1 **High revivability of vitrified-warmed bovine mature oocytes**

2 **after recovery culture with α-tocopherol**

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13 *Running title:* α-tocopherol rescues vitrified bovine oocytes

Abstract

The objective of this study was to investigate whether developmental competence of vitrified-warmed bovine oocytes can be improved by antioxidant treatment during recovery culture. In experiment 1, one of two antioxidants (either L-ascorbic acid or α-tocopherol) was supplemented into the recovery culture medium to which postwarming oocytes were exposed for 2 h prior to in vitro fertilization (IVF). The exposure to α-tocopherol had a positive effect on rescuing the oocytes as assessed by the blastocyst yield 8 days after the IVF (35.1–36.3% versus 19.2–25.8% in untreated postwarming oocytes). Quality of expanding blastocysts harvested on Day 8 was comparable between α-tocopherol-treated vitrification group and fresh control group in terms of total cell number and chromosomal ploidy. In experiment 2, level of reactive oxygen species, mitochondrial activity and distribution of cortical granules in α-tocopherol-treated postwarming oocytes were assessed. No obvious differences from the control data were found in these parameters. However, the treatment with α-tocopherol increased the percentage of zygotes exhibiting normal single aster formation (90.3% versus 48.0% in untreated postwarming oocytes; 10 h post-IVF). It was concluded that α-tocopherol treatment of vitrified-warmed bovine mature oocytes during recovery culture can improve their revivability, as shown by the high blastocyst yield and the higher mean total cell number in the blastocysts.

Introduction

Oocyte cryopreservation has become an important tool for gamete banking and assisted reproductive technology. Revivability of cryopreserved oocytes from small rodents and humans is extremely high, adapting well to the maintenance of huge number of transgenic strains and the efficient use in therapies for human infertility (Fabbri *et al.* 2000). However, in bovine species, the fertilization rate and subsequent developmental competence of cryopreserved oocytes still need improvement (Ledda *et al.* 2001). Ultrarapid vitrification procedure, originally reported using electron microscope grid as cryodevice (Martino *et al.* 1996), has become a standard approach for cryopreservation of cytoplasmic lipid droplet-enriched bovine oocytes. Due to the development of novel cryodevice such as open-pulled straw (OPS: Vajta *et al.* 1998) or Cryotop (Kuwayama *et al.* 2005) and/or the preloading with low concentration of permeable cryoprotective agent (CPA) (Dinnyés *et al.* 2000, Papis *et al.* 2000), blastocyst yields at >10% have been commonly achieved with vitrified-warmed bovine oocytes (Hwang and Hochi 2014).

Two recent attempts to improve cryosurvival of bovine oocytes include the qualitative improvement of oocytes during in vitro maturation (IVM) prior to the vitrification and the short-term recovery culture of vitrified-warmed oocytes prior to the subsequent in vitro fertilization (IVF). Supplementation of L-carnitine to the IVM medium of bovine oocytes has been reported to redistribute cytoplasmic lipid droplets and improve the cryotolerance of the oocytes after Cryotop vitrification with blastocyst yield of 34%, which was significantly higher than 20% in untreated control (Chankitisakul *et al.* 2013). However there are conflicting reports on the positive effect of L-carnitine on cryosurvival of bovine oocytes

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56   (Phongnimitr *et al.* 2013). Incidence of multiple aster formation, a possible cause for low

57   developmental potential of vitrified-warmed bovine oocytes (Hara et al. 2012), can be

58   inhibited by a short-term culture of the postwarming oocytes in the presence of

59   Rho-associated coiled-coil kinase (ROCK) inhibitor, with a significantly higher blastocyst

60   yield of 21% compared to 14% in untreated control (Hwang *et al.* 2013).

61   High sensitivity of oocytes to cryopreservation is probably due to the large cell size and

62   low permeability of water and CPA (Saragusty and Arav 2011). Depolymerization of

63   microtubules induced by CPA treatment and cryopreservation resulted in disassembly of

64   meiotic spindles and misalignment of chromosomes (Coticchio *et al.* 2009). Treatment with

65   CPA induced a transient increase of intracellular free calcium level, premature exocytosis of

66   cortical granules (CG), and hardening of zonae pellucidae (Larman *et al.* 2006, Kohaya *et al.*

67   2011). Impaired ability of vitrified-warmed bovine oocytes to support the function of

68   microtubule-organizing center (MTOC) was responsible for delay or arrest of pronuclear

69   development (Hara *et al.* 2012). In addition, oxidative stress by reactive oxygen species

70   (ROS) must be one of the causes that may induce lipid peroxidation and/or organelle damage

71   in bovine oocytes (Gutnisky *et al.* 2013). Mechanism of radical scavenger might be involved

72   in the effect of antioxidants (L-ascorbic acid and α-tocopherol) that can improve the

73   blastocyst yield from IVM/IVF oocytes in cattle (Olson and Seidel 2000) and pigs (Kiatagawa

74   *et al.* 2004, Jeong *et al.* 2006, Hossein *et al.* 2007).

75   This study was designed to investigate whether antioxidant L-ascorbic acid or

76   α-tocopherol can rescue vitrified-warmed bovine mature oocytes by the treatment during

77   recovery culture prior to IVF (based on blastocyst yield). Quality of the blastocysts was

78   investigated by counting total cell number and by analyzing chromosomal ploidy.

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79 Furthermore, changes in ROS level, mitochondrial activity, CG distribution, and microtubule

80 assembly were investigated in the α-tocopherol-treated oocytes.

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82 **Materials and Methods**

83

84 **Experimental design**

85 In experiment 1, vitrified-warmed oocytes were randomly allocated to 2-h recovery culture

86 with or without one of the two antioxidants (50 μg/ml L-ascorbic acid or 10 μM α-tocopherol)

87 and then blastocyst formation rates after IVF were investigated. Next, effect of different

88 concentration of α-tocopherol (0, 10, 30, 100 or 300 μM) on developmental potential of the

89 post-warm oocytes into blastocysts was investigated. As a separate experiment in the 300-μM

90 α-tocopherol group, quality of the resultant blastocysts was assessed in terms of total cell

91 number and chromosomal constitution. Chemically untreated postwarming oocytes served as

92 controls in each replicate. In experiment 2, several parameters in oocytes prior to IVF (ROS

93 level, mitochondrial activity, and CG distribution) and in pronuclear-stage zygotes

94 (microtubule assembly: 10 h after IVF) were investigated to clarify the positive effect of the

95 α-tocopherol treatment on rescuing the vitrified-warmed oocytes. Data were compared

96 between 0 and 300-μM α-tocopherol groups, as well as non-vitrified fresh control group.

97

98 **Preparation of mature oocytes**

99 Unless otherwise indicated, all chemicals used in the present study were purchased from

100 Sigma-Aldrich Chemicals (St. Louis, MO, USA). Local abattoir-derived bovine ovaries were

101 transported to the laboratory in 20–24 ˚C saline within 6 h after slaughter. The contents of

102   follicles (diameter, 2–8 mm) were aspirated with an 18-G needle connected to a 10-ml syringe.

103   Oocytes surrounded with at least two layers of compact cumulus cells were matured in 100-μl

104   microdrops of Hepes-buffered Tissue Culture Medium (TCM)-199 (Earle salts; Gibco-BRL,

105   Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; SAFC

106   Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/ml follicle-stimulating

107   hormone (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 μg/ml 17β-estradiol, and

108   50 μg/ml gentamicin sulfate for 22 h at 38.5 ˚C under 5% CO_2 in air (10–12

109   oocytes/microdrop). Then, cumulus cells were removed by vortex-mixing for 3 min in the

110   Hepes-buffered TCM-199 supplemented with 3 mg/ml bovine serum albumin (BSA), 0.2 mM

111   sodium pyruvate, 1,000 IU/ml hyaluronidase, and 50 μg/ml gentamicin sulfate. Oocytes were

112   comprehensively checked for extrusion of the first polar body, and oocytes with an extruded

113   first polar body were defined as matured.

114

115   **Vitrification and warming**

116   Mature oocytes were subjected to a vitrification procedure according to the method described

117   previously (Hwang *et al.* 2013). Briefly, oocytes were equilibrated with 7.5% ethylene glycol

118   (EG; Wako Pure Chemical Industries, Osaka, Japan) and 7.5% dimethyl sulfoxide (DMSO;

119   Wako Pure Chemical Industries) in Hepes-buffered TCM-199 with 20% FBS base medium

120   for 3 min at room temperature (23–28 ˚C) and then transferred into a vitrification solution

121   consisting of 15% EG, 15% DMSO, and 0.5 M sucrose in the base medium for approximately

122   60 s at the room temperature. Within this 60-s period, up to 15 oocytes were loaded onto the

123   polypropylene strip of a Cryotop device (Kitazato BioPharma, Shizuoka, Japan) with a

124   minimal amount of the vitrification solution (< 0.1 µl) and then quickly plunged into liquid

125   nitrogen (LN$_2$).

126   After storage for more than 1 week in the LN2, oocytes were warmed by immersing the

127   polypropylene strip of a Cryotop into 3 ml of the base medium containing 1 M sucrose

128   prewarmed to 38.5 ˚C for 1 min. The oocytes were transferred to the base medium at the room

129   temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 5, and 5 min,

130   respectively). According to the manufacturer's instruction, the predicted cooling and warming

131   rates of the Cryotop procedure were 23,000 and 42,000 ˚C /min, respectively.

132

133   **Recovery culture**

134   Postwarming oocytes were rinsed three times and cultured in 500-µl of Hepes-buffered

135   TCM-199 plus 5% FBS, 0.2 mM sodium pyruvate, and 50 µg/ml of gentamicin sulfate in a

136   4-well dish for 2 h at 38.5 ˚C under 5% CO_2 in air (15–30 oocytes/well). Depending on the

137   experimental series, the culture medium was supplemented with or without 50 µg/ml

138   L-ascorbic acid ([+]-sodium L-ascorbate; $C_6H_7NaO_6$, molecular weight = 198.11), or 10, 30,

139   100 or 300 µM α-tocopherol ([±]-α-tocopherol; $C_{29}H_{50}O_2$, molecular weight = 430.71).

140

141   **In vitro fertilization and culture**

142   Commercially available frozen semen of a Japanese black bull was used for IVF. After

143   thawing at 37 ˚C for 30 s, the contents of a 0.5-ml straw were layered on the top of a Percoll

144   density gradient consisting of 2 ml of 45% Percoll above 2 ml of 90% Percoll in a 15-ml

145   conical tube and then centrifuged for 20 min at $700 \times g$. The sperm pellet was resuspended in

146   4 ml of modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional

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147   Peptides, Yamagata, Japan) supplemented with 5 mM theophylline, washed twice (5 min at

148    $300 \times g$ each time), and then resuspended in the mBO medium supplemented with 5 mg/ml

149   BSA and 10 µg/ml heparin (IVF medium) to yield a concentration of 4×10^7 sperm cells/ml.

150   Ten to 12 oocytes in the IVF medium were coincubated with the above-mentioned sperm

151   suspension at a final concentration of 8×10^6 sperm cells/ml for 6 h in a 100-µl microdrop

152   under mineral oil at 38.5 ˚C under 5% CO_2 in air.

153   Up to 30 presumptive zygotes (6 h postinsemination [hpi]) were cultured in a 250-µl

154   microdrop of modified synthetic oviduct fluid (mSOF; Holm *et al.* 1999) supplemented with

155   30 µl/ml of essential amino acids solution (50 ×; 11130; Gibco), 10 µl/ml of non-essential

156   amino acids solution (100 ×; 11140; Gibco), and 5% FBS (defined hereafter as mSOFaa with

157   5% FBS) at 39.0 ˚C under 5% CO_2, 5% O_2, and 90% N_2. The cleavage rate was determined

158   on Day 2 (Day 0 = day of IVF), and the appearance of expanded blastocysts was recorded on

159   Day 7 and Day 8.

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161   **Blastocyst cell number**

162   Fully expanded blastocysts harvested on Day 8 were analyzed for total cell number.

163   Blastocysts were washed three times in PBS supplemented with 0.1% polyvinylpyrrolidone

164   (PVP) and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. The

165   blastocysts were then stained with 10 µg/ml Hoechst 33342 for 10 min in a dark condition.

166   After being washed three times in PBS with 0.1% PVP and mounted with coverslip in

167   antifade agent (100 mg 1,4-diazabicyclo[2.2.2]octane in 1 ml glycerol), the total cell number

168   was determined under an epifluorescence microscope.

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Blastocyst ploidy

For chromosomal preparation, Day 8 expanded blastocysts were further cultured for 17 h in the mSOFaa with 5% FBS containing 30 ng/ml vinblastine sulfate as a mitotic inhibitor at 38.5 ˚C under 5% CO_2 in air (Yoshizawa *et al.* 1998). The blastocysts were then transferred in a 400-µl hypotonic solution of 1% sodium citrate for 15–23 min, and fixed mildly by adding 20-µl acetic acid:ethanol mixture (1:1) into the hypotonic solution. After 5 min, each blastocyst was placed onto a slide glass, and blastom

eres were separated by adding a small amount of acetic acid. Finally, chromosome preparations were fixed with several drops of acetic acid:ethanol mixture, air-dried overnight, and stained with 4% Giemsa solution (diluted with PBS [pH6.8]; Wako) for 10 min. The chromosomal preparations were observed for ploidy under a light microscope with a magnification of × 400.

ROS level

To measure the change in ROS level, oocytes after recovery culture were washed three times in PBS with 0.1% PVP and incubated for 30 min with 10 µM 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) suspended in PBS in a dark condition. Then, the oocytes were rinsed three times in PBS with 0.1% PVP and mounted with coverslips in the antifade agent. Digital images were collected at 2 µm distance and stacked using a confocal laser scanning microscope (FV1000-D; Olympus, Tokyo, Japan). The fluorescence intensity of each oocyte was measured using Image-J software (National Institutes of Health, Bethesda, ML, USA; accessed online). In each replicate, the mean value

192 in the vitrified-warmed control group was defined as 1.0, and the relative values were given

193 for those in the fresh control and 300 µM α-tocopherol groups.

194

195 **Mitochondrial activity**

196 To measure mitochondrial activity of oocytes after recovery culture, oocytes were washed

197 three times in PBS with 0.1% PVP, fixed in 4% PFA for 30 min at room temperature, and then

198 incubated for 15 min in PBS with 0.1 µg/ml of MitoTracker Red CMXRos (Lonza

199 Walkerscille Inc., Walkerscille, MD, USA) in a dark condition. Then, the

200 mitochondria-labeled oocytes were rinsed three times in PBS with 0.1% PVP and mounted

201 with coverslips in the antifade agent. The fluorescence intensity of each oocyte at the largest

202 diameter was measured using the Image-J analysis software under the confocal laser-scanning

203 microscope. In each replicate, the mean value in the vitrified-warmed control group was

204 defined as 1.0, and the relative values were given for those in the fresh control and 300 µM

205 α-tocopherol groups.

206

207 **CG distribution**

208 Oocytes after recovery culture were fixed in 4% PFA for 30 min at room temperature, after

209 zonae pellucidae had been removed in M2 medium (Quinn *et al.* 1982) with 0.75% protease

210 (Pronase™; Calbiochem, Darmstadt, Germany). Membranes of the oocytes were

211 permeabilized overnight in PBS with 0.1% Triton X-100 and 0.3% BSA at 4 ˚C, and then the

212 oocytes were incubated for 15 min in PBS with 100 ng/ml fluoresceine isothiocyanate

213 (FITC)-conjugated *lens culinari*s agglutinin (LCA; Vector Laboratories, Burlingame, CA,

214 USA) in a dark condition. The oocytes were rinsed three times in PBS with 0.1% PVP and

215   mounted with coverslips in the antifade agent. Digital images were collected using the

216   confocal laser-scanning microscope. According to the categories previously reported (Goud *et*

217   *al*. 2005), distribution of the CGs periphery to oolemma of each oocyte was classified either

218   as intact, minor loss, or major loss (Fig. 1).

219

220   **Microtubule assembly**

221   To assess microtubule assembly in pronuclear-stage zygotes, inseminated oocytes were

222   cultured for an additional 4 h in TCM-199 with 20% FBS at 38.5 ˚C under 5% CO_2 in air, and

223   then immunostained according to the method described previously (Hara *et al*. 2011). The

224   zygotes were extracted for 15 min by buffer M (25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$

225   0.1 mM EDTA, 1 mM EGTA and 50 mM imidazole hydrochloride, pH 6.8) that contained

226   5% methanol and 1% Triton X-100, after zonae pellucidae had been removed in M2 medium

227   with 0.75% protease. The zygotes were then fixed in PBS with 4% PFA for 45 min and

228   permeabilized overnight in PBS with 0.1% Triton X-100. Microtubules were labeled with a

229   monoclonal antibody against α-tubulin (T5168; diluted 1:1000). The primary antibodies were

230   detected by FITC-conjugated goat anti-mouse IgG (F1010; diluted 1:200). Nuclear DNA was

231   visualized by counterstaining with 2.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI).

232   Preparations were mounted with coverslips in antifade agent, and digital images collected at 2

233   µm distance were stacked using the confocal laser scanning microscope and assessed with the

234   Image-J software. Zygotes with 2 pronuclei (2PN) were defined as those that fertilized

235   normally, and classified either those with single sperm aster or multiple sperm asters (Fig. 2).

236

237   **Statistics**

238   Experiments were replicated at least four times in each group. Percentage data for cleavage

239   and blastocyst development were arcsin-transformed and compared to untreated postwarming

240   controls by paired Student's *t*-test following Bonferroni correction. Blastocyst cell number

241   data, relative data for ROS level and mitochondrial activity, and arcsin-transformed

242   percentage data for intact CG status and single aster formation were compared by one-way

243   analysis of variance (ANOVA). When the ANOVA was significant, differences among means

244   were analyzed by Tukey's test. A value of $P < 0.05$ was defined as a significant difference,

245   except in cases of Bonferroni correction.

246

247   **Results**

248

249   **Blastocyst development of oocytes after recovery culture**

250   All the postwarming oocytes in 5 replicates (n = 320) appeared morphologically normal and

251   were cultured for 2 h with or without antioxidant supplementation prior to IVF. In addition to

252   these vitrified oocytes, fresh oocytes (n = 105) were subjected to the IVF protocol. A

253   significant difference ($P < 0.017$) was detected in Day 8 blastocyst yield between the

254   untreated control and α-tocopherol-treated groups (25.8% versus 36.3%), while no significant

255   differences were detected in Day 2 cleavage rate (Table 1). Supplementation of L-ascorbic

256   acid to recovery culture medium had no effect on rescuing the vitrified-warmed oocytes

257   (blastocyst yield at Day 8, 27.5%).

258   In the next experiment to examine the higher concentrations of α-tocopherol, all the

259   postwarming oocytes (n = 638) were subjected to 6 replicates of recovery culture, and IVF

260   with fresh control oocytes was carried out in parallel (n = 130). Blastocyst yield on Day 8 was

261 significantly higher (P < 0.01) than the control when the highest concentration of α-tocopherol

262 (300 μM) was supplemented (35.1% versus 19.2%; Table 2). Supplementation of the lower

263 concentrations of α-tocopherol (10, 30 and 100 μM) resulted in improved yields, but with no

264 significant differences from the control (26.9 to 30.8% versus 19.2%, P = 0.104 to 0.251).

265

266 **Quality analyses of the blastocysts**

267 Mean total cell number of blastocysts derived from postwarming oocytes after

268 α-tocopherol-free recovery culture (107.8, n = 21) was significantly lower (P < 0.05) than that

269 of fresh control blastocysts (158.0, n = 25), as shown in Fig. 3. Mean total cell number of

270 blastocysts derived from postwarming oocytes with recovery culture in 300 μM α-tocopherol

271 (143.4, n = 14) did not differ from that of the fresh control blastocysts. The difference in mean

272 total cell number between 0 μM α-tocopherol-treated and 300 μM α-tocopherol-treated

273 groups (both for vitrified oocytes) was significant (P < 0.05).

274   In chromosomal ploidy analysis, the proportions of normal diploid blastocysts (2n = 60)

275 were 84.6% (11/13), 81.3% (13/16) and 100% (15/15) in fresh control, 0 μM α-tocopherol

276 and 300 μM α-tocopherol groups, respectively. Abnormal blastocysts included 1 tetraploid

277 and 1 mixploid (tetraploid/diploid) in the fresh control group, and 1 triploid, 1 mixploid (2n =

278 50/60) and 1 aneuploid (2n = 83) in the 0 μM α-tocopherol group.

279

280 **Changes detected in oocytes or pronuclear zygotes**

281 Three parameters were traced in oocytes immediately after recovery culture. Postwarming

282 oocytes treated for 2 h with 300 μM α-tocopherol showed decreased level of ROS (relative

283 value 0.73, n = 30) when compared with 0 μM α-tocopherol group (1.00, n = 31), but oocyte

13

284   vitrification procedure employed here was not the critical factor increasing the intracellular

285   ROS level as fresh control value (0.98, n = 30) (Fig. 4A). The relative mitochondrial activities

286   were comparable among fresh reference (0.89, n = 26), 0 μM α-tocopherol (1.00, n = 23) and

287   300 μM α-tocopherol (0.84, n = 23) groups (Fig. 4B). In addition, distribution pattern of the

288   CG was similar among the fresh reference (intact 57.1%, intact + minor loss 82.0%, n = 51), 0

289   μM α-tocopherol (intact 57.6%, intact + minor loss 79.1%, n = 52) and 300 μM α-tocopherol

290   (intact 58.0%, intact + minor loss 84.0%, n = 50) groups (Fig. 4C).

291   Pronuclear-stage zygotes at 10 hpi were immunostained against α-tubulin to assess their

292   cytoplasmic potential for microtubule assembly. Normal fertilization rates in the

293   vitrified-warmed oocytes (57.7 and 59.1% in 0 and 300 μM α-tocopherol groups,

294   respectively) were not different from that in the fresh reference group (60.6%; Table 3).

295   Regardless of vitrification, proportions of the 2PN zygotes forming one or more sperm asters

296   were also comparable among the groups (94.2 to 98.3%). A higher incidence of multi-aster

297   formation in vitrified-warmed oocytes (52.0% versus 15.3% in fresh reference group; $P <$

298   0.05) was observed when the oocytes were not treated with α-tocopherol during the recovery

299   culture. However, the multi-aster formation in vitrified-warmed oocytes was inhibited when

300   the oocytes were treated with α-tocopherol in the postwarming culture (9.7%). Inversely, the

301   single-aster formation rate of vitrified-warmed oocytes in the α-tocopherol treated group

302   (90.3%) was similar to that of fresh reference oocytes (84.7%).

303

304   **Discussion**

305

306   A short-term culture of vitrified-warmed bovine oocytes with α-tocopherol contributed to

307   inhibit high incidence of multiple aster formation (Table 3) and improve development into

308   normal blastocysts (Tables 1, 2 and Fig. 3). These results are similar to those described in our

309   previous report (Hwang *et al.* 2013) where ROCK inhibitor was used to rescue the

310   vitrified-warmed bovine oocytes. The high revivability obtained here (maximum Day-8

311   blastocyst yield at 36.3%) is partially due to the improved baseline of blastocyst yield by

312   availability of the fresh (=within 6 h after slaughter) bovine ovaries, when compared to the

313   previous report using 1-day-stored ovaries (21.4%; Hwang *et al.* 2013). Higher incidence of

314   multiple-aster formation was observed in vitrified-warmed bovine oocytes after IVF, and

315   pronuclear development and migration were delayed in the zygotes with multiple-aster

316   formation (Hara *et al.* 2012). In most mammalian species except for rodents, spermatozoal

317   centrosome plays a critical role in assembly of microtubule network (sperm aster) that brings

318   both male and female pronuclei to the center of the newly formed zygote (Navara *et al.* 1996).

319   Thus, the centrosome is considered to be the MTOC, with duplication during the pronuclear

320   stage and the subsequent separation to serve as mitotic centers anchoring the chromosomes

321   during the first cleavage (Chen *et al.* 2003, Schatten and Sun 2009). Abnormalities of the

322   spindle, MTOC function and sperm aster have been shown to directly correlate with the loss

323   of developmental potential after IVF, because they are crucial for completion of the second

324   meiosis, extrusion of the polar body, migration of the pronuclei, and formation of the first

325   mitotic spindle (Schatten *et al.* 1985).

326   Interestingly in contrast to α-tocopherol, supplementation of L-ascorbic acid into the

327   recovery culture medium had no effect on improvement of blastocyst yield from

328   vitrified-warmed mature bovine oocytes (Table 1). We have also reported that chemical

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329   treatment of bovine oocytes to increase intracellular glutathione level, which also acts as a

330   radical scavenger, did not alter their cryotolerance (Hara *et al.* 2014). These differences might

331   be associated with the affinity of antioxidants, since the hydrophobic α-tocopherol protects

332   lipid bilayer of the plasma membrane from oxidative stress (Gutnisky *et al.* 2013). Oxidative

333   stress increases membrane fluidity through cleavage of the lipid acyl chain (Tai *et al.* 2010).

334   Such alterations in membrane property might disturb uptake of extracellular components

335   essential for supporting physiological aster formation. On the other hand, no significant

336   changes were detected in intracellular ROS level between fresh and untreated postwarming

337   oocytes although α-tocopherol treatment suppressed the ROS formation (Fig. 4A). In some

338   independent experiments for comparison between fresh control oocytes and postwarming

339   non-cultured oocytes, no differences were found in the ROS level (data not shown). This

340   phenomenon is not coincident with recent report where intracellular ROS level increased in

341   mouse blastocysts vitrified-warned using nylon loop as cryodevice (Martino *et al.* 2013).

342   During vitrification of bovine oocytes using Cryotop device, oxidative stress might

343   concentrate only to the plasma membrane.

344   Two other parameters (mitochondrial activity and CG distribution) were investigated to

345   understand the possible role of α-tocopherol in rescuing vitrified-warmed bovine oocytes.

346   Mitochondrial activity in postwarming bovine oocytes was fully restored during the recovery

347   culture (Fig. 4B), as shown by our previous time-dependent observation (Hwang *et al.* 2013).

348   Maintenance of ATP contents in bovine IVM oocytes by cyclosporine treatment can improve

349   parthenogenetic development into blastocysts after oocyte vitrification (Zhao *et al.* 2011). No

350   significant changes were also detected between α-tocopherol-treated and untreated

351   postwarming oocytes in CG distribution (Fig. 4C). Premature release of CG has been

352 observed in cryopreserved oocytes and considered to be responsible for the failure of sperm

353 penetration or the incidence of polyspermic fertilization (Carroll *et al.* 1990, Fuku *et al.* 1995).

354 In preliminary trials, CG distribution pattern difficult to be categorized as in Fig. 1 was

355 observed in bovine oocytes immediately after warming (data not shown). Ultrastructure of

356 bovine oocytes immediately after OPS vitrification and warming indicated the presence of

357 small membrane-bound vesicles and the partial lack of CGs aligned along the oolemma

358 (Hyttel *et al.* 2000). Same authors also noted that postwarming oocytes after additional 2 h

359 culture had less abundant small vesicles and more degenerating CG clusters.

360   Two quality analyses of blastocysts (total cell number and chromosomal ploidy) did not

361 show any disadvantageous features in Day 8 blastocysts derived from α-tocopherol-treated

362 postwarming oocytes when compared to fresh control blastocysts. There might be a larger

363 part of blastocysts developed from multiaster-formed zygotes, which had suffered delayed

364 pronuclear development and migration, in untreated control group. However, increased total

365 cell number of Day 8 blastocysts derived from α-tocopherol-treated versus untreated

366 postwarming oocytes (Fig. 3) is not explained by their different developmental kinetics until

367 Day 8, because Hoechst-staining was performed only for fully expanding blastocysts. This

368 difference may be due to improved ooplasmic environment including elevation of MPF

369 activity (Motlik & Kubelka 1990), which can organize normal microtubule assembly after the

370 subsequent IVF. Any possible epigenetic differences, which might result from α-tocopherol

371 treatment, have not been investigated using the resultant blastocysts in the present study, and

372 remained to be investigated in future. Ultimate quality analysis of vitrified-warmed bovine

373 oocytes is undoubtedly the production of live calves following transfer of the resultant

374 blastocysts into uteri of recipient cows. Proof whether the α-tocopherol treatment for

375 post-warming oocytes is not only supportive for higher formation of blastocysts, but also for

376 normal offspring production would be of great impact in the veterinary field.

377   In conclusion, α-tocopherol treatment of bovine IVM oocytes during recovery culture

378 after Cryotop vitrification can improve their revivability, as shown by the high blastocyst

379 yield and the higher mean total cell number in the blastocysts.

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385 **Declaration of interest**

386 The authors have no conflict of interest to declare.

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Table 1 Effect of L-ascorbic acid and α-tocopherol during recovery culture on developmental competence of vitrified-warmed oocytes

Chemicals	No. (%) of oocytes		No. (%) of blastocysts	
	Inseminated	Cleaved	Harvested on Day 7	Harvested on Day 7+8
Untreated control	108	89 (83.3 ± 6.0)	22 (20.5 ± 6.0) [a]	28 (25.8 ± 6.6) [a]
L-ascorbic acid	106	75 (70.9 ± 2.7)	30 (22.6 ± 2.7) [a]	30 (27.5 ± 2.5) [a]
α-tocopherol	106	73 (69.6 ± 2.9)	24 (23.0 ± 2.9) [a]	38 (36.3 ± 5.2) [b]
Fresh control	105	79 (75.4 ± 1.9)	43 (41.3 ± 1.9) [b]	55 (52.6 ± 5.5) [b]

Percentages are expressed as mean ± SE of 5 replicates in each group.

[a,b] Different superscripts within columns denote significant difference from untreated control group (paired Student's t-test with Bonferroni correction, $P < 0.017$).

Table 2 Effect of different α-tocopherol concentrations during recovery culture on developmental competence of vitrified-warmed oocytes

Concentration of α-tocopherol (μM)	No. (%) of oocytes		No. (%) of blastocysts	
	Inseminated	Cleaved	Harvested on Day 7	Harvested on Day 7+8
0 (untreated control)	125	103 (82.5 ± 2.2)	15 (12.1 ± 3.5) [a]	24 (19.2 ± 4.7) [a]
10	127	108 (85.1 ± 2.2)	24 (17.3 ± 1.5) [a]	39 (30.8 ± 4.9) [a]
30	129	108 (83.7 ± 1.1)	26 (20.2 ± 4.8) [a]	36 (27.9 ± 3.6) [a]
100	131	104 (79.5 ± 4.6)	24 (18.4 ± 3.9) [a]	35 (26.9 ± 4.4) [a]
300	126	110 (87.4 ± 3.1)	30 (23.8 ± 2.1) [a]	44 (35.1 ± 3.0) [b]
Fresh control	130	101 (77.7 ± 4.3)	66 (50.6 ± 5.9) [b]	78 (60.0 ± 2.7) [b]

Percentages are expressed as mean ± SE of 6 replicates in each group.

[a,b] Different superscripts within columns denote significant difference from untreated control group (paired Student's t-test with Bonferroni correction, $P < 0.01$).

Table 3 Effect of α-tocopherol treatment during recovery culture on sperm aster formation of vitrified-warmed and in vitro-fertilized oocytes

Groups	No. (%) of oocytes		No. (%) of aster-forming zygotes		
	Inseminated	Fertilized: 2PN	Total	Single aster	Multiple asters
Untreated control	52	28 (57.7 ± 2.5)	27 (96.0 ± 4.0)	13 (48.0 ± 2.0) [a]	14 (52.0 ± 2.0) [a]
α-tocopherol-treated	50	33 (59.1 ± 3.4)	31 (94.2 ± 3.6)	28 (90.3 ± 4.1) [b]	3 (9.7 ± 4.1) [b]
Fresh control	94	58 (60.6 ± 5.4)	67 (98.3 ± 1.7)	58 (84.7 ± 4.5) [b]	9 (15.3 ± 4.5) [b]

Percentages are expressed as mean ± SE of 6 replicates in each group.

[a,b] Different superscripts within columns denote significant differences among groups (Tukey's test, $P < 0.05$).
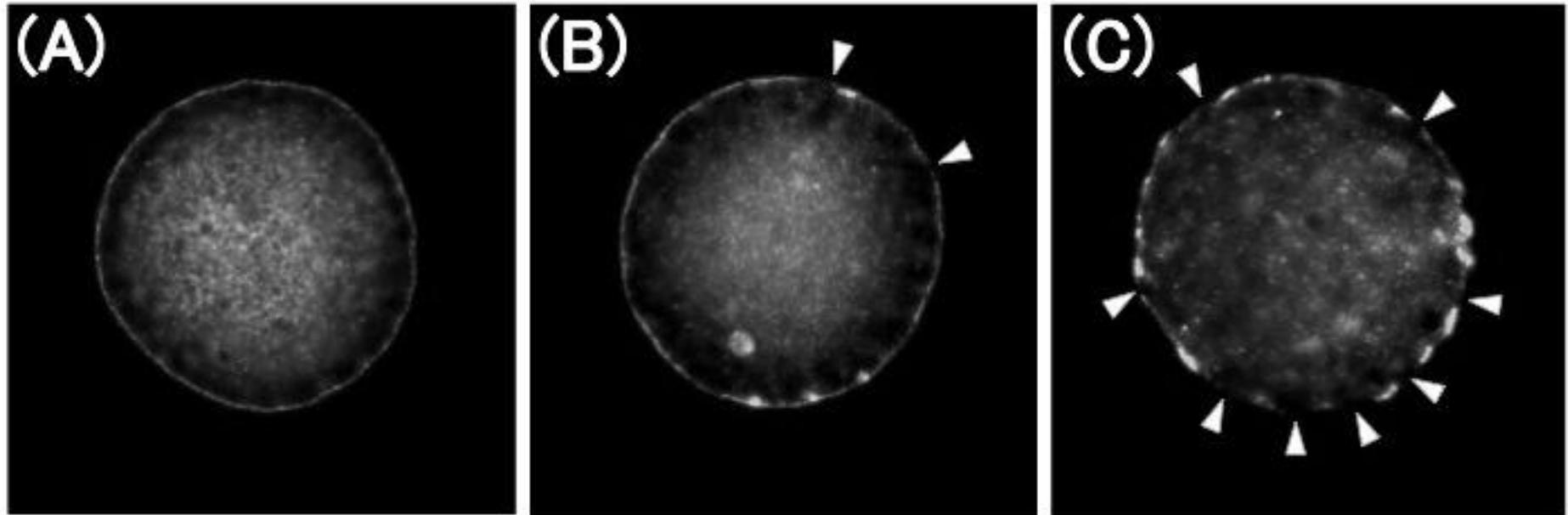
Figure legends

Figure 1 Classification of peripheral cortical granule (CG) distribution. A: Intact CG alignment periphery to oolemma (100% stained). B: Minor CG loss ($\geq$ 90% stained). C: Major CG loss (< 90% stained). Arrowheads indicate periphery areas without CG alignment.

Figure 2 Pronuclear zygotes immunostained against α-tubulin (green) and counterstained with DAPI (blue). A: Normal zygote with a single sperm aster. B: Abnormal zygote with multiple sperm asters.
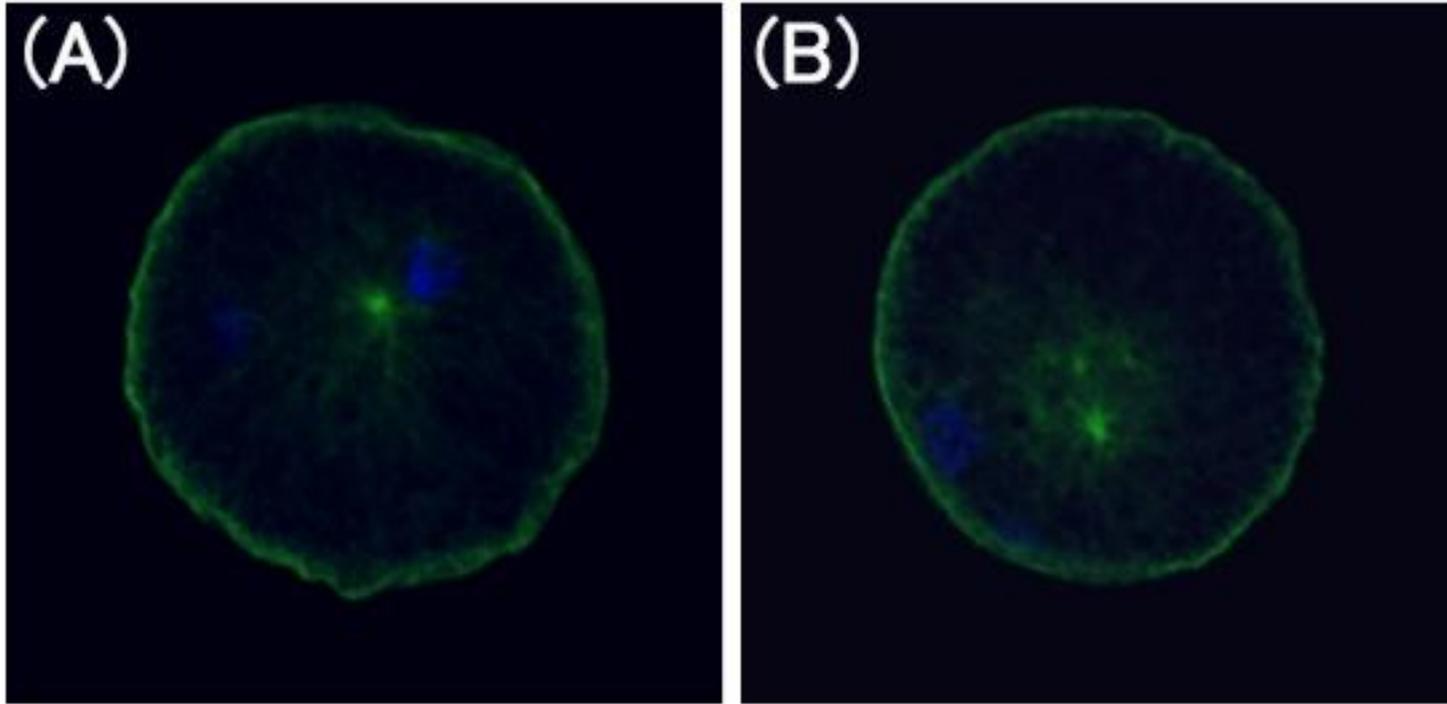
Figure 3 Total cell number of Day 8 expanded blastocysts derived from fresh oocytes, as well as vitrified-warmed oocytes after recovery culture with or without α-tocopherol. Different letters show significant differences (P < 0.05).

Figure 4 Level of reactive oxygen species (A), mitochondrial activity (B), and distribution of cortical granules (C) in vitrified-warmed oocytes after recovery culture with or without α-tocopherol. Categories in panel-C; Black column: Intact, Gray column: Minor loss, White column: Major loss.
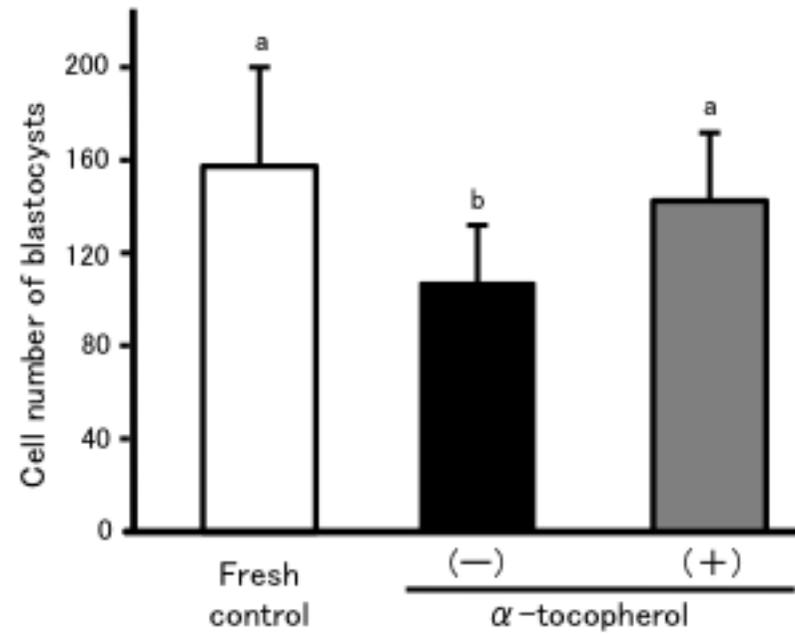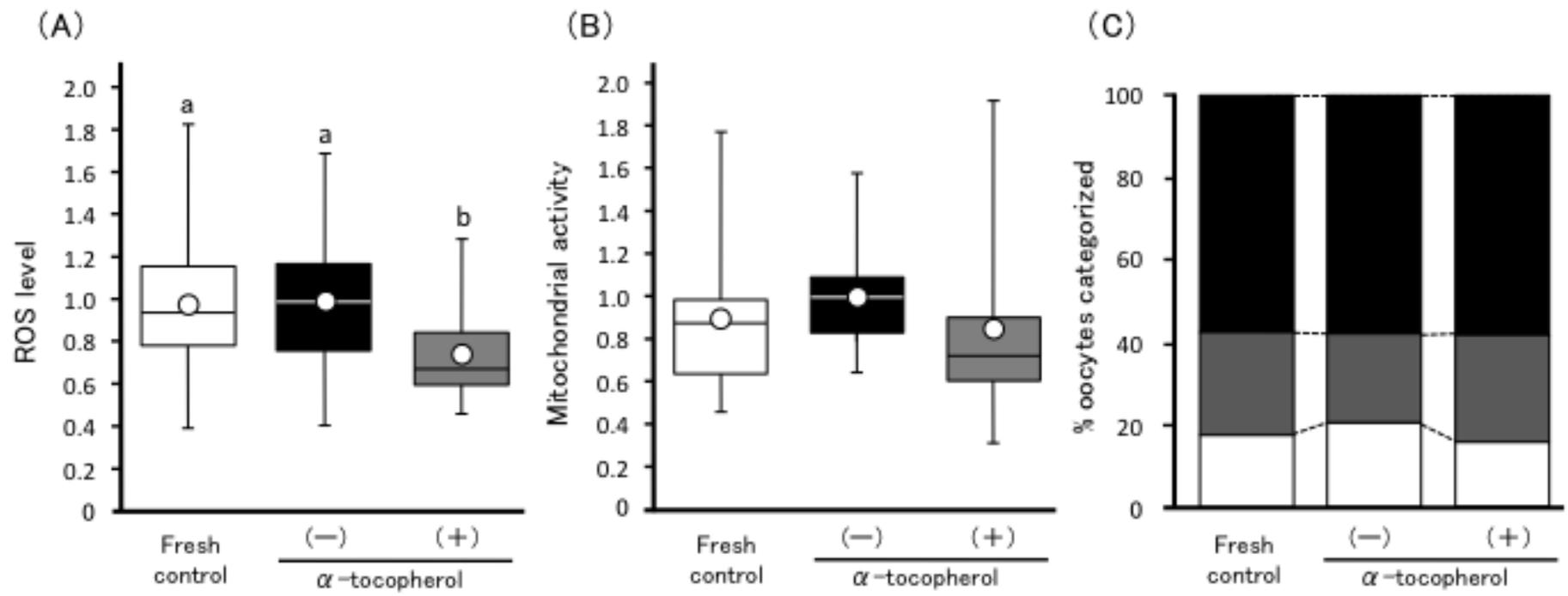
(Figure 1)

28

(Figure 2)

(Figure 3)

（A）

（B）

（C）

(Figure 4)