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

Elisa Caroline da Silva SANTOS[1], Tamas SOMFAI[1], Ruth APPELTANT[2], Thanh Quang DANG-NGUYEN [2], Junko NOGUCHI[2], Hiroyuki KANEKO[2] and Kazuhiro KIKUCHI[2]

[1] Institute of Livestock and Glassland Science, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki 305-0901, Japan

[2]Division of Animal Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki 305-8602, Japan

Correspondence: Tamás SOMFAI, Ph.D
Institute of Livestock and Glassland Science,
National Agriculture and Food Research Organization (NARO),
Ikenodai 2, Tsukuba, Ibaraki 305-0901, Japan
Tel & Fax: +81-298-38-8637, E-mail: somfai@affrc.go.jp

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.

38 **Introduction**

39 Cryopreservation of gametes and embryos keeps cell metabolism quiescent during storage,
40 allowing the subsequent use in programs of assisted reproduction and gene banks formation. Porcine
41 oocyte cryopreservation has potential agricultural and biomedical importance (Zhou & Li 2009).
42 However, this technique in pigs is considered much more difficult comparing with other domestic animal
43 species (Mullen & Fahy 2012) and is yet to be applied in practice (Nohalez *et al.* 2015). Recently it was
44 demonstrated that blastocysts obtained from porcine oocytes cryopreserved at the immature germinal
45 vesicle (GV) stage by solid surface vitrification could develop to term, despite of reduced embryo
46 development (Somfai *et al.* 2014). Vitrification of oocytes at the GV stage is considered as an alternative
47 way to prevent spindle depolymerization or damage often observed during the preservation of matured
48 oocytes, owing to the absence of the meiotic spindle (Moward *et al.* 2012). Matured porcine oocytes are
49 known to survive cryopreservation at higher rates compared with immature ones (Rojas *et al.* 2004;
50 Gupta *et al.* 2007). Nevertheless, previous studies have demonstrated that high rates (over 80 %) of
51 oocyte survival can be achieved even after the vitrification at the GV stage by careful optimization of
52 cryoprotectant treatment regimen (Somfai *et al*. 2013,2015) and warming temperatures (Somfai *et al.*
53 2014). Although reasonable survival rates have been reported after vitrification of the GV stage porcine
54 oocyte (Gupta *et al.* 2007; Nohalez *et al.* 2015; Somfai *et al.* 2014,2015) the embryo developmental
55 ability of surviving oocytes remained low underlining the need to further improvements in vitrification
56 protocols. For this purpose, one possible approach is the application of alternative CPAs in existing
57 vitrification protocols. Polyethylene glycol (PEG) and synthetic ice blockers such as Supercool X-1000
58 (SC) have been used as alternative additives during vitrification to improve survival and developmental
59 rates of oocytes in mice (Fahy *et al.* 2004; O'Neil *et al.* 1997) and horses (de Leon *et al.* 2012). However,
60 to our knowledge PEG and synthetic ice-blockers have not been tested for the vitrification of immature

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

65

66 **Materials and Methods**

67 *Collection of cumulus- oocyte complexes (COCs)*

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

76

77 *Vitrification and warming of COCs*

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

82 µM ß-mercaptoethanol. The medium was further supplemented with 4 mg/mL bovine serum albumin
83 (Fraction V, Sigma-Aldrich) and 7.5 µg/mL cytochalasin B (C-6762, Sigma-Aldrich). Then they were
84 transferred into equilibration solution (ES) comprised of BM supplemented with 7.5 µg/mL cytochalasin
85 B and 4% (v/v) of a permeating CPA combination [ethylene glycol (EG) + Propylene glycol (PG) = 1:1], for
86 5–15 min (Somfai *et al.* 2015) at 38.5 °C. Then, 10–12 COCs were washed 2 times in 50 µL of vitrification
87 solution (VS) in 20 seconds and then they were loaded on Cryotop sheets (Kitazato, Biopharma, Shizuoka,
88 Japan) in minimum volume of VS kept at 38.5 °C and were plunged in liquid nitrogen (LN) (Kuwayama
89 2007). VS was comprised of BM supplemented with 50 mg/mL polyvinyl pyrrolidone (P-0930, Sigma-
90 Aldrich), 0.3 M sucrose (196-00015, Wako Pure Chemical Industries, Osaka, Japan) and 35% (v/v) of EG+
91 PG (1:1, total percentage). The treatment of COCs in VS medium (including washing, loading and
92 removal of excess VS) was performed in 40 seconds. Vitrified samples were stored in LN tank until use.
93 Warming of vitrified COCs was performed according to a previous report (Somfai *et al.* 2015) with slight
94 modifications. In brief, Cryotop devices were immersed directly into 2.5 mL of warming solution (0.4 M
95 Sucrose in BM) in a 35-mm plastic dish (Falcon 351008, Becton Dickinson, Franklin Lakes, NJ, USA) for 1
96 min at 42 °C. The COCs were then consecutively transferred for periods of 1 min (each) to 500-µL
97 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
98 BM without sucrose at 38.0°C and then placed into maturation medium.

99

100 *In vitro maturation (IVM)*

101 After warming, all COCs were subjected to IVM. Oocytes were washed 3 times in 2ml aliquots of pre-
102 incubated IVM medium which was NCSU-37 containing 10% (v/v) porcine follicular fluid, 0.6 mM
103 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).*

125 At the end of IVF, presumptive zygotes were transferred into 2 ml of pre-incubated IVC-PyrLac
126 medium (Kikuchi *et al.* 2002). Spermatozoa and cumulus cells were removed from the surface of the
127 zona pellucida by pipetting through a fine glass pipette. At this time, the live/dead status of the oocytes
128 was assessed morphologically by observation under a stereomicroscope. Survival was evaluated based
129 on the integrity of oolema. Oocytes with normal spherical shape demarcation, smooth surface, dark and
130 eventually granulated were considered live; whereas oocytes that did not any of fit these criteria were
131 categorized as dead. Only live oocytes were subjected to IVC, which was performed in 500-µL of IVC-
132 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
133 39 °C under 5% CO_2, 5% O_2 and 90% N_2 (Kikuchi *et al.* 2002). Cleavage rates were recorded on Day 2,
134 blastocyst rate on Day 7. On Day 2, only cleaved embryos (2–4 cells) were subjected to subsequent
135 culture to obtain embryos with good quality (Dang-Nguyen *et al.* 2010). In the morning of Day 7, the
136 embryos without a visible perivitelline space containing more than 10 blastomeres and a blastocoel
137 were categorized as blastocysts (Somfai *et al.* 2013).

138

139 *Evaluation of blastocyst cell number*

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

146

147 *Experimental design*

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

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

160

161 *Statistical Analysis*

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

165

166 **Results**

167 *Experiment 1*

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

175

176 *Experiment 2*

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

187

188 **Discussion**

189 For vitrification of animal cells including oocytes, CPAs are used at high concentration combined
190 with rapid cooling to eliminate ice crystal formation. However, high concentrations of CPAs also show
191 toxicity and cause cell damage (Best 2015). The composition of CPAs in vitrification medium has a major
192 impact on the success of vitrification on mammalian oocytes as it affects the speed of dehydration, CPA
193 uptake, osmotic stress and other toxic effects (Best 2015). Accordingly, survival rates of immature
194 porcine oocytes during vitrification could be improved by optimizing CPA composition (Somfai *et al.*
195 2013, 2015). The aim of the present study was to test for the first time if supplementation in media
196 during vitrification with alternative non-permeating CPA such as PEG and SC, which were reported to act
197 positively during oocyte cryopreservation in mice and horses (de Leon *et al.* 2012; Fahy *et al.* 2004;
198 O'Neil *et al.* 1997) would affect the outcome of vitrification of GV-stage porcine oocytes.

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

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

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

235 compounds are copolymers that can prevent ice nucleation resulting in increased rates of survival (Fahy
236 *et al.* 2004). The application of synthetic ice blockers such as Supercool X-1000 or Supercool Z-1000
237 during cryopreservation showed promising results matured mouse oocytes (Fahy *et al.* 2004), immature
238 equine oocytes (de Leon *et al.* 2012), mouse ovaries tissues (Tan *et al.* 2012) and rabbit embryos
239 (Marco-Jimenez *et al.* 2014). Supercool X-1000 ice blocker is a copolymer of polyvinyl alcohol, with 20%
240 of vinyl acetate content and would prevent ice formation, in the early stages of ice nucleation, during
241 cooling or warming, even when present in very low concentrations (Wowk *et al.* 2000). While the CPAs
242 prevent ice crystal formation by interacting with the water, Supercools are believed to prevent by
243 molecular recognition of ice nucleators (Wowk 2005). In Experiment 2 of the present study, we applied
244 Supercool X-1000 at 1% (v/v) in the vitrification solution, based on the previous report (Marco-Jimenez
245 *et al.* 2014). Experiment 2 revealed that, Supercool X-1000 did not affect the ratio of post thaw survival
246 of vitrified oocytes but significantly increased the ability of surviving oocytes to cleave after IVF. This
247 suggests that Supercool X-1000 acted positively on oocytes during vitrification not by preventing
248 membrane damage but reducing sub-lethal damages which affect embryo development. The exact
249 mechanism behind this phenomenon remains unclear. Supercool X-1000 is not membrane permeable,
250 therefore is can be suspected that this CPA exerted its positive effect via acting on the extra oocyte
251 compartments of the COCs such as the cumulus cells or the gap junctions between the cumulus cells and
252 oocytes, which are essential for to oocytes to acquire their developmental competence (Nagai *et al.*
253 2006). On the other hand, despite of the significant increase in cleavage rates, Supercool X-1000 did not
254 increase the rate of blastocyst formation after vitrification, which suggests that the vitrification process
255 exerts negative effects on embryo development even beyond the 2-cell stage, irrespective of Supercool
256 X-1000. This suggestion is supported by the fact, that blastocyst developmental competence of cleaved
257 embryos obtained from vitrified oocytes was not different between the groups treated with or without
258 Supercool X-1000 but were lower than that in non-vitrified control (Table 2).

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

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

288

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295

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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)	(% 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)	(% 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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419 **Figures**

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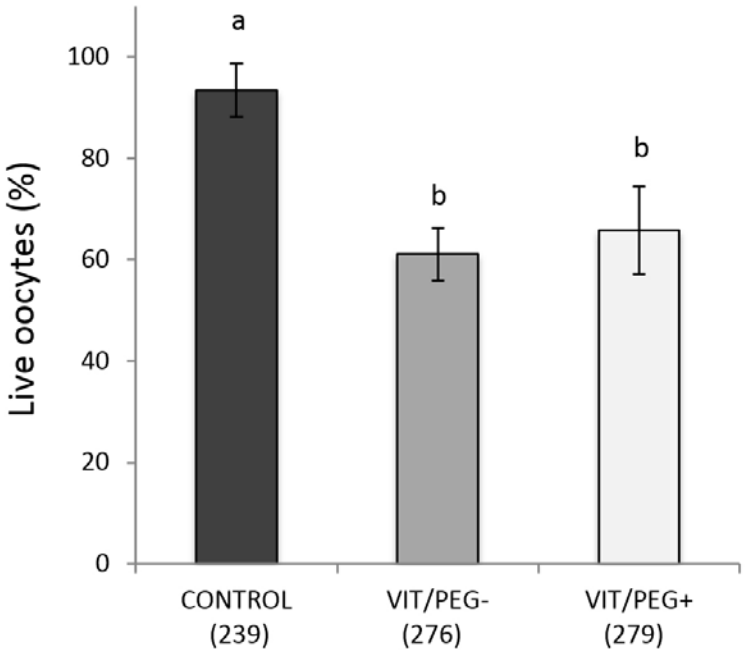
421 **Figure 1.** Survival of COCs vitrified in the presence or absence of 2% (w/v) PEG in equilibration and

422 vitrification solutions. Data are presented as mean ±SEM. Six replications were performed. Total

423 numbers of oocytes vitrified in group are given in parentheses. Percentages with different letters differ

424 significantly (P<0.05). VIT/PEG- = COCs vitrified without PEG; VIT/PEG+ = COCs vitrified with PEG.



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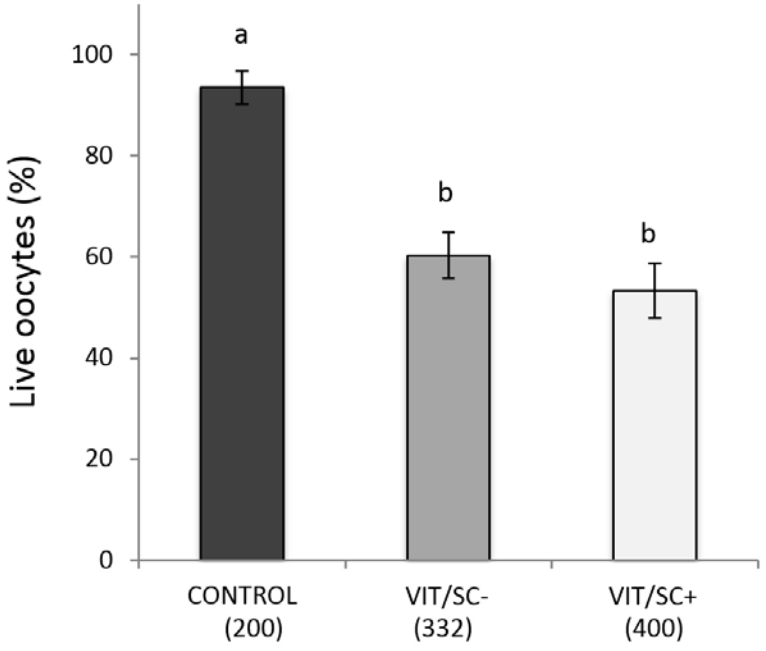
427 **Figure 2.** Survival of COCs vitrified in the presence or absence of 1% (v/v) Supercool X-1000 (SC) in

428 vitrification solution. Data are presented as mean ±SEM. Five replications were performed. Total

429 numbers of COCs vitrified in group are given in parentheses. Percentages with different letters are

430 significantly different (P<0.05). VIT/SC- = COCs vitrified without SC; VIT/SC+ = COCs vitrified with SC.



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