Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 73 (2010) 1139–1145
Theriogenology
www.theriojournal.com

Stimulatory effect of Rho-associated coiled-coil kinase (ROCK) inhibitor on revivability of in vitro-produced bovine blastocysts after vitrification

S. Hochi [a,b,*], H. Abdalla [a,c], H. Hara [b], M. Shimoda [a], H. Morita [d], M. Kuwayama [d], M. Hirabayashi [e,f]

[a] *Graduate School of Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan*
[b] *Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan*
[c] *Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia 44519, Egypt*
[d] *Kato Ladies Clinic, Shinjuku, Tokyo 160-0023, Japan*
[e] *National Institute for Physiological Sciences, Okazaki, Aichi 444-8787, Japan*
[f] *The Graduate University of Advanced Studies, Okazaki, Aichi 444-8787, Japan*

Received 20 August 2009; received in revised form 29 December 2009; accepted 8 January 2010

Abstract

Inhibition of Rho-associated coiled-coil kinase (ROCK) activity promoted recovery and growth of frozen-thawed human embryonic stem cells. The primary objective was to determine if a ROCK inhibitor (Y-27632) in post-thaw culture medium improved revivability of vitrified IVP bovine blastocysts. Expanding or expanded blastocysts (7 d after IVF) were vitrified (minimum volume cooling procedure, using a Cryotop) in 15% ethylene glycol, 15% DMSO and 0.5 M sucrose. When post-warm blastocysts were cultured in mSOF medium, survival rate (re-expansion of blastocoel at 24 h of culture) was improved ($P < 0.05$) by the addition of 10 μM Y-27632 (94.9 ± 2.4%, mean ± SEM) compared to a control (78.0 ± 6.0%). Conversely, after 48 h of culture, there were no significant differences in hatching rate (62.8 ± 11.1 vs. 59.6 ± 9.4%) and mean total cell number (135.2 ± 13.1 vs. 146.7 ± 13.3). In non-vitrified IVP bovine blastocysts, the hatching rate on Day 9 was improved by Y-27632 (91.7 ± 3.8 vs. 54.7 ± 8.9%, $P < 0.05$), with no difference in mean total cell number of blastocysts (230.0 ± 23.0 vs. 191.2 ± 22.2, P = 0.23). In an additional experiment, Y-27632 was added to culture medium on either Day 0, Day 2, or Day 4 (and remained present until Day 8), resulting in no improvement in blastocyst yield compared to a control group (7.5 ± 2.1, 31.4 ± 2.3, 36.2 ± 3.2, and 28.6 ± 6.9%, respectively). In conclusion, adding a ROCK inhibitor to post-thaw culture medium improved revivability of IVP bovine blastocysts after vitrification and warming.

Keywords: Bovine blastocysts; IVF; Rho-associated coiled-coil kinase; Vitrification; Y-27632

1. Introduction

Although *in vitro* production (IVP) of bovine embryos and subsequent transfer to recipients is practical, the survival rate of IVP embryos after cryopreservation is lower than that of their *in vivo*-derived counterparts [1,2]. Culture of presumptive

* Corresponding author. Tel.: +81 268 215350; fax: +81 268 215830.
E-mail address: shochi@shinshu-u.ac.jp (S. Hochi).

0093-691X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2010.01.015

zygotes in a serum-free medium [3], removal of cytoplasmic lipid droplets [4], a slower cooling rate during two-step freezing [5], and supplementation of linoleic acid-albumin [6] or hyaluronan [7] increased 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 improved *in vitro* development of embryos after solid-surface vitrification [8]. The protection conferred by ß-ME was attributed to its anti-oxidative action, which inhibits cell apoptosis [9,10]. Supplementation of ß-ME to culture medium for IVP-derived presumptive zygotes also increased the yield of bovine blastocysts [9,10].

Rho-associated coiled-coil kinase (ROCK) of approximately 160 kDa is a member of serine/threonine-specific kinase, and serves as a target protein of Rho proteins in the small GTP-binding protein superfamily. The Rho proteins are responsible for various cellular functions, e.g., contractile force generation, cytoplasmic division, and cell motility, which are mediated by organization of the actin cytoskeleton [11]. The ROCK (two isoforms; ROCK I and ROCK II) is comprised of a catalytic domain at the N-terminal, followed by a coiled-coil domain in which the Rho protein can bind, and a Pleckstrin-homology domain. Target proteins for phosphorylation by ROCK include the regulatory myosin light chain [11] and the LIM kinase-1 and kinase-2 [12]. Inhibition of ROCK activity promoted recovery and growth of frozen-thawed human embryonic stem cells [13,14]. The ROCK inhibitor (Y-27632) was also used to stimulate *in vitro* growth of embryonic stem cells derived from rabbits [15] and mice [16].

Since the effect of a ROCK inhibitor on preimplantation embryos has not yet been investigated, the present study was conducted to clarify whether the presence of a ROCK inhibitor (Y-27632) in the culture medium improved the revivability of bovine IVP blastocysts vitrified by a minimum volume cooling (MVC) procedure. In an additional experiment, Y-27632 was added to culture medium, starting on Days 0, 2, or 4 (and continued until Day 8), to investigate its effect on the yield of blastocysts following standard IVM and IVF.

2. Materials and methods

2.1. In vitro maturation

Unless otherwise stated, 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 saline (maintained at 10 to 12 °C) 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. Oocytes surrounded with at least two layers of compact cumulus cells were washed twice with hepes-buffered TCM-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA), supplemented with 3 mg/mL bovine serum albumin (BSA), 0.2 mM sodium pyruvate, and 50 μg/mL gentamycin sulfate. The hepes-buffered TCM-199 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 was used for IVM medium. Oocytes were matured in 100-μL microdrops of the IVM medium for 22 h at 38.5 °C under 5% CO_2 in air (10–12 oocytes per microdrop). Then, cumulus cells were removed by a brief vortex-mixing in the hepes-buffered TCM-199 supplemented with 1000 IU/mL hyaluronidase; those with an extruded first polar body were defined as matured.

2.2. In vitro fertilization and culture

Commercially available frozen semen from a Japanese Black bull (Livestock Improvement Association of Japan, Inc., Tokyo, Japan) was used. After thawing in a water bath at 37 °C for 30 s, 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 modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan), and centrifuged for 20 min at 700 × *g*. The pellet was re-suspended in mBO medium supplemented with 5 mM theophylline, washed twice (5 min at 300 × g each), and then re-suspended in the mBO medium supplemented with 3 mg/mL BSA and 10 μg/mL heparin (IVF medium), to yield a concentration of 2.5×10^7 sperm/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/mL for 6 h in 100-μL microdrops under mineral oil at 38.5 °C under 5% CO_2 in air.

Up to 30 presumptive zygotes were cultured in a 250-μL microdrop of 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 at 39.0 °C 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 (Day 0 = day of IVF).