High revivability of vitrified–warmed bovine mature oocytes after recovery culture with α -tocopherol

Ikuko Yashiro¹, Miho Tagiri¹, Hayato Ogawa², Kazuya Tashima², Seiji Takashima^{1,2}, Hiromasa Hara³, Masumi Hirabayashi³ and Shinichi Hochi^{1,2}

¹Graduate School of Science and Technology and ²Faculty of Textile Science and Technology, Shinshu University, Nagano 386-8567, Japan and ³National Institute for Physiological Sciences, Aichi 444-8787, Japan

Correspondence should be addressed to S Hochi; Email: shochi@shinshu-u.ac.jp

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 the two antioxidants (either L-ascorbic acid or α -tocopherol) was added as a supplement to the recovery culture medium to which postwarming oocytes were exposed for 2 h before 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% vs 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% vs 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. *Reproduction* (2015) **149** 347–355

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 an electron microscope grid as cryodevice (Martino *et al.* 1996), has become a standard approach for cryopreservation of cytoplasmic lipid droplet-enriched bovine oocytes. Owing to the development of novel cryodevices such as open-pulled straw (OPS; Vajta *et al.* 1998) or Cryotop (Kuwayama *et al.* 2005) and/or the preloading with a 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 & Hochi 2014).

Two recent attempts to improve cryosurvival of bovine oocytes include the qualitative improvement of oocytes

during *in vitro* maturation (IVM) before the vitrification and the short-term recovery culture of vitrified–warmed oocytes before the subsequent 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 a 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 (Phongnimitr *et al.* 2013). Incidence of multiple aster formation, a possible cause for low developmental potential of vitrified–warmed bovine oocytes (Hara *et al.* 2012), can be inhibited by a short-term culture of the postwarming oocytes in the presence of Rho-associated coiled-coil kinase (ROCK) inhibitor, with a significantly higher blastocyst yield of 21% compared with 14% in untreated control (Hwang *et al.* 2013).

High sensitivity of oocytes to cryopreservation is probably due to the large cell size and low permeability of water and CPA (Saragusty & Arav 2011). Depolymerization of microtubules induced by CPA treatment and cryopreservation resulted in disassembly of meiotic spindles and misalignment of chromosomes

(Coticchio *et al.* 2009). Treatment with CPA induced a transient increase in intracellular free calcium level, premature exocytosis of cortical granules (CGs), and hardening of zonae pellucidae (Larman *et al.* 2006, Kohaya *et al.* 2011). Impaired ability of vitrified-warmed bovine oocytes to support the function of microtubule-organizing center (MTOC) was responsible for delay or arrest of pronuclear development (Hara *et al.* 2012). In addition, oxidative stress by reactive oxygen species (ROS) must be one of the causes that may induce lipid peroxidation and/or organelle damage in bovine oocytes (Gutnisky *et al.* 2013). Mechanism of radical scavenger might be involved in the effect of antioxidants (α -ascorbic acid and α -tocopherol) that can improve the blastocyst yield from IVM/IVF oocytes in cattle (Olson & Seidel 2000) and pigs (Kitagawa *et al.* 2004, Jeong *et al.* 2006, Hossein *et al.* 2007).

This study was designed to investigate whether antioxidant α -ascorbic acid or α -tocopherol can rescue vitrified-warmed bovine mature oocytes by treatment during recovery culture before IVF (based on blastocyst yield). Quality of the blastocysts was investigated by counting total cell number and by analyzing chromosomal ploidy. Furthermore, changes in ROS level, mitochondrial activity, CG distribution, and microtubule assembly were investigated in the α -tocopherol-treated oocytes.

Materials and methods

Experimental design

In experiment 1, vitrified-warmed oocytes were randomly allocated to 2-h recovery culture with or without one of the two antioxidants (50 μ g/ml α -ascorbic acid or 10 μ M α -tocopherol) and then blastocyst formation rates after IVF were investigated. Then, effect of different concentrations of α -tocopherol (0, 10, 30, 100, or 300 μ M) on developmental potential of the postwarm oocytes into blastocysts was investigated. As a separate experiment in the 300- μ M α -tocopherol group, quality of the resultant blastocysts was assessed in terms of total cell number and chromosomal constitution. Chemically untreated postwarming oocytes served as controls in each replicate. In experiment 2, several parameters in oocytes before IVF (ROS level, mitochondrial activity, and CG distribution) and in pronuclear-stage zygotes (microtubule assembly: 10 h after IVF) were investigated to clarify the positive effect of the α -tocopherol treatment on rescuing the vitrified-warmed oocytes. Data were compared between 0 and 300- μ M α -tocopherol groups, as well as non-vitrified fresh control group.

Preparation of mature oocytes

Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals. Local abattoir-derived bovine ovaries were transported to the laboratory in 20–24 °C saline within 6 h after slaughter. The contents of follicles (diameter, 2–8 mm) were aspirated with an 18-G needle connected to a 10-ml syringe. Oocytes surrounded by at least

two layers of compact cumulus cells were matured in 100- μ l microdrops of HEPES-buffered tissue culture medium (TCM)-199 (Earle salts; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/ml follicle-stimulating hormone (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 μ g/ml 17 β -estradiol, and 50 μ g/ml gentamicin sulfate for 22 h at 38.5 °C under 5% CO₂ in air (10–12 oocytes/microdrop). Then, cumulus cells were removed by vortex-mixing for 3 min in the HEPES-buffered TCM-199 supplemented with 3 mg/ml BSA, 0.2 mM sodium pyruvate, 1000 IU/ml hyaluronidase, and 50 μ g/ml gentamicin sulfate. Oocytes were comprehensively checked for extrusion of the first polar body, and oocytes with an extruded first polar body were defined as matured.

Vitrification and warming

Mature oocytes were subjected to a vitrification procedure according to the method described previously (Hwang *et al.* 2013). Briefly, oocytes were equilibrated with 7.5% ethylene glycol (EG; Wako Pure Chemical Industries, Osaka, Japan) and 7.5% dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries) in HEPES-buffered TCM-199 with 20% FBS base medium for 3 min at room temperature (23–28 °C) and then transferred into a vitrification solution consisting of 15% EG, 15% DMSO, and 0.5 M sucrose in the base medium for ~60 s at the room temperature. Within this 60-s period, up to 15 oocytes were loaded onto the polypropylene strip of a Cryotop device (Kitazato BioPharma, Shizuoka, Japan) with a minimal amount of the vitrification solution (<0.1 μ l) and then quickly plunged into liquid nitrogen (LN₂).

After storage for more than 1 week in the LN₂, oocytes were warmed by immersing the polypropylene strip of a Cryotop into 3 ml of the base medium containing 1 M sucrose prewarmed to 38.5 °C for 1 min. The oocytes were transferred to the base medium at room temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 5, and 5 min respectively). According to the manufacturer's instruction, the predicted cooling and warming rates of the Cryotop procedure were 23 000 and 42 000 °C/min respectively.

Recovery culture

Postwarming oocytes were rinsed three times and cultured in 500- μ l of HEPES-buffered TCM-199 plus 5% FBS, 0.2 mM sodium pyruvate, and 50 μ g/ml of gentamicin sulfate in a four-well dish for 2 h at 38.5 °C under 5% CO₂ in air (15–30 oocytes/well). Depending on the experimental series, the culture medium was supplemented with or without 50 μ g/ml α -ascorbic acid ([+]-sodium α -ascorbate; C₆H₇NaO₆, molecular weight=198.11), or 10, 30, 100, or 300 μ M α -tocopherol ([$\pm$]- α -tocopherol; C₂₉H₅₀O₂, molecular weight=430.71).

IVF and culture

Commercially available frozen semen of a Japanese black bull was used for IVF. After thawing at 37 °C for 30 s, the contents of a 0.5-ml straw were layered on the top of a Percoll density

gradient consisting of 2 ml of 45% Percoll above 2 ml of 90% Percoll in a 15-ml conical tube and then centrifuged for 20 min at 700 *g*. The sperm pellet was resuspended in 4 ml of modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan) supplemented with 5 mM theophylline, washed twice (5 min at 300 *g* each time), and then resuspended in the mBO medium supplemented with 5 mg/ml BSA and 10 μ g/ml heparin (IVF medium) to yield a concentration of 4×10^7 sperm cells/ml. Ten to 12 oocytes in the IVF medium were coincubated with the above-mentioned sperm suspension at a final concentration of 8×10^6 sperm cells/ml for 6 h in a 100- μ l microdrop under mineral oil at 38.5 °C under 5% CO₂ in air.

Up to 30 presumptive zygotes (6 h postinsemination (hpi)) were cultured in a 250- μ l microdrop of modified synthetic oviduct fluid (mSOF; Holm *et al.* 1999) supplemented with 30 μ l/ml of essential amino acid solution (50 $\times$; 11130; Gibco), 10 μ l/ml of non-essential amino acid solution (100 $\times$; 11140; Gibco), and 5% FBS (defined hereafter as mSOFaa with 5% FBS) at 39.0 °C under 5% CO₂, 5% O₂, and 90% N₂. The cleavage rate was determined on Day 2 (Day 0 = day of IVF), and the appearance of expanded blastocysts was recorded on Days 7 and 8.

Blastocyst cell number

Fully expanded blastocysts harvested on Day 8 were analyzed for total cell number. Blastocysts were washed three times in PBS supplemented with 0.1% polyvinylpyrrolidone (PVP) and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. The blastocysts were then stained with 10 μ g/ml Hoechst 33342 for 10 min in a dark condition. After being washed three times in PBS with 0.1% PVP and mounted with coverslip in antifade agent (100 mg 1,4-diazabicyclo[2.2.2] octane in 1 ml glycerol), the total cell number was determined under an epifluorescence microscope.

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% CO₂ in air (Yoshizawa *et al.* 1998). The blastocysts were then transferred in a 400- μ l hypotonic solution of 1% sodium citrate for 15–23 min, and fixed mildly by adding 20- μ l acetic acid:ethanol mixture (1:1) into the hypotonic solution. After 5 min, each blastocyst was placed onto a slide glass, and blastomeres 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 (pH 6.8); Wako) for 10 min. The chromosomal preparations were observed for ploidy under a light microscope with a magnification of $\times 400$.

ROS level

To measure the change in the ROS level, oocytes after recovery culture were washed three times in PBS with 0.1% PVP and incubated for 30 min with 10 μ M

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 μ m distance and stacked using a confocal laser scanning microscope (FV1000-D; Olympus, Tokyo, Japan). The fluorescence intensity of each oocyte was measured using the Image-J software (National Institutes of Health, Bethesda, MD, USA; accessed online). In each replicate, the mean value in the vitrified-warmed control group was defined as 1.0, and the relative values were given for those in the fresh control and 300 μ M α -tocopherol groups.

Mitochondrial activity

To measure mitochondrial activity of oocytes after recovery culture, oocytes were washed three times in PBS with 0.1% PVP, fixed in 4% PFA for 30 min at room temperature, and then incubated for 15 min in PBS with 0.1 μ g/ml of MitoTracker Red CMXRos (Lonza Walkersville, Inc., Walkersville, MD, USA) in a dark condition. Then, the mitochondria-labeled oocytes were rinsed three times in PBS with 0.1% PVP and mounted with coverslips in the antifade agent. The fluorescence intensity of each oocyte at the largest diameter was measured using the Image-J analysis Software under the confocal laser scanning microscope. In each replicate, the mean value in the vitrified-warmed control group was defined as 1.0, and the relative values were given for those in the fresh control and 300 μ M α -tocopherol groups.

CG distribution

Oocytes after recovery culture were fixed in 4% PFA for 30 min at room temperature, after zonae pellucidae had been removed in M2 medium (Quinn *et al.* 1982) with 0.75% protease (Pronase; Calbiochem, Darmstadt, Germany). Membranes of the oocytes were permeabilized overnight in PBS with 0.1% Triton X-100 and 0.3% BSA at 4 °C, and then the oocytes were incubated for 15 min in PBS with 100 ng/ml FITC-conjugated *lens culinaris* agglutinin (LCA; Vector Laboratories, Burlingame, CA, USA) in a dark condition. The oocytes were rinsed three times in PBS with 0.1% PVP and mounted with coverslips in the antifade agent. Digital images were collected using the confocal laser scanning microscope. According to the categories reported previously (Goud *et al.* 2005), distribution of the CGs periphery to the oolemma of each oocyte was classified either as intact or minor loss or major loss (Fig. 1).

Microtubule assembly

To assess the microtubule assembly in pronuclear-stage zygotes, inseminated oocytes were cultured for an additional 4 h in TCM-199 with 20% FBS at 38.5 °C under 5% CO₂ in air, and then immunostained according to the method described previously (Hara *et al.* 2011). The zygotes were extracted for 15 min by buffer M (25% glycerol, 50 mM KCl, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, and 50 mM imidazole hydrochloride, pH 6.8) that contained 5% methanol and 1% Triton X-100, after zonae pellucidae had been removed in M2

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

medium with 0.75% protease. The zygotes were then fixed in PBS with 4% PFA for 45 min and permeabilized overnight in PBS with 0.1% Triton X-100. Microtubules were labeled with an MAB against α -tubulin (T5168; diluted 1:1000). The primary antibodies were detected by FITC-conjugated goat anti-mouse IgG (F1010; diluted 1:200). Nuclear DNA was visualized by counterstaining with 2.5 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI). Preparations were mounted with coverslips in the antifade agent, and digital images collected at 2 μm distance were stacked using the confocal laser scanning microscope and assessed using the free Image-J Software. Zygotes with two pronuclei (2PN) were defined as those that fertilized normally, and classified as either those with single sperm aster or multiple sperm asters (Fig. 2).

Statistical analysis

Experiments were replicated at least four times in each group. Percentage data for cleavage and blastocyst development were arcsin transformed and compared with untreated postwarming controls by paired Student's *t*-test following the Bonferroni correction. Blastocyst cell number data, relative data for ROS level and mitochondrial activity, and arcsin-transformed percentage data for intact CG status and single aster formation were compared by one-way ANOVA. When the ANOVA was significant, differences among means were analyzed by Tukey's test. A value of $P < 0.05$ was defined as a significant difference, except in cases of the Bonferroni correction.

Results

Blastocyst development of oocytes after recovery culture

All the postwarming oocytes in five replicates ($n=320$) appeared morphologically normal and were cultured for 2 h with or without antioxidant supplementation before IVF. In addition to these vitrified oocytes, fresh oocytes ($n=105$) were subjected to the IVF protocol. A significant difference ($P < 0.017$) was detected in the Day 8 blastocyst yield between the untreated control and α -tocopherol-treated groups (25.8% vs 36.3%), while no significant differences were detected in the Day 2 cleavage rate (Table 1). Supplementation of L-ascorbic acid to recovery culture medium had no effect on rescuing the vitrified-warmed oocytes (blastocyst yield at Day 8, 27.5%).

Figure 2 Pronuclear-stage 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.

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 $\pm$ 6.0)	22 (20.5 $\pm$ 6.0)*	28 (25.8 $\pm$ 6.6)*
L-ascorbic acid	106	75 (70.9 $\pm$ 2.7)	30 (22.6 $\pm$ 2.7)*	30 (27.5 $\pm$ 2.5)*
α -tocopherol	106	73 (69.6 $\pm$ 2.9)	24 (23.0 $\pm$ 2.9)*	38 (36.3 $\pm$ 5.2) [†]
Fresh control	105	79 (75.4 $\pm$ 1.9)	43 (41.3 $\pm$ 1.9) [†]	55 (52.6 $\pm$ 5.5) [†]

Percentages are expressed as mean $\pm$ S.E.M. of five replicates in each group. *[†]Different symbols within columns denote significant difference from untreated control group (paired Student's *t*-test with the Bonferroni correction, $P < 0.017$).

In the following experiment to examine the higher concentrations of α -tocopherol, all the postwarming oocytes ($n=638$) were subjected to six replicates of recovery culture, and IVF with fresh control oocytes was carried out in parallel ($n=130$). Blastocyst yield on Day 8 was significantly higher ($P < 0.01$) than the control when the highest concentration of α -tocopherol (300 μ M) was supplemented (35.1% vs 19.2%; Table 2). Supplementation of the lower concentrations of α -tocopherol (10, 30, and 100 μ M) resulted in improved yields, but with no significant differences from the control (26.9–30.8% vs 19.2%, $P=0.104$ –0.251).

Quality analyses of the blastocysts

The mean total cell number of blastocysts derived from postwarming oocytes after α -tocopherol-free recovery culture (107.8, $n=21$) was significantly lower ($P < 0.05$) than that of fresh control blastocysts (158.0, $n=25$), as shown in Fig. 3. The mean total cell number of blastocysts derived from postwarming oocytes with recovery culture in 300 μ M α -tocopherol (143.4, $n=14$) did not differ from that of the fresh control blastocysts. The difference in the mean total cell number between 0 μ M α -tocopherol-treated and 300 μ M α -tocopherol-treated groups (both for vitrified oocytes) was significant ($P < 0.05$).

In chromosomal ploidy analysis, the proportions of normal diploid blastocysts ($2n=60$) were 84.6% (11/13), 81.3% (13/16), and 100% (15/15) in fresh control, 0 μ M α -tocopherol, and 300 μ M α -tocopherol groups respectively. Abnormal blastocysts included one tetraploid and one mixploid (tetraploid/diploid) in the fresh

control group, and one triploid, one mixploid ($2n=50/60$), and one aneuploid ($2n=83$) in the 0 μ M α -tocopherol group.

Changes detected in oocytes or pronuclear-stage zygotes

Three parameters were traced in oocytes immediately after recovery culture. Postwarming oocytes treated for 2 h with 300 μ M α -tocopherol showed a decreased level of ROS (relative value 0.73, $n=30$) when compared with 0 μ M α -tocopherol group (1.00, $n=31$), but oocyte vitrification procedure employed herein was not the critical factor increasing the intracellular ROS level as a fresh control value (0.98, $n=30$) (Fig. 4A). The relative mitochondrial activities were comparable among fresh reference (0.89, $n=26$), 0 μ M α -tocopherol (1.00, $n=23$) and 300 μ M α -tocopherol (0.84, $n=23$) groups (Fig. 4B). In addition, distribution pattern of the CGs was similar among the fresh reference (intact 57.1%, intact+minor loss 82.0%, $n=51$), 0 μ M α -tocopherol (intact 57.6%, intact+minor loss 79.1%, $n=52$), and 300 μ M α -tocopherol (intact 58.0%, intact+minor loss 84.0%, $n=50$) groups (Fig. 4C).

Pronuclear-stage zygotes at 10 hpi were immunostained against α -tubulin to assess their cytoplasmic potential for microtubule assembly. Normal fertilization rates in the vitrified-warmed oocytes (57.7 and 59.1% in 0 and 300 μ M α -tocopherol groups respectively) were not different from that in the fresh reference group (60.6%; Table 3). Regardless of vitrification, proportions of the 2PN zygotes forming one or more sperm asters were also comparable among the groups (94.2–98.3%).

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

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

Percentages are expressed as mean $\pm$ S.E.M. of six replicates in each group. *[†] Different symbols within columns denote significant difference from untreated control group (paired Student's *t*-test with the Bonferroni correction, $P < 0.01$).

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 ($P < 0.05$).

A higher incidence of multiaster formation in vitrified–warmed oocytes (52.0% vs 15.3% in fresh reference group; $P < 0.05$) was observed when the oocytes were not treated with α -tocopherol during the recovery culture. However, the multiaster formation in vitrified–warmed oocytes was inhibited when the oocytes were treated with α -tocopherol in the postwarming culture (9.7%). Inversely, the single-aster formation rate of vitrified–warmed oocytes in the α -tocopherol-treated group (90.3%) was similar to that of fresh reference oocytes (84.7%).

Discussion

A short-term culture of vitrified–warmed bovine oocytes with α -tocopherol contributed to inhibit high incidence of multiple aster formation (Table 3) and improve development into normal blastocysts (Tables 1, 2 and Fig. 3). These results are similar to those described in our previous report (Hwang *et al.* 2013), where ROCK inhibitor was used to rescue the vitrified–warmed bovine oocytes. The high revivability obtained herein (maximum Day-8 blastocyst yield at 36.3%) is partially due to the improved baseline of blastocyst yield by availability of the fresh (= within 6 h after slaughter) bovine ovaries, when compared with the previous report using 1-day-stored ovaries (21.4%; Hwang *et al.* 2013). A higher incidence of multiple-aster formation was observed in vitrified–warmed bovine oocytes after IVF, and pronuclear development and migration were delayed in the zygotes with multiple-aster formation (Hara *et al.* 2012). In most mammalian species except for rodents, spermatozoal centrosome plays a critical role in assembly of microtubule network (sperm aster) that brings both male and female pronuclei to the center of the newly formed zygote (Navara *et al.* 1996). Thus, the centrosome is considered

to be the MTOC, with duplication during the pronuclear stage and the subsequent separation to serve as mitotic centers anchoring the chromosomes during the first cleavage (Chen *et al.* 2003, Schatten & Sun 2009). Abnormalities of the spindle, MTOC function, and sperm aster have been shown to directly correlate with the loss of developmental potential after IVF, because they are crucial for completion of the second meiosis, extrusion of the polar body, migration of the pronuclei, and formation of the first mitotic spindle (Schatten *et al.* 1985).

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. Different letters in panel-A show significant differences ($P < 0.05$).

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 $\pm$ 2.5)	27 (96.0 $\pm$ 4.0)	13 (48.0 $\pm$ 2.0)*	14 (52.0 $\pm$ 2.0)*
α -tocopherol-treated	50	33 (59.1 $\pm$ 3.4)	31 (94.2 $\pm$ 3.6)	28 (90.3 $\pm$ 4.1) [†]	3 (9.7 $\pm$ 4.1) [†]
Fresh control	94	58 (60.6 $\pm$ 5.4)	67 (98.3 $\pm$ 1.7)	58 (84.7 $\pm$ 4.5) [†]	9 (15.3 $\pm$ 4.5) [†]

Percentages are expressed as mean $\pm$ S.E.M. of six replicates in each group. *[†]Different symbols within columns denote significant differences among groups (Tukey's test, $P < 0.05$).

Interestingly, in contrast to α -tocopherol, supplementation of L-ascorbic acid into the recovery culture medium had no effect on improvement of blastocyst yield from vitrified–warmed mature bovine oocytes (Table 1). We have also reported that chemical treatment of bovine oocytes to increase the intracellular glutathione level, which also acts as a radical scavenger, did not alter their cryotolerance (Hara *et al.* 2014). These differences might be associated with the affinity of antioxidants, as the hydrophobic α -tocopherol protects the lipid bilayer of the plasma membrane from oxidative stress (Gutnisky *et al.* 2013). Oxidative stress increases membrane fluidity through cleavage of the lipid acyl chain (Tai *et al.* 2010). Such alterations in membrane property might disturb uptake of extracellular components essential for supporting physiological aster formation. On the other hand, no significant changes were detected in the intracellular ROS level between fresh and untreated postwarming oocytes, although α -tocopherol treatment suppressed the ROS formation (Fig. 4A). In some independent experiments for comparison between fresh control oocytes and postwarming non-cultured oocytes, no differences were found in the ROS level (data not shown). This phenomenon is not coincident with the recent report, where intracellular ROS level increased in vitrified–warmed mouse blastocysts using nylon loop as a cryodevice (Martino *et al.* 2013). During vitrification of bovine oocytes using a Cryotop device, oxidative stress might concentrate only to the plasma membrane.

Two other parameters (mitochondrial activity and CG distribution) were investigated to understand the possible role of α -tocopherol in rescuing vitrified–warmed bovine oocytes. Mitochondrial activity in postwarming bovine oocytes was completely restored during the recovery culture (Fig. 4B), as shown by our previous time-dependent observation (Hwang *et al.* 2013). Maintenance of ATP contents in bovine IVM oocytes by cyclosporine treatment can improve parthenogenetic development into blastocysts after oocyte vitrification (Zhao *et al.* 2011). No significant changes were also detected between α -tocopherol-treated and untreated postwarming oocytes in CG distribution (Fig. 4C). Premature release of CGs has been observed in cryopreserved oocytes and considered to be responsible for the failure of sperm penetration or the incidence

of polyspermic fertilization (Carroll *et al.* 1990, Fukui *et al.* 1995). In preliminary trials, CG distribution pattern difficult to be categorized as in Fig. 1 was observed in bovine oocytes immediately after warming (data not shown). The ultrastructure of bovine oocytes immediately after OPS vitrification and warming indicated the presence of small membrane-bound vesicles and the partial lack of CGs aligned along the oolemma (Hyttel *et al.* 2000). The same authors also noted that postwarming oocytes after additional 2 h culture had less abundant small vesicles and more degenerating CG clusters.

Two quality analyses of blastocysts (total cell number and chromosomal ploidy) did not reveal any disadvantageous features in Day 8 blastocysts derived from α -tocopherol-treated postwarming oocytes when compared with fresh control blastocysts. There might be a larger part of blastocysts developed from multiaster-formed zygotes, which had suffered delayed pronuclear development and migration, in the untreated control group. However, the increased total cell number of Day 8 blastocysts derived from α -tocopherol-treated vs untreated postwarming oocytes (Fig. 3) is not explained by their different developmental kinetics until Day 8, because Hoechst staining was performed only for fully expanding blastocysts. This difference may be due to improved ooplasmic environment including elevation of MPF activity (Motlik & Kubelka 1990), which can organize normal microtubule assembly after the subsequent IVF. Any possible epigenetic differences, which might result from α -tocopherol treatment, have not been investigated using the resultant blastocysts in this study and remained to be investigated in future. Ultimate quality analysis of vitrified–warmed bovine oocytes is undoubtedly the production of live calves following transfer of the resultant blastocysts into uteri of recipient cows. Proof whether the α -tocopherol treatment for postwarming oocytes is supportive not only for higher formation of blastocysts, but also for normal offspring production, would be of great effect in the veterinary field.

In conclusion, α -tocopherol treatment of bovine IVM oocytes during recovery culture after Cryotop vitrification can improve their revivability, as shown by the high blastocyst yield and the higher mean total cell number in the blastocysts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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