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Cryosurvival and pregnancy rates after exposure of IVF-derived *Bos indicus* embryos to forskolin before vitrification

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ABSTRACT

In vitro–produced (IVP) bovine embryos are more sensitive to cryopreservation than their *in vivo* counterparts due to their higher lipid concentrations, whereas *Bos indicus* IVP embryos are even more sensitive than *Bos taurus* IVP embryos. To examine the effects of a lipolytic agent, before vitrification of *Bos indicus* IVP embryos, on embryo survival, viability, and pregnancy rates, two experiments were conducted. In experiment 1, *Bos indicus* (Nelore) embryos were produced from abattoir-derived ovaries and allocated into two groups. In the treatment group, 10 μM of forskolin was added to the *in vitro* culture medium on Day 5 and incubated for 48 hours. On Day 7 of culture, IVP-expanded blastocysts from both the control (n = 101) and treatment (n = 112) groups were vitrified with ethylene glycol and DMSO via the Cryotop procedure. Although there was no significant difference between the rates of blastocoel reexpansion and hatching of the embryos exposed to forskolin (87.5% and 70.5%, respectively) compared with the control embryos (79.2% and 63.3%, respectively), the numerically superior rates of the embryos exposed to forskolin led to another experiment. In experiment 2, blastocysts produced from the ovum pick up were exposed or not exposed to the lipolytic agent and vitrified as in experiment 1. Embryos treated with forskolin had higher pregnancy rates than the control group (48.8% vs. 18.5%). In view of these results, 1908 *Bos indicus* embryos were produced from ovum pick up, exposed to the lipolytic agent, and blastocysts were transferred to recipients, and the pregnancy rates of the embryos of various breeds were compared. The mean pregnancy rate obtained was 43.2%. All data were analyzed by chi-square or by binary logistic regression ($P \leq 0.05$). In conclusion, treatment with forskolin before vitrification improved cryotolerance of *Bos indicus* IVP embryos, resulting in good post-transfer pregnancy rates.

1. Introduction

Bos indicus accounts for the majority of Brazilian cattle herds, thereby contributing to this country's place at the forefront of *in vitro* embryo production (IVEP) [1]. However, the availability of recipients is typically less than needed (more embryos are produced than are implanted). Thus, considerable effort has been invested in the development of an efficient protocol for bovine embryo cryopreservation. Low rates of pregnancy after warming are associated with low numbers of embryos that are produced *in vitro* and subjected to cryopreservation worldwide [2]. The main problems include the lack of consistency in results [3] and

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differences in the survival and developmental rates after warming across species, developmental stages, and quality of embryos [4].

Most cryopreservation methods are based on two factors, the use of cryoprotectants and cooling rate [5]. Vitrification is a widely used method [6]. The minimal-volume approach of the Cryotop method increases the rates of cooling, and especially warming (up to 40,000 °C/minute), which may contribute to consistent, improved survival rates and improved rates of development, both *in vitro* and *in vivo*. Cryotop technology has been successfully used for cryopreservation of oocytes from various species [7,8] and *in vitro* fertilization (IVF)-derived embryos [9], reconstructed embryos with somatic cell nuclei [10], blastocysts produced by parthenogenic activation, or somatic cell nuclear transfer from delipidated *in vitro*-matured oocytes [10] and embryos been derived from intracytoplasmic sperm injection [11].

Vitrification is the most efficient cryopreservation method for embryos produced *in vitro* [6], which are more sensitive to cryoinjuries than their *in vivo* counterparts [12] because they contain more intracellular lipid droplets [13]. In addition, sensitivity of *Bos indicus* IVP embryos is higher than that of *Bos taurus* embryos [14]. The extent of cryoinjury is dependent on the size and shape of the embryonic cells, as well as on membrane permeability and quality and sensitivity of the embryos [5]. Triacylglycerols make up most of the intracellular lipids of embryos [15], and lipolysis can be induced by lipolytic agents, such as norepinephrine, dibutyryl cAMP, isoproterenol, forskolin, and theophylline [16,17]. The diterpene forskolin, which is derived from the roots of *Coleus forskohlii* [17], is used to induce chemical delipidation of IVP-derived embryos [18,19].

Data concerning the effect of forskolin on *Bos indicus* IVP embryos are scarce, and the effect of forskolin on pregnancy rates of cryopreserved IVP bovine embryos is currently unknown. Addition of forskolin to culture medium of *Bos indicus* IVP embryos before vitrification could improve survival rates, providing satisfactory pregnancy rates after the transfer of these embryos.

The aim of this study was to evaluate the effect of forskolin added during *in vitro* embryo culture on cryosurvival by assessing the rates of blastocoel reexpansion and hatching after vitrification of *Bos indicus* IVP embryos; in addition, its effect on pregnancy rates following embryo transfer was assessed and compared among zebu breeds.

2. Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise.

2.1. Experimental design

In experiment 1, conducted in two replications, the blastocysts produced from abattoir-derived ovaries were cultured until Day 7 (fertilization = Day 0) when they were vitrified and rewarmed at the expanded blastocyst stage by the Cryotop procedure. On Day 5, the embryos were separated into two groups, those that were exposed and not exposed to 10 μM forskolin (7β-acetoxy-8,13-epoxy-lα,6β,9α-trihydroxylabd-14-en-11-one, $C_{22}H_{34}O_7$) for 48 hours before vitrification. Following rewarming, the blastocysts were cultured for 24 hours to assess cryosurvival. Embryos that survived vitrification were cultured for an additional 48 hours to assess hatching ability. For both the assessments, nonvitrified fresh blastocysts were used as controls.

In experiment 2, embryos produced from ovum pick up were cultured with or without exposure to forskolin, as in experiment 1. Embryos were transferred at the blastocyst stage to female recipients (two replications) and the pregnancy rates were compared. Results indicating the forskolin-induced embryo survival after vitrification led to the production of 1908 *Bos indicus* embryos after ovum pick up, all of them exposed to forskolin and vitrified. These embryos were transferred to recipients, and the pregnancy rates obtained from the embryos of various breeds were compared.

2.2. Oocyte recovery

2.2.1. In vitro

Abattoir-derived ovaries from Nelore cows were collected at a local abattoir stored in saline solution at 25 °C to 30 °C and transported to the laboratory within 25 minutes after slaughter. Briefly, each follicle was punctured with a disposable 19-gauge 1/2″ hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 20-mL syringe.

2.2.2. In vivo

Follicular aspiration was performed as described [20,21]. Briefly, each visible follicle ≥2 mm in diameter was aspirated using a real-time B-mode ultrasound scanner (Scanner 200 Vet, Pie Medical, Maastricht, The Netherlands), a 7.5-MHz convex array transducer fitted into the intravaginal device (Pie Medical), and a stainless steel guide. Follicular puncture was performed using a disposable 19 gauge × 12 mm hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 50-mL conical tube (Corning, Acton, MA, USA) via a silicon tube (0.8 m long; 2 mm internal diameter). Aspiration was performed using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10 to 12 mL of water/minute. The collection medium was TCM-199 (Gibco Life Technologies, Grand Island, NY, USA), supplemented with 25 mM HEPES (Sigma H-0763), 5% fetal calf serum (FCS), 50 μL/mL gentamycin sulfate (Schering-Plough, São Paulo, SP, Brazil), and 10,000 IU/L sodium heparin (Sigma H-3149).

2.3. In vitro maturation

Oocytes with at least three layers of compact cumulus cells were classified as grade 1 [20] and were matured for 24 hours in 100 μL drops of TCM-199 (Gibco Life Technologies) that were supplemented with 10% FCS (Gibco Life Technologies), 1 μg/mL FSH (Folltropin, Bioniche Animal Health, Belleville, ON, Canada), 50 μg/mL hCG(Profasi, Serono, São Paulo, SP, Brazil), 1 μg/mL estradiol (estradiol-17β), 0.2 mM sodium pyruvate, and 83.4 μg/mL amikacin

(Instituto Bioquímico, Rio de Janeiro, Brazil) under mineral oil (D'Altomare, Santo Amaro, SP, Brazil) at 39 °C and 5% atmospheric CO_2 (25–30 oocytes per microdrop). Before *in vitro* maturation, cumulus oocyte complexes were washed three times in TCM-199 HEPES that was supplemented with 10% FCS, 0.20 mM sodium pyruvate, and 83.4 μg/mL amikacin.

2.4. IVF and in vitro culture

Freeze-thawed sperm (2×10^7/dose) from sires of known fertility (based on previous utilization for IVF) were used. Straws were thawed for 30 seconds in a 35 °C water bath. Sperm were washed twice by centrifugation at 200 × *g* for 5 minutes in 2 mL TALP medium that was supplemented with 10 mM HEPES (Gibco Life Technologies), 0.2 mM sodium pyruvate, and 83.4 g/mL amikacin. Sperm were capacitated using heparin, and motility was stimulated by the addition of penicillamine, hypotaurine, and epinephrine.

After visual assessment of motility, the sperm concentration was adjusted to 25×10^6 motile sperm/mL, and each fertilization drop containing 90 μL TALP-IVF medium that was supplemented with 10 g/mL heparin, 18 M penicillamine, 10 M hypotaurine, and 8 M epinephrine received 4 μL of sperm (final concentration of 1×10^5 sperm per drop) [20]. After maturation, the cumulus oocyte complexes were washed three times in TCM-199 prefertilization medium that was supplemented with 25 mM HEPES and 0.3% BSA and washed once in TALP fertilization medium that was supplemented with 10 μg/mL heparin and 160 μL of penicillamine, hypotaurine, and epinephrine solution [22,23].

Presumptive zygotes had their cumulus cells removed and were transferred to 100 μL drops of embryo culture medium (SOFaa BSA containing 0.5% BSA and 2.5% FCS) under the same temperature and gaseous atmospheric conditions that were used for IVF. After 3 days (D3) and 5 days (D5) of culture, 50% of the culture medium was replaced with fresh medium (feeding) from a stock of the same medium that was used at the beginning of the culture. The cleavage and blastocyst production rates were recorded at D3 and D7 of culture, respectively.

2.5. Vitrification and warming

In experiment 1, embryos cultured with (n = 112) or without (n = 101) 10 μM forskolin were subjected to Cryotop vitrification, as described [24]. Expanded blastocysts of excellent quality were equilibrated with 10% ethylene glycol (WakoPure Chemical Industries Co., Osaka, Japan) and 10% DMSO (WakoPure Chemical Industries Co.) in TCM-HEPES base medium (TCM-199, 25 mM HEPES that was supplemented with 20% FCS) for 1 minute at room temperature. Then, the embryos were transferred into a vitrification solution consisting of 20% ethylene glycol, 20% DMSO, and 0.5 M sucrose in the base medium and incubated for 20 seconds at room temperature. During this incubation, blastocysts were loaded onto the top of the polypropylene strip of a Cryotop (three to five embryos; Kitazato BioPharma Co., Shizuoka, Japan) with a minimal amount of vitrification solution. They were then quickly immersed in liquid nitrogen (N_2). As a control, randomly selected fresh embryos (n = 96) were evaluated at D7, D8, and D9 of culturing. In experiment 2, embryos were vitrified using the same protocol.

2.6. Postwarming assays

After storage for >2 hours in liquid N_2, blastocysts were exposed to air for 4 seconds, warmed by immersing the polypropylene strip of a Cryotop into the base medium (TCM-HEPES and sucrose) at ~35 °C and then held for 1 minute. Then, the blastocysts were transferred to the base medium at room temperature in a stepwise manner (0.3 and 0.15 M sucrose for 5 minutes each) [25,26]. In experiment 1, the blastocysts were cultured in 100 μL drops of SOF medium containing 2.5% FCS and 0.5% BSA under mineral oil at 39 °C and 5% atmospheric CO_2. Cryosurvival was assessed by reexpansion of the blastocoel after 24 hours of culturing. Thereafter, the surviving embryos and the corresponding fresh control D9 embryos were randomly assigned to further 48-hour cultures to determine their abilities to hatch. Embryos with more than half of their embryonic cells emerging from the zona pellucida were defined as hatched. In experiment 2, the embryos were transferred to recipients after warming.

2.7. Protocol for embryo transfer

In experiment 2, all vitrified embryos were transferred to Nelore and Nelore crossbred recipients after warming. A fixed-time embryo transfer protocol was used to ensure recipient estrus synchrony. Each recipient received an intravaginal progesterone implant (CIDR, Pfizer, Hamilton, New Zealand) and 2 mg of estradiol benzoate (Estrogin, Farmavet, São Paulo, SP, Brazil) on Day 0. The progesterone implants were removed on Day 8, at which time the animals were given 300 IU of eCG (Novormon, Syntex, Buenos Aires, Argentina), 150 μg of D-cloprostenol (Preloban, Intervet, São Paulo, SP, Brazil), and 1 mg of estradiol cypionate (Pfizer, Guarulhos, SP, Brazil). No detection of estrus was performed; Day 10 was considered the day of estrus. One embryo was transferred per recipient on Day 17. Before embryo transfer, each recipient was subjected to a transrectal ovarian examination (Aloka SSD 500, 5 MHz linear transducer, Tokyo, Japan) to confirm the presence and size of CLs. Only recipients with a CL ≥ 13 mm received an embryo. Pregnancy status was determined by ultrasonography on Day 30.

2.8. Statistical analyses

Cleavage, blastocyst, and pregnancy rates were compared between forskolin and control groups by a binary logistic regression model in a two-factorial design (treatment and replicate). Pregnancy rates were compared between different breeds by the chi-square test. For all analyses, $P \leq 0.05$ was considered statistically significant.

3. Results

In experiment 1, there were no differences in blastocyst rates across groups ($P > 0.05$). Out of the 314 IVF-derived

Table 1
Cryosurvival and hatching abilities of IVP *Bos indicus* blastocysts after treatment with or without forskolin for 48 hours in culture.

Treatment	Oocytes, no.	Cleavage rate, no. (%)	Blastocyst rate, no. (%)	Reexpanded blastocysts 24 h, no. (%)	Hatched blastocysts 48 h, no. (%)
No forskolin vitrification	348	253 (72.7)	139 (39.9)	80 (79.2)[a]	64 (63.3)[b]
10 μM forskolin vitrification	314	247 (78.8)	142 (45.3)	98 (87.5)[a]	79 (70.5)[b]
Nonvitrified (fresh) control	331	241 (72.7)	132 (39.9)	-	82 (85.4)[a]

[a,b] Within a column, rates without a common superscript differed ($P \leq 0.05$).

zygotes cultured with forskolin, 247 (78.8%) cleaved on Day 3, whereas 142 (45.3%) developed to expanded blastocysts on Day 7. Out of the 348 IVF-derived zygotes cultured without forskolin, 253 (72.7%) cleaved on Day 3 and 139 (39.9%) developed to expanded blastocysts on Day 7 (Table 1).

There were no differences between the rates of blastocoel reexpansion and hatching of the embryos exposed to forskolin (87.5% and 70.5%, respectively) compared with embryos that were not treated with the lipolytic agent before vitrification (79.2% and 63.3%, respectively). Hatching rates of both groups were still lower ($P < 0.05$) than those in the fresh control embryos (85.4%; Table 1).

In experiment 2, the average cleavage rate was 78% and the average blastocyst rate was 42%. Pregnancy rates were higher for the embryos that were exposed to forskolin (48.8%) than for the embryos that were not (18.5%; Table 2). After transfer of the 1908 vitrified-warmed embryos that had been treated with forskolin, pregnancy rates were not different ($P > 0.05$) among various zebu breeds (rates varied from 40.3% to 45.5%, with an average rate of 43.2%; Table 3).

4. Discussion

To our knowledge, this was the first study to report pregnancy rates from vitrified *Bos indicus* embryos treated with a stimulator of lipolysis. Herein, we report that exchanging genetic material for research or commercial purposes was possible with the use of the lipolytic agent forskolin before embryo vitrification using the Cryotop procedure. These data should be of great interest to the embryo industry, as reasonable pregnancy rates were obtained following cryopreservation of IVP zebu embryos.

There is a scarcity of literature regarding the pregnancy rates of *Bos indicus* IVP embryos, and even less information describing cryopreserved embryos. Our mean pregnancy rate with forskolin (43.2%) seemed much higher than those that had been reported by Block et al. [27] (27.7%) and Stewart et al. [28] (25.7%) with vitrified IVP Holstein and Holstein and Jersey embryos, respectively. It was also higher than that obtained by Dochi et al. [29] with frozen IVP Holstein embryos (29.5%). Interestingly, the pregnancy rates that were obtained in this study are very similar to or even higher than those obtained by our team after transfer of fresh IVP *Bos indicus* embryos (33.5% [30] and 36.57% [31] with Nelore cattle and 37% to 40% with Holstein, Gir, and Holstein-Gir crossbred females [32]). It is important to emphasize that achievement of such encouraging pregnancy rates requires only cryopreservation of embryos of excellent quality.

Few studies have reported the use of forskolin for chemical delipidation of *Bos indicus* embryos before cryopreservation and its effect on cryosurvival of vitrified embryos. In a recent study [33], rates of blastocoel reexpansion in IVP Nelore-sired expanded blastocysts that had been cultured with or without 10 μM forskolin (81.09% vs. 75.07%) did not differ from rates of expansion of *in vivo*–derived Nelore embryos (86.40%). Although those rates are similar to our results (87.5% vs. 79.2%), the duration of exposure to forskolin was different (12 vs. 48 hours of culture). Additionally, in agreement with our study, there was no significant improvement in reexpansion rates after cryopreservation after exposure to forskolin. Similarly, Pryor et al. [19] reported no differences between *Bos indicus* embryos that had been cultured with or without forskolin in terms of survival to freezing (59.4% vs. 49.0%) and blastocyst hatching rates (29.1% vs. 48.5%) when forskolin was added on Day 6 of culture. In a study by Paschoal et al. [34], forskolin was also added on Day 6; it did not improve reexpansion rates after vitrification, but apparently reduced the number of damaged cells after cryopreservation when serum was used. In the present study, the addition of 10 μM forskolin on D5 of culture, which was 48 hours before vitrification, did not significantly improve rates of blastocoel reexpansion and hatching (89.5% vs. 79.2% and 70.5% vs. 63.3%, respectively). Nevertheless, numerically superior rates obtained with this treatment encouraged us to investigate the effects of exposure to forskolin before vitrification on pregnancy rates of IVP Nelore-sired embryos.

There were no significant differences in blastocyst rates for embryos that were treated with forskolin (45.3%) compared with those that were observed in the vitrified control embryos (39.9%). These results were in accordance with the study by Paschoal et al. [33], who reported blastocyst rates of 46.3% versus 46.8% for Nelore-sired IVP embryos that were cultured with or without forskolin before vitrification. Paschoal et al. [34] also reported no differences between blastocyst rates of embryos that were cultured with (46.3% with FCS and 34.2% with no serum) or without forskolin (46.8% with FCS and 33.3% with no serum).

Table 2
Pregnancy rates of IVP *Bos indicus* embryos after treatment with or without forskolin for 48 hours in culture before vitrification.

Treatment	Transferred embryos (no.)	Pregnancies (no.)	Pregnancy rate (%)
Control	65	12	18.5[b]
Forskolin	80	39	48.8[a]

[a,b] Within a column, rates without a common superscript differed ($P \leq 0.05$).

Table 3
Pregnancy rates of IVP *Bos indicus* embryos treated with forskolin for 48 hours in culture and submitted to vitrification.

Breed	Transferred embryos (no.)	Pregnancies (no.)	Pregnancy rate (%)
Taurus-indicus	87	37	42.5
Gir	701	314	44.8
Guzera	680	274	40.3
Nelore	440	200	45.5
Total	1908	825	43.2

No effect ($P > 0.05$) of breed on pregnancy rate.

Bos indicus IVP embryos have higher levels of intracellular lipids than those of *Bos taurus*, which increases their sensitivities to conventional freezing methods [14]. The use of lipolytic agents is a noninvasive approach to achieve the metabolic reduction of intracellular lipids, high levels of which are largely responsible for the exceptional sensitivity of these cells to cryopreservation. Lipolytic agents act on various components of the lipolytic signal transduction pathway, stimulating hydrolysis of intracellular lipids. Forskolin stimulates lipolysis by directly activating adenylate cyclase, increasing the levels of cAMP and activating lipases [35–37]. In porcine embryos, forskolin was responsible for reported reduction in levels of intracellular lipids [18]. In bovine IVP embryos, however, controversial effects were observed, whereas forskolin was able to decrease cellular lipid concentrations [38] or not [39]. Nevertheless, it is possible that forskolin may induce effects on the embryonic cells that are still unknown. By increasing cAMP concentrations, it might alter embryo metabolism or gene expression, leading to altered development. Although embryos and the offspring seemed normal, the effects of forskolin on metabolism and development were not thoroughly investigated in this study.

There are other substances that seem capable of decreasing embryo lipid concentrations, but their effects have not been well established. Pereira et al. [40,41] reported that the addition of an isomer of conjugated linoleic acid (CLA) to culture media decreased lipid concentrations and increased cryotolerance of IVP bovine embryos. However, no pregnancy rates were reported. The t10, c12 CLA seemed to act mainly by reducing the uptake and synthesis of fatty acids [42,43]. Conversely, Darwich et al. [44], reported no improvement in cryopreserved embryo survival after addition of t10, c12 CLA.

This is apparently the first report of pregnancy rates from IVP bovine embryos that were vitrified after treatment with forskolin. This represents great progress in IVEP, enabling the commercial use of the technique. Until recently, surplus embryos obtained after embryo production programs have been discarded because there was no protocol allowing for reasonable pregnancy rates after cryopreservation of these embryos.

The optimal pregnancy rates that were obtained after the use of forskolin and vitrification with the Cryotop method may simplify transport of embryos over long distances, the exportation of embryos, and the conservation of species by storing genetic material. Additionally, the surplus embryos that are obtained from IVEP programs can now be stored for subsequent use during strategic periods.

In conclusion, adding forskolin before vitrification of IVP-derived embryos improved *Bos indicus* embryo cryotolerance, resulting in optimal pregnancy rates after transfer of cryopreserved *Bos indicus* IVP embryos.

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