Theriogenology 87 (2017) 108–114

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Cell apoptosis and lipid content of *in vitro*–produced, vitrified bovine embryos treated with forskolin

Daniela Martins Paschoal [a,*], Mateus José Sudano [b], Kátia Regina Lancellotti Schwarz [a], Rosiára Rosário Dias Maziero [c], Midyan Daroz Guastali [c], Letícia Ferrari Crocomo [c], Luis Carlos Oña Magalhães [c], Alício Martins Jr [d], Claudia Lima Verde Leal [a], Fernanda da Cruz Landim-Alvarenga [c]

[a] *Departmento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, SP, Brazil*
[b] *Universidade Federal do Pampa, Faculdade de Medicina Veterinária, Uruguaiana, RS, Brazil*
[c] *Departmento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade do Estado de São Paulo, Botucatu, SP, Brazil*
[d] *Departmento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária, Universidade do Estado de São Paulo, Araçatuba, SP, Brazil*

ARTICLE INFO

Article history:
Received 20 November 2015
Received in revised form 8 August 2016
Accepted 9 August 2016

Keywords:
Apoptosis
Blastocyst
Cryopreservation
Forskolin
Lipid
Vitrification

ABSTRACT

The presence of fetal calf serum in culture medium influences embryo quality, causing a reduction in postcryopreservation survival. Forskolin has been used to induce lipolysis and increase cryotolerance, functioning as an activator of adenylate cyclase and elevating cAMP levels. In the present experiment, bovine zygotes were cultured in synthetic oviduct fluid with amino acid plus 2.5% fetal calf serum for 6 days, when forskolin was added in three concentrations: 2.5, 5, and 10 μM. Treatment with forskolin lasted for 24 hours. Blastocyst formation rate, quantification of lipid granules, total cell numbers, and apoptosis rate were evaluated. In a second assessment, embryos were vitrified, and warming, re-expansion rate, total cell numbers, and apoptosis rate were also evaluated. There was no difference due to forskolin in blastocyst formation or re-expansion rates after vitrification. However, lipid measurements were lower (control: 136.8 and F 2.5 μM: 128.5; $P < 0.05$), and number of cells per embryo higher (control: 140.1 and F 2.5 μM: 173.5; $P < 0.05$) than controls for 2.5 μM forskolin but not for higher forskolin concentrations. The number of intact cells per embryo was higher, and the rate of apoptosis was lower in fresh than in vitrified embryos (number of cells of warmed embryos, control: 104.1, F 2.5 μM: 101.3, F 5 μM: 115.4, F 10 μM: 95.1; apoptotic of fresh cells, control: 12.1%, F 2.5 μM: 16.7%, F 5 μM: 11.1%, F 10 μM: 14.2%; and apoptotic warmed embryos, control: 22.3%, F 2.5 μM: 37.3%, F 5 μM: 33.2%, F 10 μM: 30.3%; $P < 0.05$). It was concluded that forskolin is an effective lipolytic agent even at low concentrations, leading to formation of blastocysts with a comparatively larger number of cells.

1. Introduction

In 2011, Brazil produced 350,762 cattle embryos, 90.7% of which were *in vitro* produced (IVP). This represents an increase of 15.7% over the total number of embryos

* Corresponding author. Tel.: +55 19 35654306; fax: +55 19 35611689.
E-mail address: dmpaschoal@gmail.com (D.M. Paschoal).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.08.011

produced in 2010. Such recent growth in the number of embryos produced is associated with significant changes in agricultural activities, indicating the profile of future embryo technologies [1].

Such IVP embryos, however, are of inferior quality to those produced *in vivo*, as *in vitro* production conditions cannot perfectly simulate the natural environment [2]. Fetal calf serum (FCS) is a major contributor to such differences in quality, resulting in morphological [3,4] and physiological [5,6] changes in IVP embryos, such as an increase in the number and size of lipid granules [3,7–10]. This increase influences embryo quality [11,12] and survival rates following cryopreservation [9,13,14]. This factor is, however, conflicting, as vitamins, fatty acids, and growth factors found in FCS are beneficial for the morula compaction and blastocyst development [15].

Forskolin has been used in culture media to reduce lipid content by chemical lipolysis, and increase the cryotolerance and postvitrification quality of embryos [15–18]. Forskolin (7β-acetoxy-8,13-epoxi-1α,6β,9α-trihydroxy-Labd-14-en-11-one $C_{22}H_{34}O_7$) is a dipertene [19–21], which activates adenylate cyclase in intact cells. The enzyme activates endogenous lipase through cAMP and protein kinase lipolysis, causing the release of fatty acids and glycerol [16].

Vitrification has been considered as an alternative to freezing [18,22] and is used routinely both in laboratories and farms [23]. This technique is simple to execute, less costly [24,25], and faster than freezing and does not cause the formation of intracellular ice crystals [26]. The aim of the present study was to induce lipolysis by adding forskolin on the sixth day of embryo culture to reduce intracytoplasmic lipid granules and increase the survival rate of bovine IVP embryos after cryopreservation.

2. Material and methods

All chemicals were obtained from Sigma (Sigma–Aldrich Corporation, St. Louis, MO, USA), except where specified in the text.

2.1. Collection of ovaries and IVM

Slaughterhouse-derived bovine ovaries were collected and transported to the laboratory. Follicles measuring 2 to 8 mm were aspirated. Oocytes (n = 1.509, in eight replicates) with at least three intact layers of cumulus cells and homogenous cytoplasm were selected for IVM. The oocytes were transferred to drops of Tissue Culture Medium 199 (TCM 199, Gibco 11150-059, Grand Island, NY, USA) supplemented with 10% (vol/vol) fetal calf serum (FCS, Gibco 12657-029, Grand Island, NY, USA), 11-mg/mL sodium pyruvate, 1-μg/μL FSH (Pluset, Hertape Calier Saúde Animal, Juatuba, Brazil), 5 μg/μL LH (Lutropin, Bioniche Co., Belleville, Ontário, Canada), and 100-UI/mL amikacin. The oocytes were incubated for 24 hours in an incubator with humidified 5% CO_2 in air at 38.5 °C.

2.2. In vitro fertilization

Matured oocytes from all groups were fertilized *in vitro* (IVF = Day 0) with frozen Nelore bull semen (*Bos taurus indicus*). The semen was prepared using the Percoll method [27], and the final concentration was adjusted to 2×10^6 sperm/mL. The oocytes were fertilized in Human Tubal Fluid (Irvine Scientific, Santa Ana, CA, USA) supplemented with 5-mg/mL BSA (A-8806), 11-mg/mL sodium pyruvate, 0.5-mg/mL caffeine, 3-mg/mL heparin, 0.3-mg/mL penicillamine, 0.11-mg/mL hypotaurine, 0.18-mg/mL epinephrine, and 100-UI/mL amikacin.

2.3. In vitro culture

Presumptive zygotes (n = 577) were cultured in synthetic oviduct fluid (SOFaa—[28]) supplemented with 2.7-mM myo-inositol, 0.2-mM sodium pyruvate, 5-mg/mL BSA (A-8806), 2.5% (vol/vol) FCS, and 100 UI/mL amikacin. The embryos were kept in an incubator with humidified 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C until Day 6. When Forskolin (F-6886) was added as follows, embryos were divided in four groups for forskolin addition for 24 hours: group F 2.5 μM (2.5 μM forskolin), group F 5 μM (5 μM forskolin), and group F 10 μM (10 μM forskolin). A control group without forskolin treatment was also assessed. On Day 7 (Day 0 = IVF), forskolin was removed from culture, and the rate of blastocyst formation was evaluated.

2.4. Vitrification and warming of embryos

Vitrification was performed with blastocysts (Bl) and expanded blastocysts (n = 259). The protocol of Campos-Chillon et al. [23] was followed with some modifications. The embryos were exposed to vitrification medium 1 composed of 5-M ethylene glycol for 3 minutes and

Table 1
Evaluation of blastocyst production and analysis of lipid on Day 7 with different concentrations of forskolin added on Day 6.

Treatments	Evaluation of blastocysts		Analysis of lipid in blastocysts		
	N. Blast./N. Ooc.	% Blast.	N. Blast.	Gray intensity/area ($\times 10^{-3}/\mu m^2$)	Gray intensity/volume ($\times 10^{-3}/\mu m^3$)
Control	170/445	37.0 ± 4.8	10	136.8 ± 2.2^{ab}	50.6 ± 1.1^{ab}
F 2.5 μM	136/351	38.6 ± 2.5	10	128.5 ± 2.2^{c}	46.2 ± 1.1^{c}
F 5 μM	164/376	40.7 ± 4.0	10	135.6 ± 2.3^{b}	49.9 ± 1.2^{b}
F 10 μM	107/337	31.4 ± 4.2	10	142.7 ± 2.4^{a}	53.9 ± 1.2^{a}

^{abc}Values indicated by different superscript letters in the same column differ ($P < 0.05$).
Total of eight replicates for evaluation of blastocysts and five for analyses of lipid (least-squares means ± standard error mean).
Abbreviations: F, forskolin; N. Ooc., number of oocytes matured; N. Blast., number of blastocysts produced; % Blast., total blastocyst rate; N. Blast., number of blastocysts submitted to Sudan Black technique.