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 ± 34.6, 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 (Mumini 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 (Łukasiewicz 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; García-García 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 (Presel 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 implant 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® – Her- tape 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 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 twice, at 8-h intervals. The first insemination 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 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 (Embramat, 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 (Trombofol®; 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%
Embryo re-expansion and hatching rates were observed after culture in an incubator at 39°C in 5% CO₂ 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 (SOFαaci, 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–Wallis 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.
Fresh embryos (control) 85.7%/C6 vitrification with dimethylformamide. (Gr. 1) conventional freezing, (Gr. 2) vitrification with ethylene glycol, (Gr. 3) vitrification with dimethylformamide.

Table 3. Total cell number, apoptotic cells and index with the vitrification of dimethylformamide

<table>
<thead>
<tr>
<th>Groups</th>
<th>Re-expansion % (n)</th>
<th>Hatching % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Gr. 1</td>
<td>54.0 (20/37)</td>
<td>40.5 (15/37)*</td>
</tr>
<tr>
<td>Gr. 2</td>
<td>47.0 (16/34)</td>
<td>35.5 (12/34)*</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>36.6 (12/33)</td>
<td>15.5 (5/33)*</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total embryo cells (n)</th>
<th>Dead cells (n)</th>
<th>Dead cells index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh embryos (control)</td>
<td>85.7 ± 30.7</td>
<td>1.5 ± 0.8c</td>
<td>0º</td>
</tr>
<tr>
<td>Gr. 1</td>
<td>43.6 ± 16.7</td>
<td>10.1 ± 8.5b</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>Gr. 2</td>
<td>64.2 ± 21.5</td>
<td>42.6 ± 26.2*</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>68.8 ± 36.0</td>
<td>63.2 ± 34.6a</td>
<td>0.9 ± 0.6a</td>
</tr>
</tbody>
</table>

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.

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 Inès, 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 (Agea 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. Chiariini-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.

References


