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In vitro survival of *in vitro*-produced bovine embryos cryopreserved by slow freezing, fast freezing and vitrification

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Abstract

In order to compare the survival rate of bovine embryos cryopreserved with different protocols, oocytes obtained from abattoir-derived ovaries were used for *in vitro* maturation (IVM), fertilization (IVF) and co-culture. Expanded blastocysts were cryopreserved by two slow-freezing [(i) 0.5 and (ii) 1.2°C/min], (iii) one quick-freezing and two vitrification [(iv) ethylene glycol + ficoll + sucrose (EFS), and (v) glycerol (Gly) + ethylene glycol (EG)] protocols. After thawing, embryos were co-cultured on a granulosa cell monolayer and evaluated after 24 h for re-expansion. At 24, 48, 72, and 96 h the hatching rates were determined. The *in vitro* survival rate (hatching at 96 h) of embryos cryopreserved by the slow-freezing method with cooling rate of 0.5°C/min (58.8%) was similar to that obtained for control group (68.4%; not cryopreserved). Vitrification using Gly + EG yielded similar survival rates when compared to a slow-freezing at 1.2°C/min (36.9 and 39.4%, respectively) and higher rates than those for quick freezing and vitrification in EFS (7.0 and 14.0%, respectively). In conclusion, slow-freezing with 0.5°C/min was the best method to cryopreserve *in vitro* produced bovine embryos.

Keywords: bovine, IVF, quick-freezing, slow freezing, vitrification.

Introduction

In vitro embryo production (IVP) is a valuable tool for providing genetically superior animals (Ward *et al*., 2000). An efficient and practical technique for bovine embryo cryopreservation is a fundamental issue in the widespread use of embryo transfer. Storage of IVP embryos in liquid nitrogen is essential to make full use of this technology. Rall and Fahy (1985) established the vitrification protocol; since that, embryo cryopreservation has been a valuable technique to allow a larger application of embryo transfer in commercial farms (Moreira-da-Silva and Metelo, 2005).

Studies on embryo cryopreservation aim to simplify methods and increase non-returning rates after transfer of cryopreserved embryos. Thus, it is necessary to improve cryoprotectants composition and removal, cooling and warming rates and also cryopreservation method (Visintin *et al*., 2002).

In vitro-produced embryos differ considerably from their *in vivo* counterparts. In the context of freezing, they show an increased sensitivity to chilling and freezing probably due to different ratios of lipids to proteins in these two types of embryos (Leibo and Loskutoff, 1993; Pollard and Leibo, 1993). Increased intracellular lipid content and also the relatively smaller inner cell mass may play a crucial role for *in vitro* embryos and both are probably attributed to the inappropriate environment of the *in vitro* oocyte and embryo culture system (Iwasaki *et al*., 1990; Greve *et al*., 1993; Leibo and Loskutoff, 1993).

There are two main methods for cryopreserving embryos: conventional slow cooling/freezing and vitrification. Several studies conducted on cryobiology have shown that slow freezing using glycerol or ethylene glycol are the most efficient for IVP bovine embryos (Hasler *et al*., 1997; Sommerfeld and Niemann, 1999). However, slow freezing protocols are slow and laborious.

Takahashi and Kanagawa (1985) described a quick freezing protocol with high rates of embryo survival after quick exposition of the straw to nitrogen vapor followed by immersion in nitrogen.

Vitrification was another advance on embryo cryopreservation technology that permits rapid cooling of liquid medium in absence of ice crystal formation by using high levels of cryoprotectants (Vajta *et al*., 1997; Dobrinsky, 2001). Vitrification is a simpler and cheaper technique than embryo slow freezing. Conversely, embryo exposure to high levels of cryoprotectant additives may cause deleterious effects on embryo development after re-warming (Leoni *et al*., 2003). To minimize this effect, different cryoprotectors can be associated to reduce individual toxic effect or the addition of macromolecules and/or sugar may increase the intracellular osmolarity (Kuwayama *et al*., 1994; Kasai, 1996; Martinez *et al*., 1998). Therefore, studies with *in vivo*-produced embryos have shown that non-returning rates after transfer of vitrified embryos

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(44.5%) did not differ from those cryopreserved by slow freezing (45.1%; Van Wagtendonk-De Leeuw *et al*., 1995, 1997).

The aim of this work was to compare the *in vitro* survival of IVP bovine embryos cryopreserved by two slow-freezing [(i) 0.5 and (ii) 1.2°C/min], (iii) one quick-freezing and two vitrification [(iv) ethylene glycol + ficoll + sucrose (EFS), and (v) glycerol (Gly) + ethylene glycol (EG)] protocols.

Material and Methods

Chemicals and reagents

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Tissue culture media (TCM), Hepes and sodium bicarbonate, and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA). Emcare® holding media was obtained from Immuno Chemical Products Ltd, USA.

Collection of oocytes

A total of 3,486 oocytes were recovered from bovine ovaries collected from a local slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) at 30°C. After aspiration with 21-g needle, cumulus-oocytes complexes (COCs) were allowed to settle for 10 min. Selection of COCs was based on morphological appearance, according to cytoplasm aspect and the number of granulosa cell layer (De Loss *et al*., 1989).

In vitro-production of bovine embryos

Procedures, reagents, and media formulation for oocyte maturation, fertilization, and embryo culture were performed as described elsewhere (Parrish *et al*., 1988). All incubations were performed in an atmosphere of CO_2 in humidified air. Selected oocytes were matured for approximately 22-24 h and then inseminated with 1 x 10^6 Percoll-purified spermatozoa from one Nelore bull. At 18 h post-insemination (hpi), putative zygotes were mechanically denuded of cumulus cells by repeated pippeting. Presumptive zygotes (groups of ~30) were co-cultured in a granulosa cell monolayer in 90 µl droplets of tissue culture medium (TCM-199) supplemented with 10% of FCS, pyruvate (22 µg/ml) and gentamycin (50 µg/ml), for 9 d In an atmosphere of 5% CO_2, cleavage and blastocyst rates were recorded 48 h post-insemination and on following days 7 to 9 of culture, respectively.

Day 7, 8, and 9 expanded blastocysts cultured *in vitro* were randomly distributed among experimental groups. Non-cryopreserved IVP embryos were used as the control group.

Embryo cryopreservation

Slow-freezing at 0.5 ºC/min or 1.2ºC/min

Expanded blastocysts were transferred into a 1.5 M EG in Emcare® holding solution and loaded in a 0.25 ml straw at room temperature (24°C) for 10 min. Columns of 85 µl Emcare® holding medium + 0.3 M sucrose solution were placed at the edges of the straw. Straws were placed into an alcohol bath of a programmable freezer (PG20, HAACKE, Germany) at −7°C and seeded by touching the straw with cold forceps after 10 min. The straws were then cooled from −7 to −31°C at a rate of 0.5 C/min or 1.2°C/min, according to the experimental group. After reaching the target final temperature of −31°C, straws were transferred into LN_2.

Quick-freezing

Embryos were pre-equilibrated at room temperature (22-23°C) in 1.5 M EG + Emcare® holding medium for 5 min. Pre-equilibrated embryos were directly loaded into a 0.25 ml straw containing a 1.5 M EG + 1.36 M Gly + 0.3 M sucrose with Emcare® holding solution in the middle column and in the edge columns an Emcare® holding solution + 0.3 M sucrose solution. After equilibration, straws were placed horizontally on a Styrofoam rack with dimensions 14.5 x 12 x 0.8 cm in the vapor phase of a nitrogen bath at approximately -170°C (Mello *et al*., 2001). After 5 min the straws were plunged directly into LN_2 and loaded into racks in the LN_2 container.

Vitrification in EFS

Expanded blastocysts were equilibrated with the cryopreservation solutions in three steps at room temperature. First, the embryos were placed in 1.5 M EG in Emcare® holding solution for 5 min and then placed in 3 M EG in Emcare® holding solution for 5 min. After this, the embryos were transferred into a 40% ethylene glycol + 18% Ficoll 70 + 0.3 M sucrose in Emcare® holding solution (EFS) for 30 s, loaded in 0.25 ml straws and finally plunged directly in LN_2.

Vitrification in Gly + EG

Expanded blastocysts were first equilibrated in a 1.36 M Gly and then in a 2.72 M Gly in Emcare® holding solution, both for 5 min. Embryos were transferred into 25% EG + 25% Gly + Emcare® holding solution for 30 s, loaded in 0.25 ml straws and plunged directly in liquid nitrogen.

Thawing and cryoprotectant dilution

Thawing was accomplished by holding the frozen straw for 10 s in air and 10 s in a 25°C water bath. Straws contents were emptied into a Petri dish and embryos were recovered and transferred into 0.3 M sucrose in Emcare® holding solution for 5 min and then into Emcare® (isotonic solution) for re-hydration

for 4-5 min. Embryos were washed two or three times and transferred into culture media (TCM-199 + 10% FCS). Re-expansion and development of embryos and hatching of blastocysts were recorded at 24, 48, 72, and 96 h post-thawing.

Statistical analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models (GLM) procedure of SAS (1989). Embryo straw was considered the experimental unit. Percentage data were transformed using the arcsin transformation before analysis in order to meet the assumptions of normality and homogeneity of variances. The mathematical model included main effects and all interactions. All main effects were considered fixed. Independent variables were treatment and replicate and dependent variables were percentage of expanded and percentage of hatched blastocysts. Orthogonal contrasts and a mean separation procedure of SAS called pdiff were performed when appropriate to determine differences between levels of individual treatments. $P < 0.05$ was considered significant.

Results

There was no difference in expanded blastocyst rates after 24 h of *in vitro* culture (IVC) between both slow-freezing methods. However, these rates were higher than vitrification in EFS ($P < 0.05$). Hatched blastocyst rates after 48 and 72 h of IVC were similar for slow freezing and vitrification in Gly + EG. Moreover, these rates were higher than those for quick freezing and vitrification in EFS (Table 1).

Table 1. Mean ± SEM of expanded and hatched blastocysts at 24, 48, and 72 h of *in vitro* culture post-thawing.

Group	IVC (n)	24 h		48 h	72 h
		Expanded % (n)	Hatched % (n)	Hatched % (n)	Hatched % (n)
Slow-freezing (0.5ºC/min)	122	41.53 ± 6.67^{c} (60)	33.88 ± 4.6^{a} (27)	43.28 ± 5.31^{a} (42)	53.52 ± 5.86^{a} (52)
Slow-freezing (1.2ºC/min)	144	36.99 ± 5.27$^{b, c}$ (56)	22.67 ± 3.9$^{a, b}$ (21)	27.92 ± 4.20^{a} (33)	31.90 ± 4.63^{a} (38)
Quick-freezing	94	20.82 ± 7.09$^{a, b}$ (23)	11.49 ± 4.9$^{b, d}$ (2)	12.20 ± 5.65^{b} (3)	12.94 ± 6.23^{b} (4)
Vitrification EFS	136	17.40 ± 6.24^{a} (24)	9.32 ± 4.28^{d} (2)	11.06 ± 4.97^{b} (6)	13.29 ± 5.48^{b} (9)
Vitrification Gly + EG	125	23.15 ± 6.67$^{a, b}$ (29)	30.28 ± 4.6^{a} (22)	37.97 ± 5.31^{a} (29)	42.25 ± 5.86^{a} (35)

^{a,b,c,d}Different superscript letters within columns indicate statistical difference ($P < 0.05$).

Hatched blastocyst rates after 96 h of IVC do not differ between the slow freezing with cooling rate of 1.2ºC/min and vitrification in Gly + EG. As for 48 and 72 h of IVC, these methods presented higher rates of hatched blastocyst than those for quick freezing and vitrification in EFS. Hatched blastocyst rate at 96 h of IVC did not differ between control groups (not cryopreserved) and slow freezing with cooling rate of 0.5ºC/min (Table 2).

Table 2. Mean ± SEM of hatched blastocysts at 96 h of *in vitro* culture post-thawing.

Group		Blastocyst (n)	96 h
Slow-freezing	0.5°C/min	62/122	58.80 ± 7.28^{a}
	1.2°C/min	48/144	39.44 ± 6.06^{b}
Quick-freezing		3/94	7.02 ± 7.73^{c}
Vitrification	EFS	15/136	14.07 ± 6.85^{c}
	Gly + EG	35/125	36.86 ± 7.28^{b}
Control		95/138	68.37 ± 5.61^{a}

^{a,b,c}Different superscript letters within columns indicate statistical difference ($P < 0.05$).

Discussion

There was no difference in expanded blastocyst rates between the two slow-freezing protocols (cooling rates of 0.5ºC or 1.2ºC/min). However, higher hatched blastocyst rate was obtained with cooling rate of 0.5ºC/min. These results are in accordance with those by Hochi *et al.* (1994) in which, comparing *in vitro* development after thawing of bovine embryos cryopreserved by slow freezing in EG 1.5 M, obtained better results with cooling rates of 0.6ºC than 1.2ºC/min.

Karlsson *et al.* (1996) described that intracellular ice formation is related to cooling rates, temperature, and cryoprotectant concentration. Cooling slowly does not freeze embryo cytoplasm because it remains in equilibrium with an increasing concentration of extracellular solution (Mazur, 1990). According to the present results, cryoprotectant concentration was efficient to protect a great number of embryos from cryoinjury in both slow freezing protocols. The reduction of cryopreservation procedure by 28 min using a more rapid cooling rate (0.5°C/min x 1.2°C/min), without losing embryo viability after thawing has great importance on routine use of this technique.

Quick freezing in nitrogen vapor presented the lowest expansion and hatching embryo rates after thawing. Even for *in vivo*-produced embryos, this method presents lower embryo survival rates when compared to slow freezing (Mezzalira *et al*., 2002). In this method, a higher cryoprotectant concentration (3.16 M) was used because of the quick temperature reduction. Lower embryo survival rates with this method may be due to cryoprotectant concentration, inappropriate to protect the embryos from injuries inherent to cryopreservation.

Since quick freezing is a simple and not expensive method, studies are necessary to adjust cryoprotectant concentration, time of exposure of the embryo to cryoprotectant solution and dilution (one or step wise) protocol of the cryoprotectant or associations and make this method an efficient alternative for IVP cryopreservation.

The hatched blastocyst rates obtained after vitrification in EFS were similar to those obtained by quick freezing and lower than those by slow freezing and vitrification in Gly + EG. Vitrification in EFS induces the reduction of size and number of zona pelucida pores (Moreira-da-Silva and Metelo, 2005), decreasing the transport of nutrients and resulting in loss of embryo viability (Leoni *et al*., 2003). Vitrification procedure can also produce some damages to the embryonic cells. Bovine IVF-derived embryos, after vitrification, usually become extremely vulnerable to cryo-damage due to intracellular and membrane defects caused by exchange of water and cryoprotectant agents between the intracellular and extracellular environment (Pugh *et al*., 2000). Changes due to the vitrification procedure have already been reported and are related to structural damage (Vajta *et al*., 1997), loss of function of membrane receptors (Koruji *et al*., 2004), poor activity of Na/H antiporter and HCO_3^- /Cl^- exchanger and damages in the integrity of cellular membranes and intercellular organelles (Lane *et al*., 2000).

For vitrification protocols, higher cryoprotectant concentrations are necessary which may expose the embryos to osmotic and toxic effects. These effects can be bypassed by the use of more appropriate cryoprotectants as EG or the association of two or more cryoprotectants (Massip, 2001). In this study, vitrification in Gly + EG resulted in hatched blastocyst rates similar to those obtained by slow freezing at 1.2°C/min. These results are in accordance with Kaidi *et al.* (2001) which obtained hatched rates similar for slow freezing in Gly 1.36 M and vitrification in 25% EG + 25% Gly. Also, the number of trophoectoderm cells, glycose, pyruvate, and oxygen uptake were reduced for slow freezing when compared to vitrification, showing a stress response to this procedure (Kaidi *et al.*, 2001).

In conclusion, the results suggest that IVP bovine expanded blastocysts at days 7, 8 and 9 of IVC can be efficiently cryopreserved by slow freezing in EG and vitrification in Gly + EG in Emcare® holding solution. The higher rates of embryo survival after thawing show that slow-freezing with 0.5°C/min was the best method to cryopreserve *in vitro*-produced bovine embryos.

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