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Title: Rescue of vitrified-warmed bovine oocytes with Rho-associated coiled-coil kinase inhibitor[1]

Running title: ROCK INHIBITION IN VITRIFIED-WARMED BOVINE OOCYTES

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ABSTRACT
Cryotolerance of bovine matured oocytes is not fully practical even though promising vitrification procedure with ultra-rapid cooling rate was applied. The present study was conducted to investigate whether recovery culture of vitrified-warmed bovine oocytes with an inhibitor (Y-27632) of Rho-associated coiled-coil kinase (ROCK) can improve the developmental potential after in vitro fertilization (IVF) and in vitro culture. Immediately after warming, almost oocytes appeared morphological normal. Treatment of the post-warm oocytes with 10 μM Y-27632 for 2 h resulted in the significantly higher oocyte survival rate prior to the IVF, cleavage rate and blastocyst formation rate. Quality analysis of the resultant blastocysts in terms of total cell number and apoptotic cell ratio also showed the positive effect of the Y-27632 treatment. Time-dependent change in mitochondrial activity of the vitrified-warmed oocytes was not influenced by ROCK inhibition during the period of recovery culture. However, the ability of ooplasm to support single-aster formation was improved by the ROCK inhibition. Thus, inhibition of ROCK activity in vitrified-warmed bovine oocytes during a short-term recovery culture can lead to the higher developmental competence, probably due to decreased apoptosis and normalized function of microtubule-organizing center.

Summary sentence: Inhibition of ROCK activity in vitrified-warmed bovine oocytes can lead to the higher developmental competence into blastocysts, due to decreased apoptosis and normalized function of microtubule-organizing center.

Key words: Bovine oocytes, Cryotop vitrification, ROCK inhibitor, Apoptosis, Microtubule assembly

INTRODUCTION

Successful pregnancies or birth of offspring derived from frozen-thawed oocytes have been reported in several mammalian species a few decades ago [1-3], with relatively low developmental rates. Application of vitrification, instead of the conventional two-step freezing, improved the efficacy of oocyte cryopreservation, especially in mice [4, 5] and humans [6, 7], and thus, oocyte cryopreservation has become an important tool for gamete banking and assisted reproductive technology. Various cryodevices, such as open-pulled straws [8], cryoloop [9], and cryotop [10], have been developed to accelerate the cooling rate. However, vitrification of oocytes from large domestic species enriched with cytoplasmic lipid droplets still requires substantial improvement [11-13]. Proposed reasons for high sensitivity of oocytes to cryopreservation include the large cell size and low permeability of water and cryoprotectants (CPA) [3]. Depolymerization of microtubules induced by CPA treatment and cryopreservation resulted in meiotic spindle disassembly and chromosome misalignment [14]. Treatment with CPA induced a transient rise of intracellular free calcium level, premature exocytosis of cortical granules, and hardening of zonae pellucidae [15, 16]. Recently, we have proposed a hypothesis for cryodamage of bovine oocytes that multiple aster formation frequently observed in vitrified-warmed and fertilized oocytes may be related to loss of ooplasmic function responsible for normal microtubule assembly from the sperm-aster [17].

Increased apoptosis of vitrified-warmed oocytes resulted in reduction of developmental competence [18, 19]. Rho-associated coiled-coil kinase (ROCK), belonged to the AGC (PKA, PKG, and PKC) family of serine-threonine kinases, was discovered as a downstream target of the small GTP-binding protein Rho [20], which can regulate cellular growth, adhesion, migration, metabolism, and apoptosis through controlling the actin-cytoskeletal assembly and cell contraction [21]. The ROCK is comprised of a catalytic domain at the N-terminal, followed by a coiled-coil domain for the Rho protein binding, and a Pleckstrin-homology domain. Target proteins for phosphorylation by ROCK include the regulatory myosin light chain [21] and the LIM kinase-1 and kinase-2 [22]. Inhibition of the ROCK activity was involved in reduction of apoptosis in embryonic stem cell-derived neural cells [23]. Inhibition of the ROCK activity was also effective to improve the plating efficiency of dissociated human pluripotent stem cells after cryopreservation [24-26] and the revivability of in vitro-produced bovine blastocysts after vitrification and warming [27].

The present study was designed to investigate whether short-term treatment of vitrified-warmed bovine oocytes with ROCK inhibitor can improve the survival rate and the subsequent developmental competence after in vitro fertilization (IVF). In addition, the mitochondrial activity during the short-term culture with ROCK inhibitor and the function of microtubule-organizing center after IVF were investigated.

MATERIALS AND METHODS

Experimental design

Vitrified-warmed oocytes with morphologically normal appearance were randomly allocated to recovery culture either with or without ROCK inhibitor. Fresh matured oocytes were served as controls. Morphological survival after the recovery culture, cleavage and blastocyst formation after the IVF, and cell construction of the resultant blastocysts were investigated in the first

series of experiments (Experiment 1). Time-dependent change in activity of mitochondria during the recovery culture, and the potential of oocytes to support single-aster formation after IVF were investigated in the second series of experiments (Experiment 2).

In vitro maturation

Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Abattoir-derived bovine ovaries were transported to the laboratory in cold saline (maintained at 10-12 °C) within 24 h after slaughter. The contents of 2-8 mm follicles were aspirated with an 18 G needle connected to a 10 ml syringe. Oocytes surrounded with at least two layers of compact cumulus cells were matured in 100-µl microdrops of Hepes-buffered Tissue Culture Medium (TCM)-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/ml FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 µg/ml 17 β-estradiol, and 50 µg/ml gentamycin sulfate for 22 h at 38.5 °C under 5% CO2 in air (10-12 oocytes per microdrop). Then, cumulus cells were removed by a 3-min vortex-mixing in the Hepes-buffered TCM-199 supplemented with 3 mg/ml bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 1,000 IU/ml hyaluronidase, and 50 µg/ml gentamycin sulfate. Oocytes were comprehensively checked for their extrusion of the first polar body, and the oocytes with an extruded first polar body were defined as matured and used for further experiments.

Vitrification and warming

Matured oocytes were subjected to a vitrification procedure according to the method described previously by Tsujioka et al [28], with minor modifications. Briefly, oocytes were equilibrated with 7.5% ethylene glycol (EG; Wako Pure Chemical Industries, Osaka, Japan) and 7.5% dimethylsulfoxide (DMSO; Wako) in Hepes-buffered TCM-199/20% (v/v) FBS base medium for 3 min at room temperature (23 ± 2 °C), and then transferred into a vitrification solution consisting of 15% EG, 15% DMSO and 0.5 M sucrose in the base medium for approximately 60 sec at room temperature (23 ± 2 °C). Within this 60 sec, up to fifteen oocytes were loaded onto the polypropylene strip of a Cryotop (Kitazato BioPharma, Shizuoka, Japan) with a minimal amount of the vitrification solution (less than 0.1 µl), 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 at 38.5 °C, and kept 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 of sucrose for 3, 5, and 5 min, respectively). According to the manufacture's instruction, the predicted cooling and warming rates of the Cryotop procedure are 23,000 and 42,000 °C/min, respectively.

Recovery culture with or without ROCK inhibitor

Post-warm oocytes were cultured in 100-µl microdrops of Hepes-buffered TCM-199 plus 5% FBS, 0.2 mM sodium pyruvate and 50 µg/ml gentamycin sulfate for 2 h at 38.5 °C under 5% CO2 in air (15-30 oocytes per microdrop). The culture medium was supplemented with or without an inhibitor of ROCK, Y-27632 [(R)-(+)-trans-4-(1-aminoethyl)-N-(4-

pyridyl)cyclohexanecarboxamide dihydrochloride·monohydrate (C14H21N3O·2HCl·H2O, MW=338.27)], to yield a final concentration of 10 μM.

Fertilization and culture in vitro

Commercially available frozen semen of a Japanese Black bull was used for IVF. After thawing in a water bath at 37 °C for 30 sec, the contents of a 0.5 ml straw was layered on the top of Percoll density gradient consisting of 2 ml of 45% Percoll above 2 ml of 90% Percoll in a 15 ml conical tube, and centrifuged for 20 min at 700 *g*. The sperm pellet was re-suspended 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) and then re-suspended in the mBO medium supplemented with 5 mg/ml BSA and 10 μg/ml heparin (IVF medium) to yield a concentration of 4 x 10^7 sperm cells/ml. Ten to 12 oocytes in the IVF medium were co-incubated with the above sperm suspension at a final concentration of 8 x 10^6 sperm cells/ml for 6 h in a 100-μl microdrop under mineral oil at 38.5 °C under 5% CO2 in air.

Up to 30 presumptive zygotes (6 hpi: hour post-insemination) were cultured in a 250-μl microdrop of modified synthetic oviduct fluid (mSOF) [29], supplemented with 30 μl/ml essential amino acids solution (x 50, Gibco-11,130), 10 μl/ml non-essential amino acids solution (x 100, Gibco-11,140) and 5% FBS at 39.0 °C under 5% CO2, 5 % O2 and 90 % N2. The cleavage rate was determined on Day-2 and appearance of expanded blastocysts was recorded on Day-7 and -8 (Day-0 = Day of IVF).

In an additional experiment, mean time until the first cleavage was determined by every 6-hour observation of post-warm oocytes treated with or without Y-27632 and fertilized in vitro (from 18 to 48 hpi). The time of the first cleavage in each zygote was defined as the point when the cleavage was initially observed.

Quality analysis of blastocysts

Fully expanded blastocysts harvested on Day-8 (n=20 each) were analyzed for apoptotic cell ratio and total cell number by TUNEL assay and Hoechst staining, respectively. According to the manufacture's manual for In Situ Cell Death Detection Kit (TMR Red, Roche Diagnostics, Mannheim, Germany), blastocysts were washed three times in PBS supplemented with 0.1% polyvinylpyrrolidone and fixed in 4% paraformaldehyde for 24 h at 4 °C. Membranes of the blastocysts were permeabilized with 0.5% (v/v) Triton X-100 for 30 min at room temperature. Broken DNA ends were labeled with terminal deoxyribonucleotidyl transferase and fluorecein-dUTP for 1 h at 38.5 °C in the dark. The blastocysts were counterstained with 10 μg/ml Hoechst-33342 for 15 min at room temperature in the dark, to determine the total cell number of the blastocyst. Then, the blastocysts were washed at least three times in PBS and mounted onto slides with mounting solution (Mount-Quick, Daido Sankyo, Tokyo, Japan). The apoptotic cell number and total cell number in each blastocyst were counted under an epifluorescence microscopy (Nikon, Tokyo, Japan).

Mitochondrial activity during recovery culture

Vitrified-warmed oocytes were harvested at 0, 30, 60 and 120 min of the recovery culture to determine the changes of mitochondrial activity. The oocytes were washed three times with phosphate-buffered saline (PBS, Nissui Pharmaceutical, Tokyo, Japan) and fixed in 4%

paraformaldehyde suspended in PBS for 15 min at room temperature. The oocytes were incubated for 15 min with 0.1 μg/ml MitoTracker Red CMXRos (Lonza Walkerscille, MD, USA) suspended in PBS under a dark condition. Then, the mitochondria-labeled oocytes were rinsed three times with PBS and mounted on the glass bottom dish (SPL, Seoul, Korea) as a micro-droplet under mineral oil. Each oocyte was observed under a confocal laser scanning microscope (FV1000-D, Olympus, Tokyo, Japan), and 10 sections per oocyte were captured and stacked. The fluorescence intensity of each oocyte was measured using ImageJ analysis software (National Institute of Health, Bethesda, ML, USA). In each of the total 4 replicates, the average intensities of 3 oocytes per group were calculated. The value in the fresh control group was defined as 1.0, and the relative values were given for the vitrified-warmed groups.

Function of microtubule-organizing center

Presumptive zygotes after IVF (6 hpi) were cultured for an additional 4 h in the Hepes-buffered TCM-199 plus 5% FBS at 38.5 °C under 5 % CO2 in air, and then immunostained as described previously [17]. Briefly, the zygotes at 10 hpi 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) containing 5% (v/v) methanol and 1% (v/v) Triton X-100, after zonae pellucidae had been removed by 0.75% protease in M2 medium [30]. The zygotes were then fixed with cold methanol for 10 min and permeabilized overnight in PBS containing 0.1% Triton X-100. Microtubules were labeled with a monoclonal antibody against α-tubulin (diluted 1:500). The primary antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:200). Nuclear DNA was visualized by counterstaining by 2.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI). All samples were mounted onto slides with anti-fade agent, and digital images were taken at 2 μm thickness using a confocal laser scanning microscope. The digital images were stacked and assessed with ImageJ software. Zygotes with two pronuclei (2-PN) were defined as those fertilized normally.

Statistics

Experiments were replicated at least 4 times in each group. All the data were analyzed with a software for analysis, Origin 8 (OriginLab Corporation, Northhampton, MA, USA) by applying one-way analysis of variance with Turkey's honest significance test. A value of $P < 0.05$ was considered as a significant difference.

RESULTS

Experiment 1

All the 371 vitrified-warmed oocytes were harvested and 368 oocytes among them (99.2%) appeared intact. After recovery culture for 2 h, survival rate of post-warm oocytes cultured with Y-27632 (97.8%, 181/185) was significantly higher than that of those cultured without Y-27632 (90.2%, 165/183, $P < 0.05$).

Cleavage rate of post-warm oocytes treated with Y-27632 (72.4%) was significantly higher than that of those treated without Y-27632 (56.2%, $P < 0.05$), but comparable to that of non-treated fresh control oocytes (71.4%), as shown in Table 1. Based on a separate series of experiment (three replicates), the mean time until the first cleavage was found almost comparable between the post-warm oocytes treated with Y-27632 (31.3 ± 1.3 hpi, number of cleaved zygotes = 66)

and without Y-27632 (32.6 ± 1.6 hpi, number of cleaved zygotes = 55). On Day-8, blastocyst yield from post-warm oocytes treated with Y-27632 (21.4%) was significantly higher than that of those treated without Y-27632 (13.9%, $P < 0.05$), but significantly lower than that of fresh control oocytes (34.4%, $P < 0.05$). There was no difference in the proportion of Day-7 blastocysts versus Day-8 blastocysts among the three groups, ranging from 2.8 to 3.7.

Quality analysis of expanded blastocysts harvested on Day-8 by Hoechst staining showed that mean total cell number of blastocysts in the group cultured with Y-27632 (124.6 cells) was significantly higher than that of those in the group cultured without Y-27632 (97.5 cells, $P < 0.05$), but comparable to that of those in fresh control group (135.7 cells), as shown in Fig. 1A. TUNEL assay also showed that the proportion of apoptotic cells per blastocyst in the group cultured without Y-27632 (4.0%) was significantly higher than those in the group cultured with Y-27632 (2.2%) and fresh control group (1.8%), as shown in Fig. 1B.

Experiment 2

Mitochondrial activity of vitrified-warmed oocytes regained to the original (non-vitrified control) level in a time-dependent manner during the recovery culture for 2 h, as shown in Fig. 2. Both time course and extent in the recovery of mitochondria activity were not different between post-warm oocytes cultured with and without Y-27632.

At 10 hpi, proportions of oocytes fertilized normally with 2-PN (73.3-92.6%) were statistically comparable among the three groups, as shown in Table 2. Regardless of vitrification, proportions of zygotes formed aster(s) were also comparable among the groups (80.7-83.3%). A higher incidence of multiple aster formation in vitrified-warmed oocytes (49.4 versus 30.2% in fresh control group, $P < 0.05$) was observed when the oocytes were not treated with Y-27632 during the recovery culture (Fig. 3A). However, the multiple aster formation in vitrified-warmed oocytes was inhibited when the oocytes were treated with Y-27632 in the post-warm culture (32.9%). Mean aster number per zygote exhibiting multiple asters was 2.5 ± 0.2, 2.6 ± 0.1 and 2.5 ± 0.2 in vitrified/Y-27632-treated, vitrified/non-treated and fresh control groups, respectively. Inversely, the single aster formation rate of vitrified-warmed oocytes in the Y-27632 treated group (67.1%, Fig. 3B) was similar to that of fresh control oocytes (69.8%).

DISCUSSION

Inhibition of ROCK by a short-term treatment with Y-27632 improved both morphological survival of vitrified-warmed bovine oocytes prior to IVF (97.8 vs. 90.2%) and apoptotic cell ratio in the Day-8 blastocysts after oocyte vitrification and IVF (2.2 vs. 4.0%; Fig. 1B). It is well-known that the ROCK pathway is closely related to induction of cell apoptosis [31-34]. Under a stressful situation where caspase activity is increased, caspase-mediated cleavage and consequent activation of ROCK-1 may trigger to accelerate the apoptosis process [33, 35]. The ROCK inhibitor, Y-27632, has been used to improve the cryosurvival of human neural stem cells [36], human embryonic stem cells [24] and in vitro-produced bovine blastocysts [27], while Bueno et al. [37] reported the negative effect of Y-27632 on expansion and survival rate of fresh and cryopreserved hematopoietic stem cells. The Y-27632 does not act directly as a protector for anoikis (dissociation-induced apoptosis) [38]. Alternative mechanism for ROCK-dependent cell death has been proposed in human embryonic stem cell research. It was demonstrated that actin-myosin contraction is a major mechanism promoting the death of cells [39], and that the ROCK-

dependent hyperactivation of myosin is involved in the apoptosis of cells [40].

Improved developmental competence of vitrified-warmed bovine oocytes by a short-term culture with Y-27632, the main finding of the present study, may be explained by prompt recovery of ooplasmic function to support formation of single aster (Table 2). Mitochondria in the eukaryotic cells play many roles, including ATP production, redox, calcium homeostasis, and apoptosis [41]. However, the recovery of ooplasm to support the MTOC function did not match with the time-dependent kinetics of mitochondrial activity (Fig. 2). Except for rodents in which multiple cytoplasmic asters function as MTOC [42, 43], paternal centrosome organizes only a single sperm aster and functions as an MTOC in many mammalian species including humans and bovine species. 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 [17]. Very recently, we failed to inhibit the occurrence of multiple aster formation in bovine oocytes by increasing intracellular glutathione level prior to vitrification and IVF [44]. Since the ROCK regulates microtubule acetylation via phosphorylation of the tubulin polymerization promoting protein 1, inhibition of the ROCK activity resulted in increased cellular microtubule acetylation [45, 46]. Molecular mechanisms by which the ROCK inhibition improves the tolerance of bovine oocytes after vitrification and warming remain to be clarified and need further research.

Some attempts have been conducted to improve the developmental competence of vitrified-warmed oocytes using chemical reagents. Cyclosporine, an immune suppression reagent, was applied to maintain the potential of mitochondria (ATP contents) and to decrease the level of reactive oxygen species in bovine oocytes, resulting in an improved yield of parthenogenetic blastocysts after oocyte vitrification [47]. Pretreatment of bovine oocytes with Taxol, a mitotic inhibitor, had beneficial effect on early embryonic cleavage of post-warm oocytes through stabilization of spindle configuration [48]. Recently, the supplementation of L-carnitine, a quaternary ammonium compound, into maturation medium improved the cryotolerance of bovine oocytes through redistribution of lipid droplets and no reduction of ATP contents [49]. For mouse oocytes, antifreeze protein-III supplemented directly into vitrification solution resulted in an improved developmental competence through preserving spindle forming ability and membrane integrity [50]. On the other hand, the Y-27632 employed in the present study has been used for a short-term treatment after (not before and/or during) vitrification and warming procedure.

In conclusion, we demonstrated for the first time the beneficial effects of ROCK inhibitor on developmental competence of vitrified-warmed bovine oocytes. The improved blastocyst yield from vitrified-warmed oocytes may be due to decreased apoptosis and normalized function of microtubule-organizing center.

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FIGURE LEGENDS

Fig. 1. Total cell number and apoptotic cell ratio in Day-8 expanded blastocysts derived from vitrified-warmed bovine oocytes. The error bars are ± SD. [a,b] The significance is less than 0.05 ($P < 0.05$).

Fig. 2. Time-dependent change of mitochondrial activity in vitrified-warmed bovine oocytes during 2 h of recovery culture. Four replicates. The error bars are ± SD. [a-d] The significance is less than 0.05 ($P < 0.05$).

Fig. 3. Fluorescent images of vitrified-warmed and in vitro-fertilized bovine oocytes after immunostaining against α-tubulin and nuclear staining with DAPI. A) 2-PN zygote with multiple asters. B) 2-PN zygote with single aster. Bar = 25 μm.

TABLE 1. Effect of ROCK inhibition on developmental competence of vitrified-warmed bovine oocytes.

		No. of oocytes (%)*			
				Developed to blastocyst on	
Group	Y-27632	Inseminated	Cleaved on Day 2	Day 7	Day 7 + 8
Vitrified-warmed	+	154	112 (72.4 ± 2.4)[a]	26 (16.9 ± 2.0)[b]	33 (21.4 ± 1.6)[b]
Vitrified-warmed	−	162	91 (56.2 ± 2.9)[b]	17 (10.1 ± 1.3)[b]	23 (13.9 ± 1.2)[c]
Fresh control		117	85 (71.4 ± 3.1)[a]	31 (26.7 ± 2.7)[a]	40 (34.4 ± 1.8)[a]

*Percentages were expressed as mean ± SEM of six replicates in each group.
[a-c] Different superscripts denote significant differences within columns ($P < 0.05$).

TABLE 2. Effect of ROCK inhibition on aster formation of vitrified-warmed bovine oocytes 10 h after IVF.

Group	Y-27632	No. of oocyes evaluated	No. of 2-PN zygotes (%)	No. of aster-formed zygotes (%)		
				Total	Single aster	Multiple asters
Vitrified-warmed	+	62	50 (78.4 ± 5.4)	40 (80.7 ± 3.2)	26 (67.1 ± 2.8)[a]	14 (32.9 ± 2.8)[b]
Vitrified-warmed	−	58	42 (73.3 ± 6.1)	35 (81.8 ± 4.4)	17 (49.4 ± 2.7)[b]	18 (50.6 ± 2.7)[a]
Fresh control		57	52 (92.6 ± 3.5)	43 (83.3 ± 4.3)	30 (69.8 ± 4.0)[a]	13 (30.2 ± 4.0)[b]

*Percentages were expressed as mean ± SEM of five replicates in each group.
[a,b] Different superscripts denote significant differences within columns ($P < 0.05$).

Figure 1

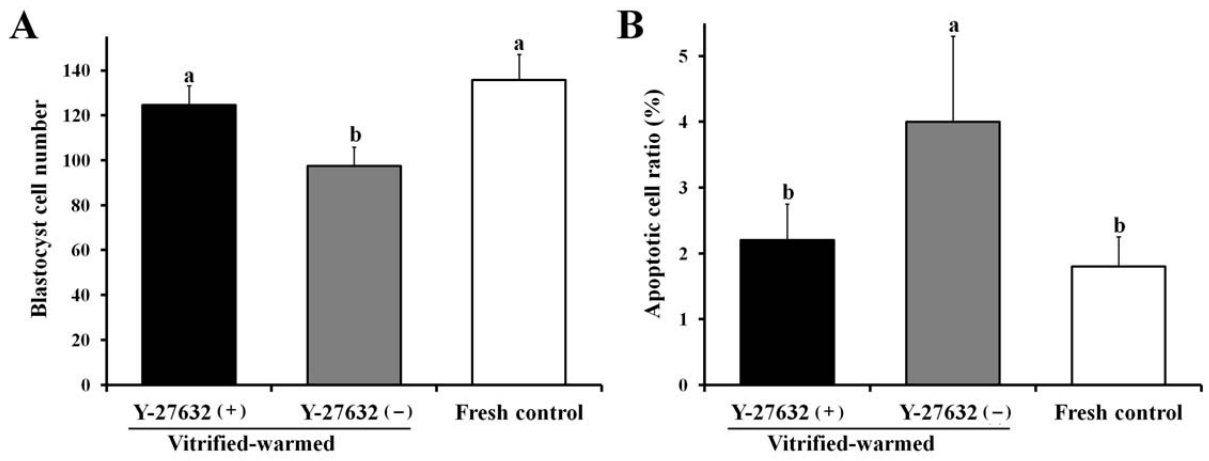
A
Blastocyst cell number
0
20
40
60
80
100
120
140
a
b
a
Y-27632 (+)
Y-27632 (−)
Fresh control
Vitrified-warmed
B
Apoptotic cell ratio (%)
0
1
2
3
4
5
b
a
b
Y-27632 (+)
Y-27632 (−)
Fresh control
Vitrified-warmed

Figure 2

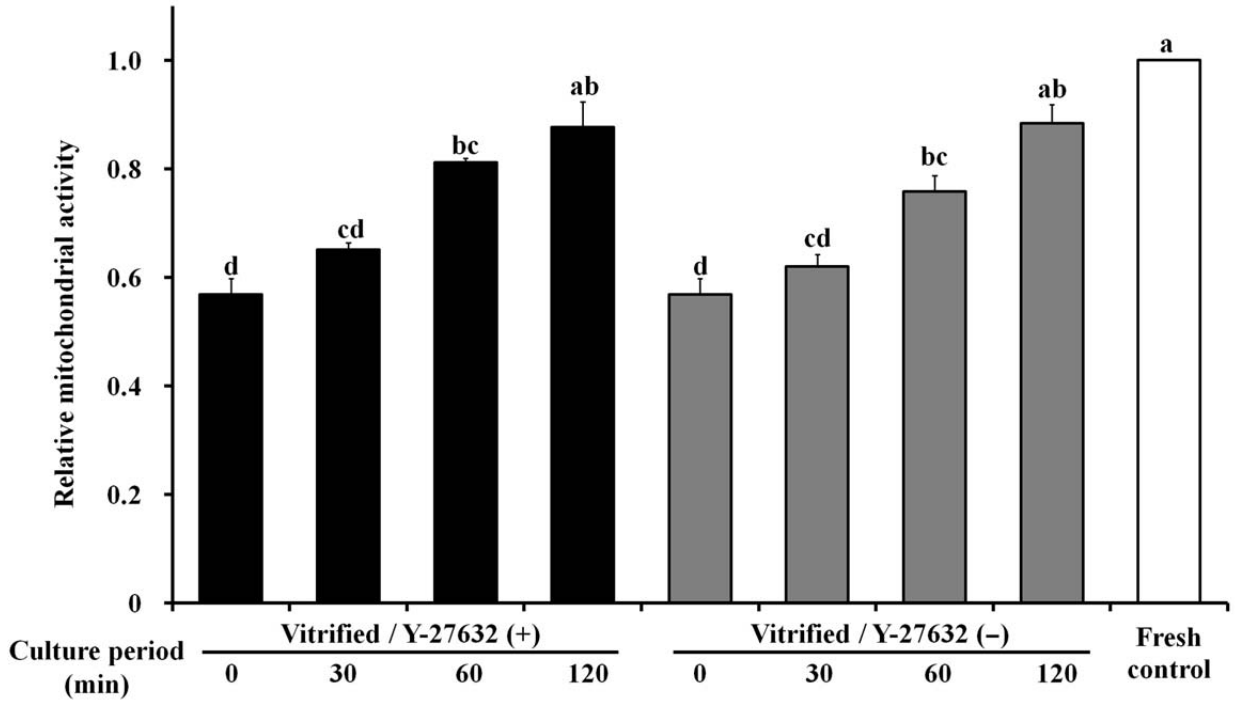
Relative mitochondrial activity
1.0
0.8
0.6
0.4
0.2
0
d
cd
bc
ab
d
cd
bc
ab
a
Culture period (min)
Vitrified / Y-27632 (+)
Vitrified / Y-27632 (–)
0
30
60
120
0
30
60
120
Fresh control

Figure 3