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Comparison of Conventional Freezing and Vitrification with Dimethylformamide and Ethylene Glycol for Cryopreservation of Ovine Embryos

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Contents
The aim of this work was to evaluate the efficiency of the cryoprotectants dimethylformamide and ethylene glycol for cryopreservation of ovine embryos using vitrification and conventional freezing. The recovered embryos were distributed randomly in three treatment groups: Gr. 1: conventional freezing (n = 44), Gr. 2: vitrification with ethylene glycol (n = 39) and Gr. 3: vitrification with dimethylformamide (n = 38). Quality of fresh embryos in control group as well as of frozen and vitrified embryos was examined by three methodologies: staining with propidium iodide and Hoechst 33258 and evaluation under fluorescent microscopy, evaluation of re-expansion and hatching rates after culture, and determination of apoptotic index with TUNEL technique. It was established that re-expansion rate in all treatment groups was similar. In the same time, hatching rates were higher in Gr. 1 (40.5%) and Gr. 2 (35.3%) in comparison with Gr. 3 (15.5%, $p < 0.05$). The number of dead cells in vitrified embryos of Gr. 2 and Gr. 3 was higher (42.6 ± 26.2 and 63.2 ± 34.65, respectively) in comparison with Gr. 1 (conventional freezing, 10.1 ± 8.5, $p < 0.05$). Embryos vitrified with dimethylformamide included the same quality of apoptotic cells that Gr. 1 (conventional freezing) and fresh embryos. In conclusion, the dimethylformamide and ethylene glycol used as cryoprotectant to vitrify ovine embryos, in the concentrations and exposition time tested in this work, were not as efficient as the conventional freezing for cryopreservation of ovine embryos Thus, the conventional freezing with ethylene glycol was the most efficient method to cryopreserve ovine embryos in comparison with vitrification.

Introduction

Since the first lambs were born after transfer of frozen embryos in 1976 (Willadsen et al. 1976), embryo cryopreservation techniques have continually improved (Baril et al. 2001; Bettencourt et al. 2009; Shirazi et al. 2010). There are basically two embryo cryopreservation methods: conventional and vitrification. The freezing of embryos by conventional method is based on the use of programmable machines to execute pre-established freezing curves (Hasler et al. 1997), and the cryoprotectants more commonly used in this method for ovine embryos are ethylene glycol and glycerol (Fogarty et al. 2000; Isachenko et al. 2003). In vitrification, known as an 'ice-free' method, a solution containing high cryoprotectant concentration passes directly from liquid state to a vitrified and amorphous state when plunged directly in liquid nitrogen. Therefore, this method avoids the formation of ice crystals (Rall and Fahy 1985; Pugh et al. 2000). Ethylene glycol has been the most frequently used cryoprotectant and has already been used to vitrify equine (Lagares et al. 2009), bovine (Varago et al. 2006), swine (Misumi et al. 2003) and as well as ovine embryos (Dattena et al. 2001). However, the gestation rate using both methods is quite lower than the one using fresh embryo transfer (30–50% vs 90%) (Martinez and Matkovic 1998; Dattena et al. 2000; Fogarty et al. 2000; Papadopoulos et al. 2002). The lower fertility of frozen embryos is partially attributed to cryoprotectant toxicity, especially in vitrification in which the cryoprotectants are used in high concentrations, establishing one of the critical points of the technique of embryo freezing. Therefore, less toxic cryoprotectants have been investigated. Amides, for example, have been tested as cryoprotectants for stallion (Squires et al. 2004), boar (Bianchi et al. 2008), dog (Lopes et al. 2009), rabbit (Hanada and Nagase 1980) and rooster (Lukaszewicz 2002) sperm freezing. The success was attributed to lower molecular weight and reduced amide toxicity to the sperm cell (Squires et al. 2004). To our knowledge, until now, no results have been reported using dimethylformamide or other amides in ovine embryo cryopreservation.

Cryopreservation induces embryo damage, although the causes have not totally been elucidated (Ali and Shelton 1993). Morphological evaluation using stereomicroscopy has been the method most routinely used to evaluate cryo-damages (Slade et al. 1985). However, this technique does not identify or differentiate embryo lesions. Besides, morphology is not always related to embryo developmental competence (Bettencourt et al. 2009). According to Dobrinsky et al. (1996), cryopreservation success depends on the reaction of the cellular organelles and interactions of the membranes with the cryoprotectants. Therefore, the use of biological trials which allow detection, characterization and quantification of embryo lesions is extremely important for the progress of embryo cryopreservation (Coutinho et al. 2007). These studies include the association of stains such as Hoechst and propidium iodide used to evaluate bovine (Hurtt et al. 2000), equine (Oberstein et al. 2001; Lagares et al. 2009) and ovine embryo viability (Leoni et al. 2002; Garcia-Garcia et al. 2006). Propidium iodide binds to the DNA of cells with damaged plasma membrane. Hoechst 33258 and 33342 permeate the plasma membrane and bind to the DNA of all embryo cells (Pursel et al. 1985). The diagnosis of embryo apoptosis can be performed through a technique of nuclear stain with propidium iodide combined with TUNEL (Otoi et al. 1999; Paula-Lopes and Hansen 2002).

Data reporting the use of different techniques to assess ovine embryo quality in the same study can be useful to choose the most appropriate methodology or the one producing more reliable results. For this reason, this study aimed to experiment different methods to evaluate ovine embryo survival after vitrification using ethylene glycol and dimethylformamide as cryoprotectants and the conventional technique with ethylene glycol.

Materials and Methods

All experimental procedures were performed according to the Brazilian ethical and animal welfare principles to utilization and care of animals used in research and were approved by the ethical Committee 'Comitê de Ética em Experimentação Animal (CETEA)' at Federal University of Minas Gerais (UFMG).

Animals and hormonal protocols

Embryos were obtained from 68 ewes submitted to superovulation protocol, by insert of vaginal sponge containing 60 mg of medroxy progesterone acetate (MAP) for 12 days (Progespon® – MSD Animal Health, São Paulo, SP, Brazil) and 250 IU of porcine Hormone Stimulating Follicle (pFSH) (Pluset® – Hertape Calier, Juatuba, MG, Brazil) provided in six decreasing doses at a 12-h interval. The superovulatory treatment initiated 72 h before vaginal sponge removal, which coincided with the application of the fifth pFSH dose. Females received, together with the last pFSH dose, 200 IU of eCG (Novormon® – MSD – MSD Animal Health, São Paulo, SP, Brazil) and 0.5 ml of PGF2α (Ciosin® – MSD Animal Health, São Paulo, SP, Brazil). One day after sponge removal, oestrous detection was carried out in the presence of rams. Those females showing oestrus were inseminated using the transcervical technique with fresh semen of rams of proven fertility. Insemination doses were prepared with a minimum of 750×10^6 sperm. Each donor was inseminated twice, at 8-h intervals. The first insemination was accomplished 38 h after progesterone sponge removal. The day of oestrus was considered as D0 of the oestrous cycle.

Embryo recovery

The embryo recovery was performed by hemi-laparoscopy 6 days after oestrous beginning. The ewes were submitted to general inhalation anaesthesia, and a 10-mm-diameter optic was introduced in the abdomen with a guide parallel to the Alba line, approximately 10 cm cranial to the mammary gland. A Bab Cok tweezer (33533BL; Karl Storz, Tuttlingen, Germany) was introduced by another guide at the opposite side 5 cm cranial to the mammary gland. Only donors reacting to the super ovulatory treatment with three or more corpus luteum were submitted to surgical embryo recovery. The uterine horns were exposed through an incision of approximately 5 cm where the Bab Cok tweezer had been introduced before. Each uterine horn was flushed with 40 ml of phosphate-buffered solution (PBS) with 10% of foetal calf serum (FCS). The flushing solution was introduced in the uterus by its extremity with an intravenous catheter 18G (Becton Dickinson, New Jersey, NJ, USA) and recovered from the uterine horn base using a silicon urethral number 10 catheter (Embramac, São Paulo, SP, Brazil). During the procedures, the uterine horns were constantly irrigated with 0.9% physiological solution at 37°C. After flushing, they were lubricated with a sodium heparin cream (Trombofob®; Abbott Laboratórios do Brasil Ltda, São Paulo, SP, Brazil) and, next, they were introduced back into the abdominal cavity, which was irrigated with 0.9% physiological solution with 20 UI/ml sodium heparin at 37°C.

Embryo classification

Embryos were classified according to the *International Embryo Transfer Society* (IETS) (Robertson and Nelson 1998), and only morula, initial blastocyst, blastocyst and expanded blastocyst stage classified as grade I embryos were cryopreserved. A total of 121 embryos were distributed randomly in three cryopreservation groups according to stage of development: Gr. 1) conventional freezing (n = 44, morula: n = 22, initial blastocyst: n = 3, blastocyst: n = 11, expanded blastocyst: n = 8); Gr. 2) vitrification with ethylene glycol (n = 39, morula: n = 19, initial blastocyst: n = 0, blastocyst: n = 12, expanded blastocyst: n = 8); Gr. 3) vitrification with dimethylformamide (n = 38, morula: n = 13, initial blastocyst: n = 3, blastocyst: n = 13, expanded blastocyst: n = 9) and 18 fresh embryos (control).

Conventional embryo freezing

For the conventional freezing treatment (Gr. 1 – control group), a commercial medium of 1.5 M ethylene glycol added with 0.05 g/l gentamicin (Nutricell®, Campinas, SP, Brazil) was used. The embryos were loaded in 0.25 ml straws. A PBS solution with 0.4% bovine serum albumin (BSA) was used on each extremity of the columns inside the straws. Before starting freezing curve, the embryos were kept in freezing solution for 10 min. The straws were cooled and frozen in a freezing machine CL 5500 (Cryologic, Sidney, Australia). Cooling rate was 1°C/min (from 20 to −7°C). Seeding was performed at −7°C, and embryos were maintained at this temperature for 15 min. After a freezing rate of −0.3°C/min to −35°C during 10 min, embryos were immersed in liquid nitrogen and stored in cryogenic containers.

Embryo vitrification

Before vitrification, embryos were submitted to the holding medium (HM) with TCM199+ Hepes (Cultilab, Campinas, SP, Brazil) and 20% FCS without cryoprotectant for 1 min to wash the embryos and remove PBS, and in another drop containing the same medium for additional 5 min. The embryos were immersed at room temperature into vitrification solution 1 (Gr. 2: 80% HM + 20% ethylene glycol; Gr. 3: 80% HM + 20%

dimethylformamide) for 5 min and then into vitrification solution 2 (Gr. 2: 40% ethylene glycol, 18% Ficoll and 10% sucrose (0.3 M); Gr. 3: 40% dimethylformamide, 18% Ficoll and 10% sucrose (0.3 M)) for 20 s, respectively. The embryos were immediately loaded into the open-pulled straw (OPS) (Vajta et al. 1997) and immersed into liquid nitrogen. The OPS was loaded into 0.5-ml straws previously cooled and identified, and then stored in liquid nitrogen.

Embryo thawing

The conventionally frozen embryos were thawed in water bath at 35°C for 30 s (Garcia-Garcia et al. 2006) and rehydrated in solution 1: 90% HM + 10% sucrose solution for 10 min and solution 2: 100% HM for 5 min.

The vitrified embryos were warmed at room temperature in air for 3 s and rehydrated in sucrose of decreasing concentration: step 1) 80% HM + 20% sucrose solution for 1 min (this step was performed to wash embryos and remove vitrification solution), step 2) 80% HM + 20% sucrose solution for 5 min, step 3) 90% HM + 10% sucrose solution for 5 min and step 4) 100% HM for 5 min (Vajta 2000).

Embryo *in vitro* evaluation

Fresh embryos (control group), frozen and vitrified embryos were evaluated by three quality methods: embryo viability by fluorescent microscopy, re-expansion and hatching rates after culture, and determination of apoptotic index by fluorescent microscopy.

Embryo viability evaluation by fluorescent microscopy

To assess the percentage of viable cells, immediately after thawing, embryos were stained with propidium iodide and Hoechst 33258 dyes (Sigma Chemical Company, St. Louis, MO, USA). After 15-min incubation in PBS with 1% BSA and 125 μg/ml propidium iodide, the embryos were loaded in a 90% glycerol prepared on PBS with 100 μg/ml Hoechst 33258 for 5 min (Lagares et al. 2009). The stained embryos were pressed on to a glass slide and then examined under a fluorescence microscope (BX-51; Olympus, Tokyo, Japan). This resulted in all (Hoechst positive) nuclei fluorescing blue (excitation filter 330–385 nm) and non-vital nuclei fluorescing red (PI positive) (excitation filter 510–550 nm). To obtain the percentage of embryonic viable cells for each treatment, the images obtained of the embryos stained with both dyes were overlaid by the program Confocal Assistant. The non-viable cells (red) were subtracted from the total blue-stained cells. The Program Image J® (version 1.41, National Institutes of Health, EUA, http://rsbweb.nih.gov/ij/download.html) was used to calculate the viable and non-viable cells.

Embryo re-expansion and hatching rates

Embryo re-expansion and hatching rates were observed after culture in an incubator at 39°C in 5% CO_2 in air and humidity for 24 and 48 h, respectively, and classified with a stereo microscope (40×). The culture medium used was synthetic oviduct fluid enriched with amino acids (SOFaaci, Holm et al. 1999). After culture, the embryo apoptotic index was verified by TUNEL technique.

Apoptotic index

The apoptotic index with the TUNEL technique was performed with the use of the kit DeadEnd™Fluorometric TUNEL System® (Promega, Madison, WI, USA), modified by Paula-Lopes and Hansen (2002). After culture, the embryos were fixed in 4% formaldehyde and cooled in refrigerator at 4°C for 25 min, washed with PBS solution with BSA and permeated with 2% Triton solution. After another washing with PBS solution and BSA, the embryos were loaded in a buffer solution and incubated with an enzymatic solution of rTdT (Terminal Deoxynucleotidyl Transferase recombinant) added with a nucleotides mix at 37°C. Some embryos were used as positive and negative control of the technique; however, all of them were washed with SSC2X, in PBS solution with BSA and loaded on a slide. The embryos were stained with DAPI dye, covered with a deck glass and observed by fluorescent microscopy (400×, Motic BA400; Motic Instruments Inc., Richmond, BC, Canada, excitation: 350–460 nm) and by a FITC filter (excitation: 480–535 nm) to obtain two images of each embryo, respectively. The captured images were used to calculate the number of apoptotic cells with the program Image J® – Image Processing and Analysis in Java (http://rsbweb.nih.gov/ij/). All blastomers were stained blue with DAPI, and the cells observed green by the FITC filter were apoptotic. The apoptotic index was obtained by the proportion of apoptotic cells of the total calculated cells.

Statistical analysis

Re-expansion and hatching rates were compared with chi-square. Total cell number, dead cells, apoptotic cells, dead and apoptotic cells index were verified to normality (Lilliefors test) and homoscedasticity (Cochran and Bartlett test) and submitted to logarithmic and arccosine transformations. The total cell number was normally distributed, and after logarithmic transformation, apoptotic cell number and dead cell number were also presented normal distribution. They were analysed by ANOVA, and mean values were compared with the SNK test. The dead and apoptotic cell indexes were not normally distributed and were analysed by the Kruskal–Wallis test. A level of 5% was considered significant.

Results

There was no difference among the expanded embryos of all three groups ($p > 0.05$, Table 1). Nevertheless, hatching rates were higher in Gr. 1 and Gr. 2 compared with Gr. 3 ($p < 0.05$).

The total cell number of embryos distributed in the three groups was not significantly different (Table 2). The fresh embryos showed lower number of dead cells

Table 1. Re-expansion and hatching rates of ovine embryos cryopreserved by conventional freezing with ethylene glycol and vitrification in OPS with ethylene glycol and dimethylformamide

Groups	Re-expansion % (n) 24 h	Hatching % (n) 48 h
Gr. 1	54.0 (20/37)	40.5 (15/37)[a]
Gr. 2	47.0 (16/34)	35.3 (12/34)[a]
Gr. 3	36.6 (12/33)	15.5 (5/33)[b]

[a,b]Mean values in the same column with different superscripts differ significantly ($p < 0.05$).
(Gr. 1) conventional freezing, (Gr. 2) vitrification with ethylene glycol, (Gr. 3) vitrification with dimethylformamide.

Table 2. Cell viability evaluated with Hoechst/PI staining after thawing and warming of ovine embryos cryopreserved by conventional freezing with ethylene glycol and vitrification with ethylene glycol and dimethylformamide

Groups	Total embryo cells (n)	Dead cells (n)	Dead cells index (%)
Fresh embryos (control)	85.7 ± 30.7	1.5 ± 0.8[c]	0[a]
Gr. 1	43.6 ± 16.7	10.1 ± 8.5[b]	0.2 ± 0.1[a]
Gr. 2	64.2 ± 21.5	42.6 ± 26.2[a]	0.6 ± 0.2[b]
Gr. 3	68.8 ± 36.0	63.2 ± 34.6[a]	0.9 ± 0[b]

[a, b]Mean values in the same column with different superscripts differ significantly ($p < 0.05$).
(Gr. 1) conventional freezing, (Gr. 2) vitrification with ethylene glycol, (Gr. 3) vitrification with dimethylformamide.

observed by Hoechst/PI, compared with all cryopreserved embryos (Gr. 1, Gr. 2 and Gr. 3, $p < 0.05$). The vitrified embryos presented higher number of dead cells compared with the conventional frozen ($p < 0.05$), and on the other hand, dead cell index was similar ($p > 0.05$) between fresh and conventional frozen embryos (Table 2).

No difference was observed among the groups with regard to the total cell number of embryos with the TUNEL technique ($p > 0.05$, Table 3). The dimethylformamide-vitrified embryos showed no apoptotic cells, different to the other treatments, which had similar apoptotic index (control: 0.08 ± 0.05%, Gr. 1: 0.10 ± 0.13%, and Gr. 2: 0.30 ± 0.32%, $p > 0.05$, Table 3).

Table 3. Total cell number, apoptotic cells and index with the TUNEL technique of ovine embryos cryopreserved with conventional freezing with ethylene glycol and vitrification with ethylene glycol and dimethylformamide

Groups	Total cells (n)	Apoptotic cells (n)	Apoptotic index (%)
Fresh embryos (control)	68.2 ± 28.1	6.0 ± 4.4[B]	0.08 ± 0.05[a,b]
Gr. 1	98.2 ± 47.0	12.1 ± 10.3[A,B]	0.10 ± 0.13[a,b]
Gr. 2	72.6 ± 29.0	15.7 ± 9.5[A]	0.30 ± 0.32[b]
Gr. 3	56.0 ± 52.0	0[C]	0[a]

[A, B]Mean values in the same column with different superscripts differ significantly ($p < 0.05$), [a, b] ($p < 0.01$).
(Gr. 1) conventional freezing, (Gr. 2) vitrification with ethylene glycol, (Gr. 3) vitrification with dimethylformamide.

Discussion

In the present work, ethylene glycol was beneficial for ovine embryo by the conventional cryopreservation. Although the ethylene glycol-vitrified embryos re-expansion rate was similar to the reported by Zhu et al. (2001), the hatching rates were lower. Nevertheless, the ovine breed used in the present work was Santa Inês, which differed from the study described above which was used Dorset and Suffolk breeds. It is possible that the donor breed influences in cryopreservation results as already demonstrated in bovine (Visintim et al. 2002). Moreover, exposition time of the embryos to the vitrification solution was longer (30 s) than in the present work (20 s). This could have led to a better dehydration time resulting in higher hatching rates. The favourable effects of ethylene glycol may be attributed to its low molecular weight and cytotoxicity, as it penetrates quicker into the cell than other cryoprotectants such as glycerol and propylene glycol, and can also be removed quickly from the embryo after warming (Jackowski et al. 1980; Szell et al. 1990). However, this effect was observed in the embryos cryopreserved with the conventional method, conversely to the ethylene glycol-vitrified embryos. Such difference was also reported in a study with ethylene glycol conventional frozen immature ovine oocytes compared with ethylene glycol-vitrified oocytes with the OPS (Bhat et al. 2014). In contrast, as the percentage of non-viable blastomeres was similar between the ethylene glycol and dimethylformamide vitrification groups, the hatching rate was lower for the dimethylformamide-vitrified embryos. The toxicity of dimethylformamide was reported for cryopreserved ovine sperm (Moustacas et al. 2011), the opposite to stallion sperm (Squires et al. 2004; Alvarenga et al. 2005). Dimethylformamide molecules have higher vitrification ability and stability when diluted in water at the same concentrations of ethylene glycol and glycerol (Baudot and Boutron 1998). However, the cells present different sensitivity to cryopreservation, cryoprotectant toxicity and protection ability (Agca and Crister 2002). The low viability of the vitrified embryos in the medium containing dimethylformamide could be due to an association of factors such as high sensitivity of the blastomeres and low ability of dimethylformamide to preserve the embryo cells. The dimethylformamide-vitrified embryos presented lower apoptotic index than the other groups, which could have been suggested that they were submitted to moderate stress conditions. Nevertheless, it is possible that the high toxicity of dimethylformamide could have promoted such a high stress that the embryos died immediately due to necrosis, running over the apoptotic mechanisms. Necrosis is defined as the beginning of accidental cellular death, which starts with alterations in the environment as severe hypoxia, ischaemia, oxygen reactive species, cold shock and biological factors such as virus and bacteria (Manjo and Joris 1995).

As cellular injuries can occur through different pathways during the cryopreservation process, it is recommended to apply different techniques to verify the *in vitro* embryo viability. Moreover, although according to the results of the present work the use of dimethyl-

formamide to cryopreserve ovine embryos is not recommended, the option to test the dimethylformamide with other concentrations and exposure times to promote ovine embryo survival cannot be excluded. The choice of ethylene glycol as the quite universal cryoprotectant because of its low toxicity and high permeability associated with the vitrification as cryopreservation method has been suggested (Massip 2001). Nevertheless, in the present work, the conventional freezing with ethylene glycol showed similar results to the control group in all embryo characteristics analysed and thus was the most efficient method to cryopreserve ovine embryos in comparison with vitrification. In conclusion, the dimethylformamide and the ethylene glycol used as cryoprotectant to vitrify ovine embryos, in the concentrations and exposition time tested in the present experiment, were not efficient. Thus, other cryoprotectants should be applied to ovine embryo vitrification as an alternative to the conventional freezing.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

F.C.Varago did the ewes surgeries to recover the embryos, their evaluation and criopreservation; V.S. Moutacas helped by the ewes surgeries to recover the embryos, B.C. Carvalho did the experimental design and statistical analysis, R.V. Serapião evaluated the ovine embryos by the fluorescent microscopy, F. Vieira evaluated the embryos quality, H. Chiarini-Garcia evaluated the embryos quality, F.Z.Brandão helped by the ewes surgeries to recover the embryos, L.S. Camargo evaluated the ovine embryos by the fluorescent microscopy, M. Henry was the co-adviser and M.A. Lagares was the adviser and wrote the project, guide the experiments and review the manuscript.

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