Revised

Stimulatory effect of ROCK inhibitor on revivability of in vitro-produced bovine blastocysts after vitrification

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Abstract

Inhibition of Rho-associated coiled-coil kinase (ROCK) activity has been reported to promote recovery and growth of frozen-thawed human embryonic stem cells. Since in vitro-produced and/or manipulated bovine embryos are sensitive to cryopreservation, the present study was conducted to investigate whether the presence of ROCK inhibitor

(Y-27632) in post-thaw culture medium improve the revivability of bovine blastocysts produced in vitro and vitrified by minimum volume cooling procedure. Expanding or fully-expanded blastocysts were harvested 7 days after in vitro fertilization, and vitrified in the presence of 15% ethylene glycol, 15% DMSO and 0.5 M sucrose using Cryotop as cryodevice. When the post-warm blastocysts were cultured in mSOF medium supplemented with 10 μM Y-27632, the survival rate (94.9 ± 2.4%), assessed by re-expansion of blastocoel at 24 h of the culture, was higher than that cultured without Y-27632 (78.0 ± 6.0%; $P < 0.05$). Conversely, hatching rate and mean total cell number of surviving embryos at 48 h of the culture were comparable regardless of the Y-27632 supplementation (62.8 ± 11.1 vs 59.6 ± 9.4%, and 135.2 ± 13.1 vs 146.7 ± 13.3, respectively). In non-vitrified in vitro-produced blastocysts, hatching rate on Day-9 was improved by the presence of Y-27632 (91.7 ± 3.8 vs 54.7 ± 8.9%; $P < 0.05$), but the increase in mean total cell number of blastocysts was not significant (230.0 ± 23.0 vs 191.2 ± 22.2; $P = 0.23$). In an additional experiment, the Y-27632 was supplemented to culture medium on either Day-0, -2 or -4 until Day-8, resulting in no increase in the blastocyst yield (7.5 ± 2.1 to 36.2 ± 3.2% vs 28.6 ± 6.9%). In conclusion, the supplementation of ROCK inhibitor to post-thaw culture medium improved the revivability of in vitro-produced bovine blastocysts after vitrification and warming.

Keywords: Bovine blastocysts; IVF; ROCK inhibitor; Vitrification; Y-27632

1. Introduction

Although in vitro production (IVP) of bovine embryos and subsequent transfer to recipients became practical, the survival rate of the IVP embryos after cryopreservation has been reported not to be fully comparable with that of their in vivo-derived counterparts [1,2]. Culture of presumptive zygotes in a serum-free medium [3], removal of cytoplasmic lipid droplets [4], application of slower cooling rate during two-step freezing [5] and supplementation of linoleic acid-albumin [6] or hyaluronan [7] have resulted in a higher revivability of bovine IVP embryos after cryopreservation. In addition, supplementation of ß-mercaptoethanol (ß-ME), a low molecular weight thiol compound, to the culture medium for post-thaw bovine IVP blastocysts was found effective to improve the in vitro development of the embryos after solid-surface vitrification [8]. The protective mechanism of ß-ME was considered to involve in their anti-oxidative action, which inhibits cell apoptosis [9,10]. Supplementation of ß-ME to culture medium for IVF-derived presumptive zygotes also showed a stimulatory effect on the yield of bovine blastocysts [9,10]. In an earlier report, stimulatory effect of ß-ME on the growth of mouse embryonic carcinoma cells was described [11]. Removal of ß-ME from culture medium also induced activation of apoptotic program in mouse embryonic stem cells [12].

Rho-associated coiled-coil kinase (ROCK) is associated with many cellular functions including the organization of the actin cytoskeleton [13], and inhibition of the

ROCK activity has been reported to promote recovery and growth of frozen-thawed human embryonic stem cells [14,15]. The ROCK inhibitor (Y-27632) is also used to stimulate in vitro growth of rabbit embryonic stem cells [16]. Since the effect of the ROCK inhibitor on preimplantation embryos has not yet been investigated, the present study was conducted to clarify whether the presence of ROCK inhibitor in the culture medium improve the revivability of bovine IVP blastocysts vitrified by minimum volume cooling (MVC) procedure. In an additional experiment, the Y-27632 was supplemented to culture medium on either Day-0, -2 or -4 until Day-8, in order to investigate its effect on the yield of blastocysts.

2. Materials and methods

2.1. Chemicals and media

Unless otherwise stated, all chemicals used in this study, including Y-27632, were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Hepes-buffered TCM-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA) containing 0.2 mM sodium pyruvate and 50 µg/mL gentamycin sulfate was supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.002 AU/mL FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan) and 1 µg/mL 17ß-estradiol for in vitro maturation (IVM) medium, or with 3 mg/mL bovine serum albumin (BSA) with or

without 1000 IU/mL hyaluronidase for denuding or handling oocytes in atmosphere condition, respectively. Modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan) was used for sperm preparation. For in vitro fertilization (IVF), the mBO medium supplemented with 3 mg/mL BSA and 10 μg/mL heparin was used. The modified synthetic oviduct fluid (mSOF) [17] supplemented with 30 μL/mL essential amino acids solution (x 50, Gibco-11130), 10 μL/mL non-essential amino acids solution (x 100, Gibco-11140) and 5% FBS was used as culture medium for presumptive zygotes.

2.2. In vitro maturation, fertilization and culture

Abattoir-derived bovine ovaries were transported to the laboratory in 10 to 12 ºC saline within 24 h after slaughter. The contents of 2- to 8-mm follicles were aspirated with an 18-G needle connected to a 10-mL syringe, and oocytes surrounded with at least two layers of compact cumulus cells were washed twice with hepes-buffered TCM-199 supplemented with 3 mg/mL BSA, 0.2 mM sodium pyruvate and 50 μg/mL gentamycin sulfate. The oocytes were matured in 100-μL microdrops of the IVM medium for 22 h at 38.5 ºC under 5% CO_2 in air (10 to 12 oocytes per microdrop). Then, the oocytes were freed from the cumulus cells by a brief Vortex-mixing, and those with an extruded first polar body were defined as matured.

Commercially available frozen semen from a Japanese Black bull was used. After

thawing in water bath at 37 ºC for 30 sec, content of 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 and centrifuged for 20 min at 700 × *g*. The pellet was re-suspended in mBO medium supplemented with 5 mM theophylline and washed twice for 5 min at 300 × *g* each, and then re-suspended in mBO/BSA/heparin medium to yield a concentration of 2.5×10^7 sperm cells/mL. Ten to 12 matured oocytes in the IVF medium were co-incubated with the above sperm suspension at a final concentration of 5×10^6 sperm cells/mL for 6 h in 100-μL microdrops under mineral oil at 38.5 ºC under 5% CO_2 in air.

Up to 30 presumptive zygotes were cultured in 250-μL microdrop of the mSOF medium at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2. The cleavage rate was determined on Day-2 and expanding/fully-expanded blastocysts were harvested on Day-7. The Day-0 was defined as the day of IVF.

2.3. Vitrification and warming

Expanding or fully-expanded Day-7 blastocysts (Fig. 1A), except for collapsing or hatching blastocysts, were subjected to the MVC vitrification according to the method described previously by Tsujioka et al. [18] with minor modifications. Briefly, blastocysts were equilibrated in base medium (TCM199 + 20% FBS) containing 7.5% ethylene glycol (EG; Wako Pure Chemical Industries Co., Osaka, Japan) and 7.5% dimethylsulfoxide (DMSO; Wako) for 3 min at room temperature, and then transferred

into a vitrification solution consisting of the base medium containing 15% EG, 15% DMSO and 0.5 M sucrose for approximately 1 min at room temperature. Within this 1 min, up to five blastocysts with a minimal amount of the vitrification solution were loaded onto the top of the polypropylene strip of a Cryotop (Kitazato Supply Co., Tokyo, Japan), and then quickly immersed into liquid nitrogen (LN_2).

After storage for at least 15 min to 1 week in LN_2 tank, the blastocysts were warmed by immersing the polypropylene strip of a Cryotop into 3 mL of the base medium containing 1 M sucrose at 38.0 ºC, and kept for 1 min. The blastocysts were transferred to the base medium in a stepwise manner (0.5, 0.25 and 0 M sucrose for 3, 5 and 5 min, respectively).

2.4. Assessment of post-warm embryos

Vitrified-warmed blastocysts (Fig. 1B) were washed twice using 700-μL of the mSOF medium supplemented with or without 10 μM Y-27632. The post-warm blastocysts were then cultured in 250-μL microdrops of the mSOF medium with or without Y-27632 for 48 h at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2 (8 to 14 embryos per microdrop). At 24 h of the culture, blastocysts with a re-expanded blastocoel cavity were considered as surviving (Fig. 1C). The hatching rate was determined at 48 h of the culture (Fig. 1D). At the end of the culture, some of the surviving embryos were fixed in cold ethanol for 5 min, and then stained with 25 μg/mL Hoechst 33342 at room

temperature. After being mounted with antifade (100 mg 1,4-diazabicyclo[2.2.2]octane in 1 mL glycerol), the total cell number was counted under epifluorescence microscope.

2.5. Blastocyst yield in Y-27632-containing medium

In an additional experiment, effect of Y-27632 supplemented to culture medium for presumptive zygotes on the yield of blastocysts was investigated. The presumptive zygotes were prepared by IVM and IVF as described above, except that frozen semen from different Japanese Black bull was used. Up to 30 zygotes were cultured in 250-μL microdrop of the mSOF medium at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2. The 250-μL microdrops were added with 2.53-μL of 1 mM Y-27632 solution to give the final concentration of Y-27632 at 10 μM on either Day-0, -2, or -4. The cleavage rate was determined on Day-2 and expanding/fully-expanded blastocysts were harvested on Day-7 and -8.

2.6. Statistical analysis

Five replicates were performed in each of four experimental groups for vitrification. All the four groups ran simultaneously in a single experiment. The survival rate on Day-8 and the proportion of surviving embryos that hatched on Day-9 were analyzed by paired *t*-test. Mean total cell number of surviving Day-9 blastocysts was compared by student's

t-test. In addition, five replicates were performed in each of four experimental groups for the effect of Y-27632 on the blastocyst yield in vitro. Proportional data for cleavage and blastocyst development were arcsin-transformed and analyzed by Tukey test. A value of $P < 0.05$ was chosen as an indication of significant difference.

3. Results

Among a total of 868 presumptive zygotes cultured, 671 (77.3%) cleaved on Day-2 and 248 (28.6%) developed to expanding, fully-expanded or hatched blastocysts on Day-7. The Day-7 blastocysts, except for collapsed or hatched blastocysts, were subjected to vitrification and warming and the subsequent 48 h of culture with or without Y-27632 (n=52 each), while non-vitrified fresh Day-7 blastocysts were served as controls (n=50 each). Survival rate of blastocysts vitrified-warmed and cultured in the presence of Y-27632 (94.9 ± 2.4%) was higher ($P < 0.05$) than that of blastocysts vitrified-warmed and cultured in the absence of Y-27632 (78.0 ± 6.0%) (Table 1). However, the proportions of the surviving blastocysts that hatched until the end of the culture were comparable regardless of the presence of Y-27632 (62.8 ± 11.1 vs 59.6 ± 9.4%; $P > 0.05$). Mean total cell number of vitrified-warmed blastocysts on Day-9 was also comparable regardless of the presence of Y-27632 (135.2 ± 13.1 vs 146.7 ± 13.3; $P > 0.05$) (Fig. 2). In the non-vitrified control blastocysts, the Y-27632 during Day-7 to Day-9 stimulated the incidence of hatching (91.7 ± 3.8 vs 54.7 ± 8.9%, $P < 0.05$; Table 1), but the increase in

mean total cell number of blastocysts (230.0 ± 23.0 vs 191.2 ± 22.2) was not significant (P = 0.23) (Fig. 2).

In an additional experiment, Y-27632 was added to the culture medium for presumptive zygotes on either Day-0, -2, or -4 until Day-8 (Table 2). There was no increase in the yield of blastocysts by the Y-27632 supplementation to the medium (Day-7 plus Day-8; 7.5 ± 2.1 to 36.2 ± 3.2% vs 28.6 ± 6.9% in control group). Conversely, the longer exposure period with Y-27632 including the initial 48 h after IVF had a harmful effect on cleavage and blastocyst development ($P < 0.05$).

4. Discussion

The kinetics and extent of recovery after warming may depend not only on the integrity of the embryo itself but also on the nature of the culture conditions for the post-warm embryos. In the present study, the presence of ROCK inhibitor in culture medium was effective to rescue some bovine IVP blastocysts from injuries caused by the MVC vitrification (94.9 vs 78.0%; Table 1). Supplementation of 100 μM ß-ME to the culture medium has improved the in vitro development of the bovine IVP blastocysts after solid-surface vitrification [8] and MVC vitrification [19], with a higher 24-h survival of 91 vs 66% and a higher 12-h survival of 85 vs 64%, respectively. Therefore, it is suggested that ROCK inhibitor, Y-27632, is a possible alternative to highly toxic ß-ME in improving the revivability of cryopreserved bovine embryos during the post-thaw

culture.

In the present study, 10 μM of Y-27632 was chosen as a concentration to be added to the culture medium, according to previous reports [14,15] where this concentration was shown optimal in the growth of human embryonic stem cells. The cellular response or sensitivity to Y-27632 in differentiating preimplantation embryos may be similar to that of undifferentiated embryonic stem cells. Stimulatory effect of ß-ME on the in vitro production of bovine blastocysts by IVF [9,10,19,20] or somatic cell nuclear transfer [21] is also known, but supplementation of ROCK inhibitor to the culture medium did not improve the yield of bovine blastocysts after IVF (Table 2). Since the presence of 10 μM Y-27632 during the initial 48 h of the culture for presumptive zygotes disturbed their first cleavage, the concentration of Y-27632 to be added should be reconsidered in future research. Modifying culture condition to mimic the in vivo microenvironment may be promising as insulin-like growth factor-1 [22] or hyaluronan [23] have been successfully added to the culture medium to increase the blastocyst yield in bovine IVF system.

Supplementation of Y-27632 at an optimal concentration promoted the in vitro hatching of non-vitrified fresh blastocysts until Day-9, while this stimulatory effect of the Y-27632 on the incidence of hatching was not observed in vitrified-warmed blastocysts (Table 1). This may be due to that the presence of Y-27632 rescue some fair-quality embryos with lower hatchability, or that the vitrified-warmed embryos require longer culture period to initiate hatching because some of them require at least several hours to regain their original sizes (re-expand). Despite of considerable difference in the mean

total cell number of surviving Day-9 blastocysts between groups, no statistical significance was detected. The highly variable total cell numbers of Day-9 blastocysts (Fig. 2) are probably due to the variety of samples including pre-hatched and collapsed blastocysts. Conversely, Nedambale et al. [8] reported that supplementation of ß-ME to SOF medium improved not only survival rate (91 vs 66%) but also hatching rate (77 vs 43%) of vitrified-warmed bovine IVP blastocysts, with a significantly higher total cell number of hatched blastocysts (>50% increase). Controversially, recent report from Hosseini et al. [19] showed that the total cell number of vitrified-warmed and hatched bovine blastocysts was comparable regardless of the presence of ß-ME in culture medium (168.0 vs 158.0).

Although both ROCK inhibitor and ß-ME play a critical role in the inhibition of apoptosis [10,12,24,25], the protective mechanism against the cryoinjuries by ROCK inhibitor may be different from that provided by ß-ME. Considering the likely action of thiol compounds, the ß-ME enhances antioxidant activity of the embryos by increasing intracellular levels of glutathione which can act as a scavenger for reactive oxygen species [20,26]. Some morphological changes such as contraction, membrane blebbing and nuclear disintegration, which are driven by ROCK-mediated actin-myosin contractile force generation [24], occur during apoptosis [27]. When vimentin, the most common protein in intermediate filaments, is phosphorylated by ROCK, formation of the filaments is inhibited [28]. Therefore, inhibition of ROCK activity may protect the vitrified embryonic cells from apoptosis by making the cytoskeletal structures quiescent or stable.

Additionally, since slightly increased caspase activity under stressed conditions activates ROCK-I and accelerates the apoptotic process [29], inhibitors or antagonists of the ROCK may have a stimulatory effect on cell survival [30].

In conclusion, this report shows for the first time that supplementation of ROCK inhibitor to culture medium can improve the revivability of vitrified-warmed bovine IVP blastocysts. Further research regarding the precise action of the ROCK inhibitor on bovine embryos and in vivo developmental ability of the treated blastocysts after transfer to recipients would be required.

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Table 1

In vitro survival and hatching of bovine IVF blastocysts after vitrification: effect of Y-27632 supplemented to culture medium for post-warm embryos

		No.(%) of embryos		
Group	Y-27632	Treated	Surviving at 24 h	Hatched at 48 h *
Vitrified	+	52	49 (94.9 ± 2.4) [a]	30 (62.8 ± 11.1)
	−	52	40 (78.0 ± 6.0) [b]	24 (59.6 ± 9.4)
Fresh control	+	50	50 (100.0 ± 0.0)	46 (91.7 ± 3.8) [a]
	−	50	49 (98.0 ± 2.0)	27 (54.7 ± 8.9) [b]

Percentage data were expressed as Mean ± SEM in five replicates.

* Percentages were calculated from surviving embryos.

[a,b] Different superscripts within a column denote significant difference ($P < 0.05$).

Table 2

In vitro development of IVF-derived bovine zygotes in culture medium added with Y-27632 at three different timings

Timing of Y-27632 addition	No.(%) of zygotes			
	Cultured	Cleaved on Day-2	Developed to blastocysts on Day-7	Developed to blastocysts on Day-7 & -8
Day-0	164	57 (34.2 ± 7.0) [b]	7 (4.6 ± 1.8) [b]	12 (7.5 ± 2.1) [b]
Day-2	164	113 (67.8 ± 4.6) [a]	35 (21.3 ± 2.5) [a]	52 (31.4 ± 2.3) [a]
Day-4	165	115 (68.7 ± 4.8) [a]	38 (23.3 ± 1.8) [a]	60 (36.2 ± 3.2) [a]
No addition	164	113 (67.0 ± 6.5) [a]	35 (20.5 ± 4.0) [a]	50 (28.6 ± 6.9) [a]

Percentage data were expressed as Mean ± SEM in five replicates.

[a,b] Different superscripts within a column denote significant difference ($P < 0.05$).

Figure legends

Fig. 1. Expanding or fully-expanded bovine IVP blastocysts harvested on Day-7 (A); Day-7 blastocysts immediately after vitrification and warming (B); Vitrified-warmed and surviving blastocysts on Day-8 (C); Vitrified-warmed and hatched blastocysts on Day-9 (D). Scale bar = 200-μm.

Fig. 2. Box-plotted total cell number of vitrified-warmed or non-vitrified control blastocysts after 48 h of culture with or without Y-27632, as well as that of fresh Day-7 blastocysts.