, the oocytes of each group were stained with 1% acetic orcein (No. 1.07100.0005, Merck, Darmstädt, Germany) for 10 to 20 minutes at room temperature; then observed at their nuclear phase.

To investigate the influence of the CPAs on the oocytes and cumulus cells, immature COs were denuded of their cumulus cells (DOs) by a vortex mixture and equilibrated. The removal of each CPA was conducted by the same method as that used for the COs.

In addition, some fresh COs and DOs were loaded into FBS-PS for 5, 15 and 30 minutes (23±1.5°C), then transferred into FBS-PS (39°C) for 5 minutes (as the CPA treatment groups). These COs and DOs were also matured as carrier solution groups.

**ATP assay**

The oocytes were loaded into a 1.5 ml Eppendorf tube with 50 µl of FBS-PS and 400 µl distilled water was added; the mixture was boiled for four minutes using a hot dry bath (No. HDB-1 type, AS-One Co., Japan) and stored at -70°C until assay. The ATP content was measured by luciferase (No. 60311, Kikkoman, Noda, Chiba, Japan) using a lumicounter (No. A-237, Advantec Co., Japan).

**Statistical analysis:**

Nuclear maturation rates and ATP content were analyzed with Duncan's Multiple Range Test.

**RESULTS**

After aspiration, 154 of 200 oocytes (77%) were at GV stage when they were denuded of their cumulus cells, fixed with acetic alcohol, and stained with acetic orcein. The nuclear maturation of the COs exposed to each CPA for 5, 15, and 30 minutes is presented in Table 1. The maturation rates of the carrier solution groups for each exposure period

**Table 1.** The effect of exposure period to various cryoprotectants at a concentration of 1.5 M on the nuclear maturation of pig oocytes *in vitro*

| Group | Nuclear maturation rate up to the M-II stage (%) | | | | | |
|---|---|---|---|---|---|---|
| | Exposure period (min) | | | | | |
| | N | 5 | N | 15 | N | 30 |
| Control | 74 | 57.1±3.36 | 89 | 53.2±6.21$^a$ | 192 | 55.7±5.42$^a$ |
| Carrier Solution | 62 | 47.5±9.44 | 98 | 52.8±3.42$^a$ | 162 | 61.5±4.69$^a$ |
| EG | 93 | 51.3±3.51 | 72 | 49.9±8.91$^a$ | 186 | 51.0±8.56$^a$ |
| PG | 79 | 52.9±7.74 | 82 | 52.3±6.11$^a$ | 194 | 64.6±9.11$^a$ |
| DMSO | 83 | 55.4±6.54 | 83 | 50.1±2.59$^a$ | 172 | 54.8±10.35$^a$ |
| G | 73 | 45.7±6.03$^A$ | 71 | 29.5±6.53$^{ABb}$ | 169 | 15.0±8.07$^{Bb}$ |

$^{a, b}$ Values with different superscripts in a column are significantly different (p<0.05).
$^{A, B}$ Values with different superscripts in a row are significantly different (p<0.05). Values are mean±SE in four replicates.

were almost the same as the control groups in all instances. Maturation rates up to the M-II stage in the 5 minute groups were not significantly different; however, the COs exposed to G for 15 and 30 minutes had significantly lower rates (p<0.05) when compared to the other CPA groups and the control groups. When the rates for each exposure period to the same CPA were compared, the COs exposed to G for 30 minutes had a significantly lower rate (p<0.05) than that of the 5 minute group.

The ATP contents of the COs and DOs of each group are presented in Table 2 and 3. The ATP contents of the carrier solution groups were the same as the control group for both COs and DOs. In regard to COs, the PG and DMSO groups were found to have no significant changes in ATP content

both during and after exposure. However, in the EG group, although the ATP content of COs exposed for any time period were significantly decreased (p<0.05) when compared to that of the control groups, their ATP content recovered to the same level as the controls after removal from EG exposure for any time period. The COs exposed to G showed a significant decrease in ATP content for those exposed for 15 and 30 minutes (p<0.05) when compared to the control groups. Furthermore, unlike the COs exposed to EG for any time period, the ATP content did not recover after removal of G.

As in the COs groups, the ATP contents of the DOs in the PG and DMSO groups were unchanged during or after exposure for any time period; however, the ATP content of

**Table 2.** The effect of exposure period to a 1.5 M concentration of each cryoprotectant on the ATP content of immature COs

| Treatment | ATP content (P mol/CO) | | | |
|---|---|---|---|---|
| | EG | PG | DMSO | G |
| Control | 2.7±0.45$^a$ | 2.5±0.21 | 2.4±0.55 | 1.9±0.35$^{ab}$ |
| Carrier solution | 2.1±0.46$^{ab}$ | 2.7±0.19 | 2.0±0.50 | 2.5±0.48$^a$ |
| Exposed for 5 min | 1.5±0.18$^b$ | 1.9±0.31 | 1.7±0.42 | 1.1±0.26$^{bc}$ |
| Exposed for 15 min | 1.5±0.30$^{BCb}$ | 1.8±0.19$^B$ | 2.7±0.44$^A$ | 0.7±0.14$^{Cc}$ |
| Exposed for 30 min | 1.2±0.17$^{ABb}$ | 1.8±0.42$^{AB}$ | 2.3±0.45$^A$ | 0.7±0.25$^{Bc}$ |
| Removed after 5 min exposure | 2.0±0.22$^{ab}$ | 2.2±0.25 | 2.3±0.61 | 1.8±0.55$^{ab}$ |
| Removed after 15 min exposure | 2.0±0.40$^{ABab}$ | 2.1±0.45$^{AB}$ | 2.6±0.47$^A$ | 0.9±0.26$^{Bbc}$ |
| Removed after 30 min exposure | 2.1±0.08$^{Aab}$ | 1.8±0.21$^A$ | 1.9±0.21$^A$ | 0.5±0.14$^{Bc}$ |

$^{a, b, c}$ Values with different superscripts in a column are significantly different (p<0.05).
$^{A, B, C}$ Values with different superscripts in a row are significantly different (p<0.05). Values are mean±SE in four replicates.

**Table 3.** The effect of exposure period to a 1.5 M concentration of each cryoprotectant on the ATP content of immature DOs

| Treatment | ATP content (P mol/DO) | | | |
|---|---|---|---|---|
| | EG | PG | DMSO | G |
| Control | 1.8±0.47$^a$ | 1.9±0.71 | 1.0±0.23 | 1.4±0.51$^a$ |
| Carrier solution | 1.5±0.24$^a$ | 1.4±0.55 | 1.0±0.21 | 1.0±0.51$^{ab}$ |
| Exposed for 5 min | 1.1±0.22$^{ABab}$ | 1.6±0.40$^A$ | 1.0±0.25$^{AB}$ | 0.3±0.15$^{Bbc}$ |
| Exposed for 15 min | 1.1±0.22$^{ABab}$ | 1.7±0.62$^A$ | 1.1±0.27$^{AB}$ | 0.3±0.14$^{Bbc}$ |
| Exposed for 30 min | 1.2±0.08$^{Aab}$ | 1.6±0.59$^A$ | 0.9±0.22$^{AB}$ | 0.1±0.02$^{Bc}$ |
| Removed after 5 min exposure | 0.7±0.12$^{ABb}$ | 1.4±0.53$^A$ | 0.8±0.16$^{AB}$ | 0.2±0.08$^{Bbc}$ |
| Removed after 15 min exposure | 0.7±0.10$^{ABb}$ | 1.5±0.64$^A$ | 0.8±0.20$^{AB}$ | 0.2±0.12$^{Bbc}$ |
| Removed after 30 min exposure | 0.8±0.10$^{ACb}$ | 1.4±0.36$^A$ | 0.7±0.19$^{BC}$ | 0.1±0.01$^{Bc}$ |

$^{a, b, c}$ Values with different superscripts in a column are significantly different (p<0.05).
$^{A, B, C}$ Values with different superscripts in a row are significantly different (p<0.05). Values are mean±SE in four replicates.

the DOs after exposure to EG for any time period were lower ($p<0.05$) than that of the control groups. In addition, DOs exposed to G for any period were found to have a significant decrease in ATP content ($p<0.05$) when compared to the control group. Furthermore, the ATP content remained significantly lower ($p<0.05$) than that of the control group after removal from G after any exposure period.

When the ATP content of COs and DOs exposed to each CPA were compared, the DOs exposed to PG were found to have high levels both during and after exposure for any time period, and they were significantly higher ($p<0.05$) than those exposed to G for all treatment groups. In addition, the ATP content of DOs exposed for 30 minutes to PG and removal were also significantly higher ($p<0.05$) than when exposed to DMSO for the same period.

## DISCUSSION

In this study, the maturation rate and ATP content of COs and DOs in the carrier solution groups were almost identical to the control. This finding indicates that the carrier solution we used did not have either a deleterious or stimulus effect on the metabolism of the COs and DOs. Moreover, Didion et al. (1990) suggested that immature (GV stage) porcine oocytes would have decreased survivability at a lower temperature (below 15°C). Huang and Holtz (2002) also indicated that porcine oocytes at GV stage could succumb to a temperature to 10°C. In this study, all COs and DOs in both the carrier solution and CPA groups were exposed at 23±1.5°C, suggesting that the survivability of immature oocytes with or without cumulus cells is not affected by this temperature.

The nuclear maturation rate up to the M-II stage of COs exposed to each CPA for 5 minutes was almost the same for all groups. The nuclear maturation rates of the COs groups, exposed to EG, PROH and DMSO for 15 and 30 minutes were found to have almost the same rates as those of the control groups; only the COs exposed to G were found to have significantly lower nuclear maturation rates than those of the control groups ($p<0.05$). Consistent with this finding, the ATP content of COs exposed to G for the same period was also significantly decreased ($p<0.05$). In addition, when the rates for each exposure period to the same CPA were compared, the COs exposed to G for 30 minutes had a significantly lower rate ($p<0.05$) than that of the 5 minute group. It has been proposed that exposure periods influence oocyte survivability (Fabbri et al., 2001; Fuller, 2004); if so, longer exposure periods to G may not be suitable for the cryopreservation of porcine immature oocytes.

Of the permeable CPAs used in this study, glycerol has the highest molecular weight (MW: 92.1, Friedler et al., 1988). For mouse oocytes matured *in vivo*, 1.33 M G was found to have the lowest permeability into the cytoplasm when compared to other permeable reagents (EG, PG, DMSO, and acetamides) at a similar molarity (Pedro et al., 2005). Wani et al. (2004) suggested that the significantly decreased maturation rates of immature buffalo oocytes vitrified with G up to the M-II stage when compared to those vitrified with PG, DMSO or EG may be caused by osmotic stress. Since this stress may be caused by the lower permeability of G to the oocytes, it might induce the decrease of oocyte survivability that resulted in the lower maturation rate and ATP contents found in this study. Another possibility is that the addition of G may inhibit glycolysis more than oxidative phosphorylation, thereby decreasing the ATP content of human platelets (Shimizu and Kouketsu, 1988). Both glycolysis and pentose phosphate pathways play key roles in the control of nuclear and cytoplasmic maturation of porcine oocytes (Herrick et al., 2006). Since glycerol may inhibit the glycolysis of the oocytes and/or cumulus cells during exposure to G, this could result in the decrease of ATP after removal of G by an inhibited glycolytic system.

In the present study, the ATP content of the DOs, but not the COs, exposed to or removed from EG was significantly lower than that of the control ($p<0.05$). Poldelski et al. (2001) reported that EG decreased the ATP content of proximal tubular segments isolated from the mouse kidney via their metabolites, glyoxylate and glycoaldehyde. We postulated in this study that EG might play a role in decreasing the oocyte ATP via these metabolites.

Both EG and G are considered less toxic than other permeable CPAs, such as PG, DMSO and acetamide, for *in vitro*-matured mouse oocytes (Mukaida and Kasai, 2004; Pedro et al., 2005). However, EG did not appear to be less toxic than PG and DMSO in our study because the ATP content of the DOs exposed to EG was lower than that of those exposed to PG and DMSO in some instances. As a vitrification solution, PG is less toxic to immature bovine oocytes when compared to DMSO and G (Arav et al., 1993). The permeability of 1.5 M DMSO is higher than that of EG at the same molarity for immature and mature bovine oocytes *in vitro* (Agca et al., 1998). In view of these reports, our findings for immature porcine oocytes suggest that EG and G may have higher toxicity secondary to their permeability than PG or DMSO.

DMSO and G may interact electrostatically with the phospholipid bilayer (Anchordoguy et al., 1987; Fuller, 2004). It has been suggested that DMSO can modulate the components of the membrane bilayers, resulting in an increase in membrane permeability (Yu and Quinn, 1998); this process may have occured in this study because the addition of DMSO can increase the membrane permeability without major osmotic stress, and this may be one of the reasons for the lack of change in the maturation rate and

ATP content of COs and DOs during and after exposure to DMSO. However, in the present study, the ATP content of the DOs during and after a 30 minute exposure to DMSO were significantly lower than those of the DOs exposed to PG or EG for the same period (p<0.05). Fahy et al. (1990) reported that DMSO can accelerate the isomerase and fructose 1,6-diphosphatase enzymes of glycolysis. If both enzymes are stimulated, the ATP content decreases. This may be one of the reasons for the decreased ATP content of the DOs after exposure for 30 minutes to DMSO.

In the present study, the change of ATP content in both the COs and DOs both during and after exposure varied with each CPA. However, the ATP content of both COs and DOs exposed to PG did not vary during and after exposure; they maintained the high ATP level when compared to those exposed to the other CPAs. In addition, the nuclear maturation rate did not decrease with PG at any exposure period. For *in vivo*-matured mouse oocytes, approximately 1.5 M PG was a more permeable reagent than either DMSO or EG at a similar molarity (Pedro et al., 2005). From this finding, we inferred that PG may be the most permeable CPA for the cryopreservation of immature porcine oocytes.

PG exposure was found to have the largest change in intracellular $Ca^{2+}$ concentration (Gardner et al., 2007). A transient fluctuation of intracellular $Ca^{2+}$ can induce many cellular events, such as the release of cortical granules, which can result in zona pellucida hardening and oocyte activation (Vincent et al., 1990). These reports suggest that further study is indicated for the analysis of the suitability of PG for oocyte development; the study should focus on the assessment of embryonic development after *in vitro* fertilization.

It is commonly accepted that a shorter CPA exposure is preferable for cryopreservation (Fuller, 2004). In our study, although the shortest exposure period (5 minutes) did not influence the maturation rate and ATP content of either COs or DOs, these parameters varied with longer exposure periods (15 and 30 minutes) for both COs and DOs exposed to each CPA. These findings correlated with reports that found that suitable exposure time was dependent upon the CPA (Friedler et al., 1988; Fabbri et al., 2001). Although the variation of the nuclear maturation rate and ATP content of each CPAs is inexplicable, a hypothesis can be proposed.

Aquaporins (AQP), classed for 13 types (from type 0 to 12), are membrane proteins influencing water and/or neutral solute membrane passage and are involved in the permeability of CPAs in both mature oocytes and embryos (Edashige and Kasai, 2007). Ford et al. (2000) reported that AQP type 9 (AQP-9) was present in immature rat oocytes. The AQP-9 can allow passage of both aqueous and neutral solutes, such as CPAs (Edashgige et al., 2000; Edasgige and Kasai, 2007). Pedro et al. (2005) postulated that each AQP may select the passage of a specific CPA. In view of these reports, it can be postulated that the specific AQP channel for PG may be present in the membrane of porcine cumulus cells and immature oocytes, facilitating the permeability of PG to a greater extent than the other CPAs.

The COs exposed to EG did not have a decrease in either their nuclear maturation rate or their ATP content regardless of the amount of ATP decrease in the DOs after removal of EG. The COs exposed for 30 minutes to DMSO had no decrease in their maturation rate; however, the DOs exposed for 30 minutes to DMSO had a lower ATP content after removal than when exposed for the same period and removed from PG. In humans and cattle, the ATP content of oocytes matured *in vivo* may be a predictable indicator of their developmental potential (Van Blerkom et al., 1995; Nagano et al., 2006). The ATP content of mature porcine oocytes may also be a useful marker for oocyte metabolism (Brevini et al., 2007). Conversely, the ATP content of immature porcine oocytes with (but not without) cumulus cells can also be a reliable marker for oocyte maturational potential *in vitro*.

Chian et al. (2004) reported that the absence of cumulus cells did not affect the survival rate of bovine oocytes after vitrification and warming. The absence of cumulus cells during exposure and removal of the CPAs in this study could not be influenced by oocyte survivability, which is related to the ATP content of the DOs. Cumulus cells play an important role in oocyte maturation in mammals, such as supplying hormonal stimulation and nutrition (Yokoo and Sato, 2004; Russell and Robker, 2007). In view of this, it can be postulated that cumulus cells support and recover oocyte metabolism which has been impacted by EG treatment. This process may recover the nuclear maturation rates for DOs exposed to EG for any period and to DMSO for 30 minutes back to the level of the control group.

In this study, the ATP content of the COs was decreased only in those exposed for 15 and 30 minutes, then removed from G; however, the ATP content of DOs exposed to EG, DMSO and G for the same periods were reduced when they were compared with the control groups. We previously suggested that attached cumulus cells might be more sensitive to 1.5 M DMSO+0.25 M sucrose than bovine oocytes matured *in vitro* (Tsuzuki et al., 2001); we noted a lower sensitivity of the cumulus cells to EG. The physiology and morphology of cumulus cells changes during maturation (Yokoo and Sato, 2004). We suggest that the discrepancy between the present results and our previous reports may be caused by a physiological state change during a different meiotic stage (present results with the immature stage, and previous results with the mature stage), different animal subjects, or different CPAs.

In conclusion, the present study suggests that PG may be suitable for oocyte cryopreservation with four different permeable CPAs. In addition, the ATP content of the

oocytes attached to cumulus cells might be more important for nuclear maturation than those of the DOs.

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