Effects of polyethylene glycol and a synthetic ice blocker during vitrification of immature porcine oocytes on survival and subsequent embryo development.

THE EFFECTS OF POLYETHYLENE GLYCOL AND A SYNTHETIC ICE BLOCKER DURING VITRIFICATION OF IMMATURE PORCINE OOCYTES ON SURVIVAL AND SUBSEQUENT EMBRYO DEVELOPMENT

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Abstract

We evaluated the effects of polyethylene glycol (PEG) and Supercool X-1000 (SC) as supplements during the vitrification of immature cumulus-enclosed porcine oocytes in a solution based on 17.5% ethylene glycol+17.5% propylene glycol. After warming, the oocytes were subjected to *in vitro* maturation, fertilization and embryo culture. In *Experiment 1*, equilibration and vitrification solutions were supplemented with or without 2% (w/v) PEG (PEG+ and PEG-, respectively). The survival rate, cleavage and blastocyst development were similar between PEG+ and PEG- groups; however, all values were lower than those in the non-vitrified control. In *Experiment 2*, vitrification solution was supplemented with or without 1 % (v/v) SC (SC+ and SC-, respectively). The percentages of survival and blastocyst development were similar between SC+ and SC- groups; however, lower than those in the non-vitrified control. The percentage of cleavage in SC- group was significantly lower than the control and the SC+ groups, which were in turn similar to one another. In both experiments, the cell numbers in blastocysts were not significantly different among the non-vitrified and vitrified groups. In conclusion, PEG did not improve oocyte survival and embryo development whereas SC improved the ability of surviving oocytes to cleave but not to develop to blastocysts.

Key words: Immature oocyte, Pig, Polyethylene glycol, Synthetic ice blocker, Vitrification.

Introduction

Cryopreservation of gametes and embryos keeps cell metabolism quiescent during storage, allowing the subsequent use in programs of assisted reproduction and gene banks formation. Porcine oocyte cryopreservation has potential agricultural and biomedical importance (Zhou & Li 2009). However, this technique in pigs is considered much more difficult comparing with other domestic animal species (Mullen & Fahy 2012) and is yet to be applied in practice (Nohalez *et al.* 2015). Recently it was demonstrated that blastocysts obtained from porcine oocytes cryopreserved at the immature germinal vesicle (GV) stage by solid surface vitrification could develop to term, despite of reduced embryo development (Somfai *et al.* 2014). Vitrification of oocytes at the GV stage is considered as an alternative way to prevent spindle depolymerization or damage often observed during the preservation of matured oocytes, owing to the absence of the meiotic spindle (Moward *et al.* 2012). Matured porcine oocytes are known to survive cryopreservation at higher rates compared with immature ones (Rojas *et al.* 2004; Gupta *et al.* 2007). Nevertheless, previous studies have demonstrated that high rates (over 80 %) of oocyte survival can be achieved even after the vitrification at the GV stage by careful optimization of cryoprotectant treatment regimen (Somfai *et al*. 2013,2015) and warming temperatures (Somfai *et al.* 2014). Although reasonable survival rates have been reported after vitrification of the GV stage porcine oocyte (Gupta *et al.* 2007; Nohalez *et al.* 2015; Somfai *et al.* 2014,2015) the embryo developmental ability of surviving oocytes remained low underlining the need to further improvements in vitrification protocols. For this purpose, one possible approach is the application of alternative CPAs in existing vitrification protocols. Polyethylene glycol (PEG) and synthetic ice blockers such as Supercool X-1000 (SC) have been used as alternative additives during vitrification to improve survival and developmental rates of oocytes in mice (Fahy *et al.* 2004; O'Neil *et al.* 1997) and horses (de Leon *et al.* 2012). However, to our knowledge PEG and synthetic ice-blockers have not been tested for the vitrification of immature

porcine oocytes to date. The objective of this study was to investigate the effects of PEG and SC for the vitrification of immature porcine oocytes on post-warming survival and subsequent embryo development. In 2 separate experiments, PEG and SC were applied in our current vitrification protocol at the concentrations based on previous studies in other species.

Materials and Methods

Collection of cumulus- oocyte complexes (COCs)

Ovaries of crossbred gilts (Landrace × Large White) were collected from a local slaughterhouse and transported to the laboratory at 35–37 °C in a Dulbecco's Phosphate Buffered Saline (PBS) within 1-2 hours. COCs were collected by scraping of 2–6 mm follicles in medium 199 (M199 with Hanks' salts; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% (v/v) of fetal bovine serum (Gibco, Thermo Fisher Scientific, Life Technologies, Carlsbad, CA, USA), 20 mM of HEPES (Dojindo Laboratories, Kumamoto, Japan), and antibiotics [100 IU/mL of streptomycin sulfate (Sigma-Aldrich), 100 IU/mL penicillin G potassium (Sigma-Aldrich)]. After dissection, COCs with multilayered compact cumulus and homogenous ooplasm were selected for further experiments.

Vitrification and warming of COCs

Cryoprotectant-treatment regimen before vitrification was performed according to previous report (Somfai *et al.* 2015). In brief, a group of 50–70 COCs were incubated in 1 mL a basic medium (BM) for 30 min, which was a modified glucose-free North Carolina State University (NCSU)-37 medium (Petters & Wells 1993) supplemented with 20 mM HEPES, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 50

µM ß-mercaptoethanol. The medium was further supplemented with 4 mg/mL bovine serum albumin (Fraction V, Sigma-Aldrich) and 7.5 µg/mL cytochalasin B (C-6762, Sigma-Aldrich). Then they were transferred into equilibration solution (ES) comprised of BM supplemented with 7.5 µg/mL cytochalasin B and 4% (v/v) of a permeating CPA combination [ethylene glycol (EG) + Propylene glycol (PG) = 1:1], for 5–15 min (Somfai *et al.* 2015) at 38.5 °C. Then, 10–12 COCs were washed 2 times in 50 µL of vitrification solution (VS) in 20 seconds and then they were loaded on Cryotop sheets (Kitazato, Biopharma, Shizuoka, Japan) in minimum volume of VS kept at 38.5 °C and were plunged in liquid nitrogen (LN) (Kuwayama 2007). VS was comprised of BM supplemented with 50 mg/mL polyvinyl pyrrolidone (P-0930, Sigma-Aldrich), 0.3 M sucrose (196-00015, Wako Pure Chemical Industries, Osaka, Japan) and 35% (v/v) of EG+ PG (1:1, total percentage). The treatment of COCs in VS medium (including washing, loading and removal of excess VS) was performed in 40 seconds. Vitrified samples were stored in LN tank until use. Warming of vitrified COCs was performed according to a previous report (Somfai *et al.* 2015) with slight modifications. In brief, Cryotop devices were immersed directly into 2.5 mL of warming solution (0.4 M Sucrose in BM) in a 35-mm plastic dish (Falcon 351008, Becton Dickinson, Franklin Lakes, NJ, USA) for 1 min at 42 °C. The COCs were then consecutively transferred for periods of 1 min (each) to 500-µL droplets of BM supplemented with 0.2, 0.1 and 0.05 M of sucrose at 38.0°C. Then COC's were washed in BM without sucrose at 38.0°C and then placed into maturation medium.

In vitro maturation (IVM)

After warming, all COCs were subjected to IVM. Oocytes were washed 3 times in 2ml aliquots of pre-incubated IVM medium which was NCSU-37 containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 µM ß-mercaptoethanol, 1 mM dibutyryl cAMP (dbcAMP; Sigma), 10 IU/mL eCG (Serotropin;

104 ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), 10 IU/mL hCG (500 units; Puberogen, Novartis Animal
105 Health, Tokyo, Japan), 0.1 mg/mL streptomycin sulfate and 100 IU/mL penicillin G. Groups of 40–50
106 COCs were cultured in 500 µL aliquots of IVM medium in 4-well dishes (Nunc, Nunclon Delta Surface,
107 Thermo Fisher Scientific, Roskilde, Denmark) without oil coverage, in an atmosphere of 5% CO_2, 5% O_2
108 and 90% N_2 at 39° C for 22 h. Then, they were subsequently cultured for an additional 22 h in IVM
109 medium without dbcAMP and hormones under the same conditions.

110

111 *In vitro fertilization (IVF)*

112 The procedures for IVF and embryo culture were performed according to a previous report (Kikuchi
113 *et al.* 2002). The medium used for IVF was a modified Pig-FM (Suzuki *et al*. 2002). The COCs after IVM
114 were partially denuded by pipetting, washed 3 times in IVF medium and then transferred into 95-µL
115 droplets of the IVF medium covered by paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan).
116 Frozen-thawed epididymal spermatozoa from a Landrace boar were pre-incubated in media 199 (with
117 Earle's salts, Gibco, and PH adjusted to 7.8) for 15 min (Kikuchi *et al.* 1998). After serial dilution in IVF
118 medium, five µL of the sperm suspension was introduced into the IVF droplets, the final sperm
119 concentration was set to 5×10^4 cells/mL. After 30 min of co-incubation with sperm at 39 °C under 5%
120 CO_2, 5% O_2 and 90% N_2, the oocytes with spermatozoa attached to the zona pellucida were carefully
121 transferred into another 100 µL drop of IVF medium without sperm and cultured for an additional 2.5 h
122 under the same conditions (Grupen, personal communication).

123

124 *Assessment of oocyte survival and subsequent in vitro embryo culture (IVC).*

At the end of IVF, presumptive zygotes were transferred into 2 ml of pre-incubated IVC-PyrLac medium (Kikuchi *et al.* 2002). Spermatozoa and cumulus cells were removed from the surface of the zona pellucida by pipetting through a fine glass pipette. At this time, the live/dead status of the oocytes was assessed morphologically by observation under a stereomicroscope. Survival was evaluated based on the integrity of oolema. Oocytes with normal spherical shape demarcation, smooth surface, dark and eventually granulated were considered live; whereas oocytes that did not any of fit these criteria were categorized as dead. Only live oocytes were subjected to IVC, which was performed in 500-µL of IVC-PyrLac, on Days 0 to 2 (Day 0= IVF) and 500-µL of IVC-Glu day 2-6 in 4-well dishes without oil coverage at 39 °C under 5% CO_2, 5% O_2 and 90% N_2 (Kikuchi *et al.* 2002). Cleavage rates were recorded on Day 2, blastocyst rate on Day 7. On Day 2, only cleaved embryos (2–4 cells) were subjected to subsequent culture to obtain embryos with good quality (Dang-Nguyen *et al.* 2010). In the morning of Day 7, the embryos without a visible perivitelline space containing more than 10 blastomeres and a blastocoel were categorized as blastocysts (Somfai *et al.* 2013).

Evaluation of blastocyst cell number

To verify the total cell numbers, blastocysts on Day 7 were placed in 25 µg/mL of Hoechst 33342 (H 33342, Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol and kept at 4 °C overnight. They were then washed in ethanol 99.5% and mounted on glass slides in glycerol droplets, flattened by cover slips and examined under UV light with an excitation wavelength of 330–385 nm, using an epifluorescence microscope (IX-71, Olympus, Tokyo, Japan). The digital image of each embryo was recorded and the total numbers of nuclei labeled by H33342 were counted.

Experimental design

Experiment 1. This experiment was performed to assess the effects of PEG applied during vitrification of immature COCs on oocyte survival and post IVF- embryo development. The media during equilibration and vitrification were supplemented with or without 2% (w/v) PEG (#6000 MW=7300–9000, Nacalai Tesque). A non-vitrified group of immature COCs served as control. Oocyte survival after IVM, IVF and subsequent IVC were compared among the PEG-treated and not treated vitrified groups and the control. The experiment was replicated six times.

Experiment 2. This experiment was performed to observe effects of Supercool X-1000 (SC, 21 st Century Medicine Inc., Rancho Cucamonga, California, USA) during vitrification of immature COCs. The vitrification medium was supplemented with or without 1% (v/v) of SC. A non-vitrified group of immature COCs was used as a control. All groups (the SC-treated and non-treated vitrified groups and control groups) were compared in terms of their survival after vitrification and embryo development after IVM/IVF. The experiment was replicated five times.

Statistical Analysis

All data were expressed as mean ± SEM values and percentage data after arcsine transformation were analyzed by one-way ANOVA followed by Tukey`s multiple comparison test using the KyPlot package (Ver. 2.0, KyensLab Inc., Tokyo, Japan). For all analysis, $P < 0.05$ was set as the significance level.

Results

Experiment 1

Survival rates of the oocytes after vitrification in the presence or absence of PEG were statistically similar to one another (65.80% and 61.12%, respectively); however, they were both significantly lower than that in the non-vitrified control (93.43%) (Fig. 1). Cleavage and blastocyst developmental rates were statistically similar between groups vitrified with or without PEG, but were significantly lower than that in the non-vitrified control (Table 1). Nevertheless, the total cells numbers in blastocysts were not significantly different among the non-vitrified and vitrified groups, irrespective of PEG treatment (Table 1).

Experiment 2

The survival rates of oocytes after vitrification in the presence or absence of SC were statistically similar to one another (53.38% and 60.32%, respectively); however, they were both significantly lower than that in the non-vitrified control (93.5%) (Fig. 2). The cleavage rate of the SC-vitrified oocytes was statistically similar to those of the non-vitrified oocytes (56.38 and 69.3% respectively); however, the group vitrified without SC showed a significantly lower rate of cleavage (39.4%) compared with the control and also with the SC vitrified treatment (Table 2). The SC-treated and non-treated vitrified groups showed similar results in terms of blastocyst developmental rates (4.7% and 2.3 %, respectively) (Table 2); however, these rates were lower than that in the non-vitrified control (13.3 %), similarly to the results of Experiment 1, the total cell numbers in blastocysts were not significantly different among the non-vitrified and vitrified groups, irrespective of SC treatment (Table 2).

Discussion

For vitrification of animal cells including oocytes, CPAs are used at high concentration combined with rapid cooling to eliminate ice crystal formation. However, high concentrations of CPAs also show toxicity and cause cell damage (Best 2015). The composition of CPAs in vitrification medium has a major impact on the success of vitrification on mammalian oocytes as it affects the speed of dehydration, CPA uptake, osmotic stress and other toxic effects (Best 2015). Accordingly, survival rates of immature porcine oocytes during vitrification could be improved by optimizing CPA composition (Somfai *et al.* 2013, 2015). The aim of the present study was to test for the first time if supplementation in media during vitrification with alternative non-permeating CPA such as PEG and SC, which were reported to act positively during oocyte cryopreservation in mice and horses (de Leon *et al.* 2012; Fahy *et al.* 2004; O'Neil *et al.* 1997) would affect the outcome of vitrification of GV-stage porcine oocytes.

It is generally accepted, that the major site of cryoinjury during cryopreservation of mammalian oocyte is the oolemma (Guetler *et al.* 2005; Horvath & Seidel Jr 2006; Brambillasca *et al.* 2013; Sprincigo *et al.* 2015). Furthermore, it has been suggested that due to the membrane structure specific at the GV-stage, high aquaporin content (Guetler *et al.* 2005) and insufficient permeation of CPA cause increased osmotic stress and oocyte mortality during the vitrification of immature porcine oocytes, especially when CPA with slow penetration speed such as EG is used (Somfai *et al.* 2013). PEG is a highly hydrated polymer that can cause dehydration of membrane surfaces (Arnold *et al.* 1983,1990) and can alter the molecular order the membrane lipid bilayer, at the point of contact between membranes, due the aggregation and dehydration (Lentz & Lee 1999; Yamazaki *et al.* 1989). This polymer has been used as a CPA during vitrification of mouse oocytes, and it reported the improved the survival and blastocyst rates (O'Neil *et al.* 1997). Also, this compound is applied in vitrification protocols for porcine blastocyst-stage embryos resulting in their improved cryotolerance (Misumi *et al.* 2013; Mito *et al.* 2015). PEG is known to depress the freezing point of solutions and due the impermeability to cells, promote their

dehydration (Banker *et al.* 1992). Also, PEG would hypothetically improve survival rates by protecting externally the oocyte membrane (O'Neil *et al.* 1997). On the other hand, it remained unclear if the structural changes in membrane caused by PEG affect the permeation of permeating CPA such as EG and PG, and therefore the survival of immature porcine oocytes. In the present study, we applied 2% (w/v) PEG during the equilibration period for 15 min and during 40 seconds of subsequent vitrification of immature porcine oocytes. This concentration and the product itself were identical to those reported previously for the vitrification of porcine embryos (Misumi *et al.* 2013). Our results demonstrated that such application of PEG using our current vitrification protocol did not alter the survival and developmental rates of vitrified immature oocytes. This suggests that, using the current vitrification protocol, insufficient dehydration or the membrane structure of the GV oocyte may not be major factors that determine the survival and embryo developmental rates after vitrification. However, it must be noted that our current protocol applies a combination of EG and PG as permeating CPAs. In this system the role of PG is to increase CPA penetration speed and thus to ease the osmotic stress during the vitrification process (Somfai *et al.* 2013). It is possible that PEG may exert a positive effect in vitrification systems, where only CPA with a permeation speed slower than that of PG (such as EG or glycerol) are used. Although O'Neil *et al* (1997) reported improved survival and developmental competence of mouse matured oocytes by the aid of PEG, the efficacy of PEG to affect cryotolerance of oocytes may vary between species and specific oocyte meiotic or maturational stages. Compared with other mammalian species, porcine oocytes have a greater hypothermic sensitivity due to the large amount of cytoplasmic lipid (Zhou & Li 2009) and, there are crucial differences between the metaphase-II and GV stage oocytes in terms of the permeability of their membrane to water and CPAs (Le Gal *et al.* 1994; Agca *et al.* 1998).

In previous studies, synthetic ice blockers had been suggested to be effective during cryopreservation process, reducing toxicity of the solutions (Wowk *et al.* 2000). These chemical

compounds are copolymers that can prevent ice nucleation resulting in increased rates of survival (Fahy *et al.* 2004). The application of synthetic ice blockers such as Supercool X-1000 or Supercool Z-1000 during cryopreservation showed promising results matured mouse oocytes (Fahy *et al.* 2004), immature equine oocytes (de Leon *et al.* 2012), mouse ovaries tissues (Tan *et al.* 2012) and rabbit embryos (Marco-Jimenez *et al.* 2014). Supercool X-1000 ice blocker is a copolymer of polyvinyl alcohol, with 20% of vinyl acetate content and would prevent ice formation, in the early stages of ice nucleation, during cooling or warming, even when present in very low concentrations (Wowk *et al.* 2000). While the CPAs prevent ice crystal formation by interacting with the water, Supercools are believed to prevent by molecular recognition of ice nucleators (Wowk 2005). In Experiment 2 of the present study, we applied Supercool X-1000 at 1% (v/v) in the vitrification solution, based on the previous report (Marco-Jimenez *et al.* 2014). Experiment 2 revealed that, Supercool X-1000 did not affect the ratio of post thaw survival of vitrified oocytes but significantly increased the ability of surviving oocytes to cleave after IVF. This suggests that Supercool X-1000 acted positively on oocytes during vitrification not by preventing membrane damage but reducing sub-lethal damages which affect embryo development. The exact mechanism behind this phenomenon remains unclear. Supercool X-1000 is not membrane permeable, therefore is can be suspected that this CPA exerted its positive effect via acting on the extra oocyte compartments of the COCs such as the cumulus cells or the gap junctions between the cumulus cells and oocytes, which are essential for to oocytes to acquire their developmental competence (Nagai *et al.* 2006). On the other hand, despite of the significant increase in cleavage rates, Supercool X-1000 did not increase the rate of blastocyst formation after vitrification, which suggests that the vitrification process exerts negative effects on embryo development even beyond the 2-cell stage, irrespective of Supercool X-1000. This suggestion is supported by the fact, that blastocyst developmental competence of cleaved embryos obtained from vitrified oocytes was not different between the groups treated with or without Supercool X-1000 but were lower than that in non-vitrified control (Table 2).

In the present study, embryo development after IVM, IVF and IVC of immature oocytes surviving the vitrification process was significantly reduced compared with that of the non-vitrified oocytes irrespective of supplementation with either of PEG or SC. The reduced competence for embryo development was indicated both by a decreased ability of the oocytes to undergo the first cleavage and the ability of cleaved embryos to reach the blastocyst stage. The reason of this phenomenon remains unclear. Theoretically, reduced embryo development could be caused by the failure of oocyte nuclear maturation, cytoplasmic maturation during IVM or the failure of normal fertilization during IVF (Nagai *et al.* 2006). However, in previous studies, we have demonstrated that, when immature porcine oocytes were vitrified in microdrops using the same CPA treatment and warming protocols as presented in this study, nuclear maturation of oocytes and fertilization were not affected (Somfai *et al.* 2014,2015). This suggests that the vitrification process causes sublethal damages in oocytes which are manifested only after fertilization, during embryo development. The exact mechanism behind this phenomenon remains to be elucidated. In the present study, instead of microdrop procedure, we used Cryotop as the carrier for vitrification because it is known to provide excellent cooling/warming rates (Liu *et al.* 2008; Spripunya *et al.* 2010; Liang *et al.* 2012; Wu *et al.* 2016). Nevertheless, the results achieved by the use of Cryotop in the present study were not improved compared with those of our previous reports using microdrops. Despite of severe reduction in blastocyst development after vitrification at the immature stage, irrespective of SC and PEG, some oocytes could develop to the blastocyst stage after IVF with cell numbers similar to those detected in the non-vitrified control. In other words, the quality of resultant blastocysts was the same as those of the control. These results are in accordance with those of our previous results (Somfai *et al.* 2010, 2013, 2014, 2015) and it suggests that these oocytes could maintain or restore the ability to develop to normal blastocysts.

In conclusion, the present study revealed that supplementation of PEG during vitrification of immature porcine oocytes did not affect the results in terms of oocyte survival and embryo development. On the other hand the synthetic ice blocker Supercool X-1000 improved the ability of surviving oocytes to cleave but not the blastocyst formation rate. Further research will be necessary to identify the reasons for reduced developmental competence to the blastocyst stage in surviving and cleaved oocytes. Such knowledge will be essential for the further optimization of the current vitrification protocol in order to minimize cryoinjuries during vitrification of immature porcine oocytes.

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413 **Tables**

Table 1. *In vitro* embryo development after IVF of cumulus-oocytes complexes vitrified in the presence or absence of 2% (w/v) polyethylene glycol (PEG) in equilibration and vitrification solutions.

Treatment Groups	Total cultured*	Cleaved embryos (% cultured)	Blastocyst (Day 6) (% cultured)	Blastocyst (Day 6) (% cleaved)	Total cells in blastocysts
Control	204	68.9 ± 2.9[a]	17.5 ± 3.1[a]	25.7 ±4.8[a]	44.1±3.5
Vitrified without PEG	173	22.2 ±5.9[b]	2.9 ± 1.3[b]	9.6 ± 8.2[b]	54.0±9.3
Vitrified with PEG	171	28.1 ± 3.6[b]	1.9 ±1.1[b]	11.1 ±3.2[b]	47.2±9.3

Data are presented as mean ±SEM.
Six replications were performed.
* After vitrification, IVM and IVF only surviving oocytes were subjected to subsequent culture.
[a,b] Percentages with different letters in the same column differ significantly (P<0.05).

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Table 2. *In vitro* embryo development after IVF of cumulus-oocytes complexes vitrified with or without 1 % (v/v) Supercool X-1000 (SC) addiction in vitrification solution.

Treatment Groups	Total cultured*	Cleaved embryos (% cultured)	Blastocyst (Day 6) (% cultured)	Blastocyst (Day 6) (% cleaved)	Total cells in blastocysts
Control	187	69.3± 2.8[a]	13.3±1.7[a]	18.9±2.1[a]	29.9±4.1
Vitrified SC-	200	39.4±5.8[b]	2.4±0.5[b]	5.9±1.4[b]	28.8±7.2
Vitrified SC+	211	56.4±10.3[a]	4.8±1.3[b]	8.9±2.4 [b]	40.7±8.0

Data are presented as mean ±SEM.
5 replications were performed.
* After vitrification, IVM and IVF only surviving oocytes were subjected to subsequent culture.
[a,b] Percentages with different letters in the same column differ significantly.

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Figures

Figure 1. Survival of COCs vitrified in the presence or absence of 2% (w/v) PEG in equilibration and vitrification solutions. Data are presented as mean ±SEM. Six replications were performed. Total numbers of oocytes vitrified in group are given in parentheses. Percentages with different letters differ significantly (P<0.05). VIT/PEG- = COCs vitrified without PEG; VIT/PEG+ = COCs vitrified with PEG.

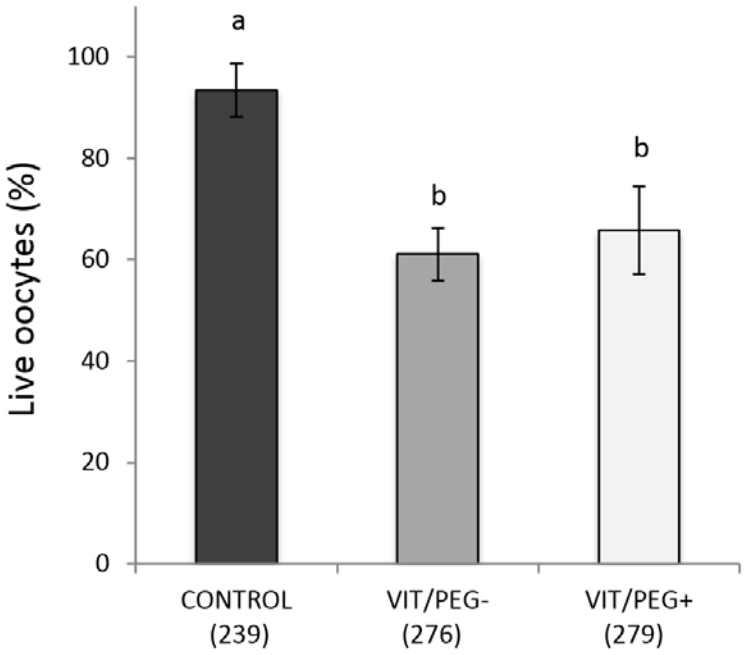

Figure 2. Survival of COCs vitrified in the presence or absence of 1% (v/v) Supercool X-1000 (SC) in vitrification solution. Data are presented as mean ±SEM. Five replications were performed. Total numbers of COCs vitrified in group are given in parentheses. Percentages with different letters are significantly different (P<0.05). VIT/SC- = COCs vitrified without SC; VIT/SC+ = COCs vitrified with SC.