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Comparison of vitrification and conventional freezing for cryopreservation of caprine embryos

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Summary

The experiment aimed to compare conventional freezing and different vitrification protocols for cryopreservation of caprine embryos at morphological, ultrastructural, and functional levels. Caprine embryos produced *in vivo* were allocated randomly to three groups: (1) conventional freezing with ethylene glycol (EG); (2) dimethyl sulfoxide + EG (DMSO/EG) vitrification; and (3) dimethylformamide + EG (DMF/EG) vitrification. All groups were scored for cell viability (propidium iodide staining and ultrastructural levels) and re-expansion rate after thawing or warming. Embryos subjected to DMSO/EG vitrification showed higher cell viability (73.33%), compared with DMF/EG vitrification and conventional freezing group embryos (40.00 and 66.66%, respectively). The ultrastructural study revealed that vitrified embryos had greater preservation of cellular structure than embryos from conventional freezing with EG. DMSO/EG vitrification resulted in higher rates of re-expansion *in vitro* (47.36%) than DMF/EG vitrification (31.58%), and conventional freezing (25.00%). In conclusion, caprine embryos produced *in vivo* are better cryopreserved after vitrification than conventional freezing, therefore we conclude that DMSO/EG vitrification is the most effective protocol for cryopreservation.

Keywords: Blastocyst, Cryobiology, Cryoprotectant, Goat, Slow freezing

Introduction

Cryopreservation allows the conservation of physical and functional properties of various cell types and tissues for indefinite periods of time. Gametes and preimplantation embryos can be cryopreserved successfully (Polge, 1951; Whittingham *et al.*, 1972; Wilmut, 1972), and the technology is largely used under scientific and commercial settings for various applications, for example to store valuable genetic material, to preserve endangered *species*, and to ease germplasm transportation while reducing disease transmission risks, among others (Dobrinsky, 2002).

Two contrasting cryopreservation methodologies have been described (Massip, 2001): conventional freezing protocols using slow freezing curves and cryoprotectants of low osmolarity (Bilton & Moore, 1976; Cognié *et al.*, 2003; Loutradi *et al.*, 2008) and vitrification, which is characterized by exposure of cells to high osmolarity cryoprotectants and direct transfer to liquid nitrogen (Vajta and Kuwayama, 2006; Dike, 2009). The simplicity and low cost have made vitrification more appealing for large-scale cryobanking and for cell types that are less amenable

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to cryopreservation, such as oocytes and *in vitro*-produced embryos (Vajta, 2000; Kuleshova *et al.*, 2001).

Several protocol and technical variations have been tested in order to improve caprine embryo survival after vitrification or conventional freezing (El-Gayar & Holtz, 2001; Guignot *et al.*, 2006; Hong *et al.*, 2007; Al Yacoub *et al.*, 2010; Morató *et al.*, 2011). Moreover, to our knowledge, dimethylformamide (DMF) has been tested to freeze goat semen (Bezerra *et al.*, 2011), but its potential for embryo vitrification remains to be tested.

The objective of the present research was to compare two different vitrification protocols with conventional freezing using caprine embryos produced *in vivo*. Methodologies were compared by embryo viability at morphological and ultrastructural levels and functionally tested for re-expansion rate of warmed/thawed embryos, respectively.

Materials and methods

Experimental location

The experiment were conducted at several Brazilian institutions: Paraíba State Agricultural Research Company (EMEPA), Universidade Federal de Pernambuco (UFPE), Universidade Federal Rural de Pernambuco (UFRPE), and Centro de Tecnologias Estratégicas do Nordeste (CETENE).

Donor selection

All experimental procedures were conducted in accordance with the rules of the local ethics review board on animal research. Thirty Boer does, with an average age of 4.1 years, and adequate nutritional characteristics (minimum body score of 3.0) were used. Animals were housed in a covered shed, fed with hay *ad libitum*, and 500 g/day/animal concentrate supplement with 18% of crude protein that contained corn, soybean, wheat, and limestone. Animals had free access to water and mineral salt.

Estrous synchronization and follicle stimulating hormone (FSH) treatment

Does had their estrous cycles synchronized at protocol day 0 by insertion of vaginal devices impregnated with 0.33 g of natural progesterone (Eazi-Breed™ CIDR, Pfizer). All controlled internal drug release (CIDR) devices were replaced by new devices on day 9 and were used over 4 days. FSH treatment was started on day 9, using 300 IU FSH (Pluset®, Hertape Calier, Brazil), divided into six decreasing doses administered in 12-h intervals. A dose of 200 IU of equine chorionic gonadotropin (eCG) was administered (Foligon, Intervet) by vaginal dispositive removal on day 13. Two doses of 0.15 mg D-cloprostenol (Prolise, Tecnopec, Brazil) were also administered on days 11 and 13. Controlled natural mating was performed on day 12 with bucks of proven fertility. At 36 h after the last mating, a new CIDR was inserted and kept until 20 h prior to embryo recovery when a shot of 0.15 mg D-cloprostenol was applied.

Embryo collection and evaluation

Embryos were collected on days 6.5 and 7.0 after estrous onset, aiming to recover embryos at morulae to expanded blastocyst stages. Animals were not fed 24 h before collection, and were anesthetized with 1.0 mg kg^{-1} oxytetracycline LA (Oxitetra, Agener Uniao, Brazil) and 0.375 mg D-cloprostenol. Embryo were collected transcervically by flushing uterine horns with Dulbecco's modified phosphate-buffered saline (DPBS, Embriocare, Cultilab, Brazil), supplemented with 1% fetal bovine serum (FBS) at 37°C. Embryos were immediately identified and placed in holding medium (TqC Holding Plus, Nutricell, Bioniche, USA). Embryos were graded as described by the International Embryo Transfer Society (IETS; Stringfellow & Seidel, 1998): grade I (excellent), II (good), III (poor), and IV (dead or degenerated). Grade I and II embryos were selected for further experiments.

Embryo cryopreservation

Conventional freezing

Embryos were kept in TqC Ethylene Glycol Freezer Plus solution (Nutricell, Bioniche, USA) for 5 min and loaded in 0.25 ml straws. Embryos were frozen using an automatic embryo freezer (TK 3000, Uberaba, Brazil). The freezing curve consisted of –1.0°C/min down to –6°C. The process of seeding was performed when –6°C was reached. After 10 min, freezing was resumed with programming reset to –0.5°C/min down to –32°C. After stabilizing at –32°C, embryos were immersed in liquid nitrogen (–196°C).

Vitrification in open-pulled straws (OPS)

All vitrification solutions were prepared using a basal solution of HEPES-containing TCM-199 medium supplemented with 20% FBS (H-TCM). Embryos were initially kept in H-TCM for 5 min (Vajta, 2000). Immediately afterwards, embryos from the DMSO/EG group were transferred to an 10% ethylene glycol (EG) solution and 10% DMSO and transferred to an 20% EG + 20% DMSO + 0.5 M sucrose solution for 1 min each. Embryos from the DMF/EG group were transferred to a 10% EG and 10% DMF solution for 1 min and moved to a 20% EG + 20% DMF + 0.5 M sucrose solution

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for an additional 1 min. After this process, embryos from both groups were aspirated into 2 μl of a second vitrification solution, solutions that contained one or two embryos were transferred by capillarity to OPS and classified. Immediately afterwards, straws were transferred to liquid nitrogen and kept until further use.

Thawing of frozen embryos

Embryos were thawed at room temperature for 10 s and immersed in a water bath at 37°C for 20 s. Thawed embryos were deposited in warm holding medium for 5 min and subsequently evaluated as described below.

Warming of vitrified embryos

After removal from liquid nitrogen, straws were immersed in warming solution with the thinner tip facing down for less than 3 s. The cryoprotectant removal was performed in a four-well dish containing H-TCM medium supplemented with 0.33 M sucrose (wells 1 and 2). Embryos were kept in wells (1 and 2) for 1 min each, transferred to well 3 containing H-TCM + 0.2 M sucrose for 1 min, and finally for 5 min in H-TCM only.

Evaluation of embryo viability

Cellular viability was scored by propidium iodide staining. Blastocysts were cultured for 10 min, incubated for 5 min in DPBS containing 1% BSA and 125 mg ml^{-1} of propidium iodide and further transferred to slides containing DPBS with 100 mg ml^{-1} Hoechst 33342. Cells that stained with propidium iodide were considered non-viable (red or pink cells). The total cell number in each embryo was determined by Hoechst 33342 staining (blue cells). Embryos with less than 50% of red/pink cells were considered to be viable. Stained embryos were examined on an inverted fluorescence microscope (Leica DM 4000). Three replicates were performed.

Ultrastructural study by transmission electronic microscopy

Embryos selected at random from control ($n = 10$), DMSO/EG ($n = 10$), and DMF/EG groups ($n = 10$) were used for ultrastructural analysis. Embryos were fixed in Karnovsky solution (2 h at 4°C), washed in 0.1 M sodium cacodylate buffer (pH 7.4), and post-fixed in 1% osmium tetroxide. After dehydration in increasing ethanol concentrations, embryos were embedded in epon resin. Ultra-thin sections were obtained with a diatome knife. Sections were mounted on copper grade and stained with uranyl acetate and lead citrate. Samples were examined and photographed using a transmission electron microscope (FEI Morgani 268D, Eindhoven, The Netherlands) at CETENE.

Analysis of re-expansion rates after embryo cryopreservation

Cryopreserved embryos were thawed or warmed accordingly and morphologically evaluated before *in vitro* culture (IVC). Embryos were cultured in synthetic oviduct fluid (SOF) medium (Nutricell, Brazil) under mineral oil, at 38.5°C with 5% of CO_2 in air. Embryo survival was scored at 6, 12, and 24 h after IVC onset. Embryo viability was determined by blastocoel expansion. Three replicates were performed.

Statistical analysis

Descriptive analysis was used for embryo morphology and ultrastructural studies. Statistical analysis for re-expansion, viability and pregnancy rates were performed using the chi-squared test for multiple comparisons. Differences with a 5% probability were considered to be significant.

Results

In total, 246 embryos were recovered from 30 does (average of 8.2 embryos per donor), of which 223 blastocysts were viable (grades I and II) and were distributed at random to the groups described above.

In order to estimate cell viability after thawing/warming based on membrane integrity, embryos were stained with propidium iodide (Fig. 1). Embryos that had at least 50% of viable cells after thawing were considered to be viable (see Material and methods). In total, 10 of 15 embryos were viable after conventional freezing (66.6%) and six of 15 embryos were viable after DMF/EG vitrification (40.0%). Remarkably, DMSO/EG vitrification showed higher cell viability, in that 11 of 15 embryos were viable (73.3%).

In order to evaluate cellular effects of cryopreservation in greater detail, blastocysts were analysed by transmission electron microscopy (TEM) (Figs. 2–4). Embryos submitted to conventional freezing displayed several aspects of damage: ruptured zona pelucida, lysed cytoplasmic and nuclear membranes, nuclei with irregular size and shape, diffused chromatin, loss of cell junctions and microvilli, and dark/enlarged mitochondria displaying disorganized crypts. Moreover, embryos after DMF/EG vitrification showed less profound effects of cryopreservation than embryos subjected to conventional freezing, with loss of cell junctions and microvilli, but with preservation of nuclear shape and intact mitochondria and ribosomes. DMSO/EG

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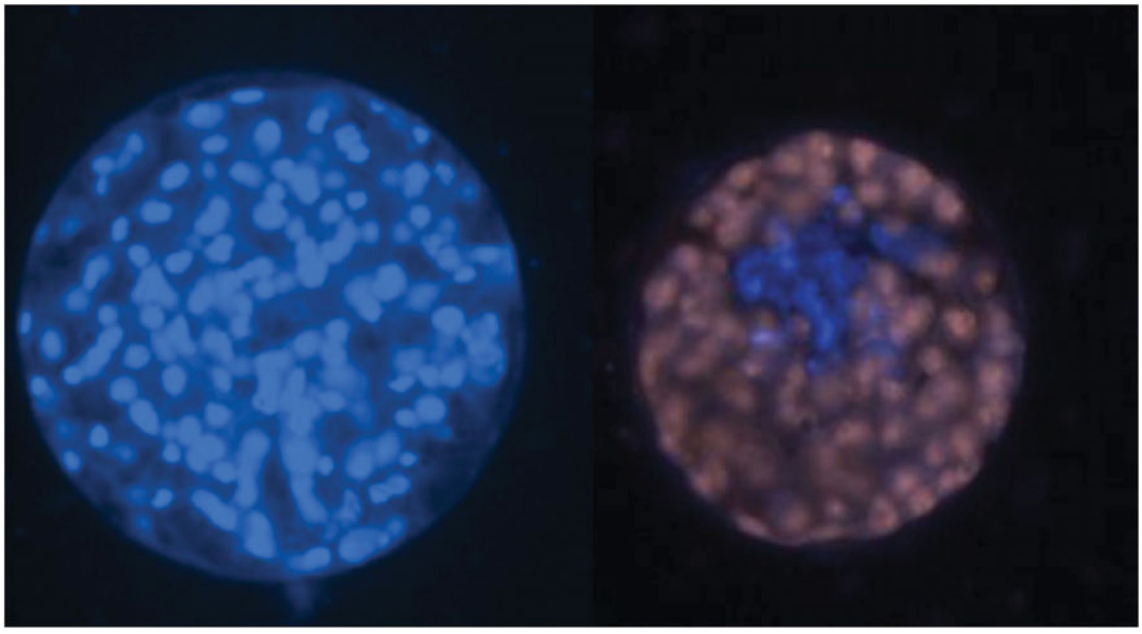

Figure 1 Caprine blastocysts stained for cell viability. Embryo with all cells viable (left) and a grade 2 embryo (right) showing damaged cells (red/pink).

vitrification embryos displayed well preserved cells, with intact zona pelucida, viable membranes with long projected microvilli, viable mitochondria with evident crypts well distributed across the cytoplasm, visible Golgi complex and ribosomes. Collectively, DMSO/EG vitrification preserved more efficiently cell and organelle structure after cryopreservation.

In order to functionally estimate embryo viability *in vitro*, re-expansion rates were recorded after thawing/warming (Table 1). No difference was observed between groups after 6 h and 12 h post-thawing/warming. However, DMSO/EG vitrification showed a higher re-expansion rate at 24 h after warming (Table 1).

Discussion

Embryo cryopreservation is of great value for livestock commercial and scientific applications. Despite routine use of embryo vitrification and particularly, conventional freezing, there are some roadblocks that need further investigation. Conventional freezing is a time-consuming process that requires expensive equipment, vitrification efficiency remains somewhat variable and recent reports have highlighted the potential for further protocol optimization (Guignot *et al.*, 2006; Hong *et al.*, 2007; Al Yacoub *et al.*, 2010). More importantly, there is a consensus that cryopreserved goat embryos have lower viability than fresh embryos.

The present report aimed to compare two different vitrification protocols (DMSO + EG and DMF + EG) with conventional freezing (EG). Direct comparisons between methods such as described here are important due to varying results from cryopreservation protocols and evaluation methodologies used by different laboratories.

Various factors determine embryo cryopreservation efficiency, such as species, cryoprotectants, embryo developmental stage, and embryo production system (*in vivo* versus *in vitro*), among others (Massip, 2001). Cryoprotectants play a major role in the cryopreservation process, and have been the focus of intense research. More recently, DMF/EG has been tested as a new cryoprotectant for semen cryopreservation of various species, including goats (Bezerra *et al.*, 2011; Moustacas *et al.*, 2011; Malo *et al.*, 2012). Although DMF/EG has proved so far to be less efficient then glycerol for semen cryopreservation (Bezerra *et al.*, 2011; Moustacas *et al.*, 2011; Malo *et al.*, 2012), sheep embryos are efficiently cryopreserved by DMF/EG vitrification.

Embryos were initially screened for cell viability, as intracellular and extracellular ice crystal formation and membrane lysis is a hallmark of cellular damage by cryopreservation (Massip, 1989; Vajta & Kuwayama, 2006). DMF/EG vitrification and conventional freezing displayed similar results, and were less efficient than DMSO/EG vitrification.

In order to better estimate the efficiency of different cryopreservation methodologies on cellular integrity,

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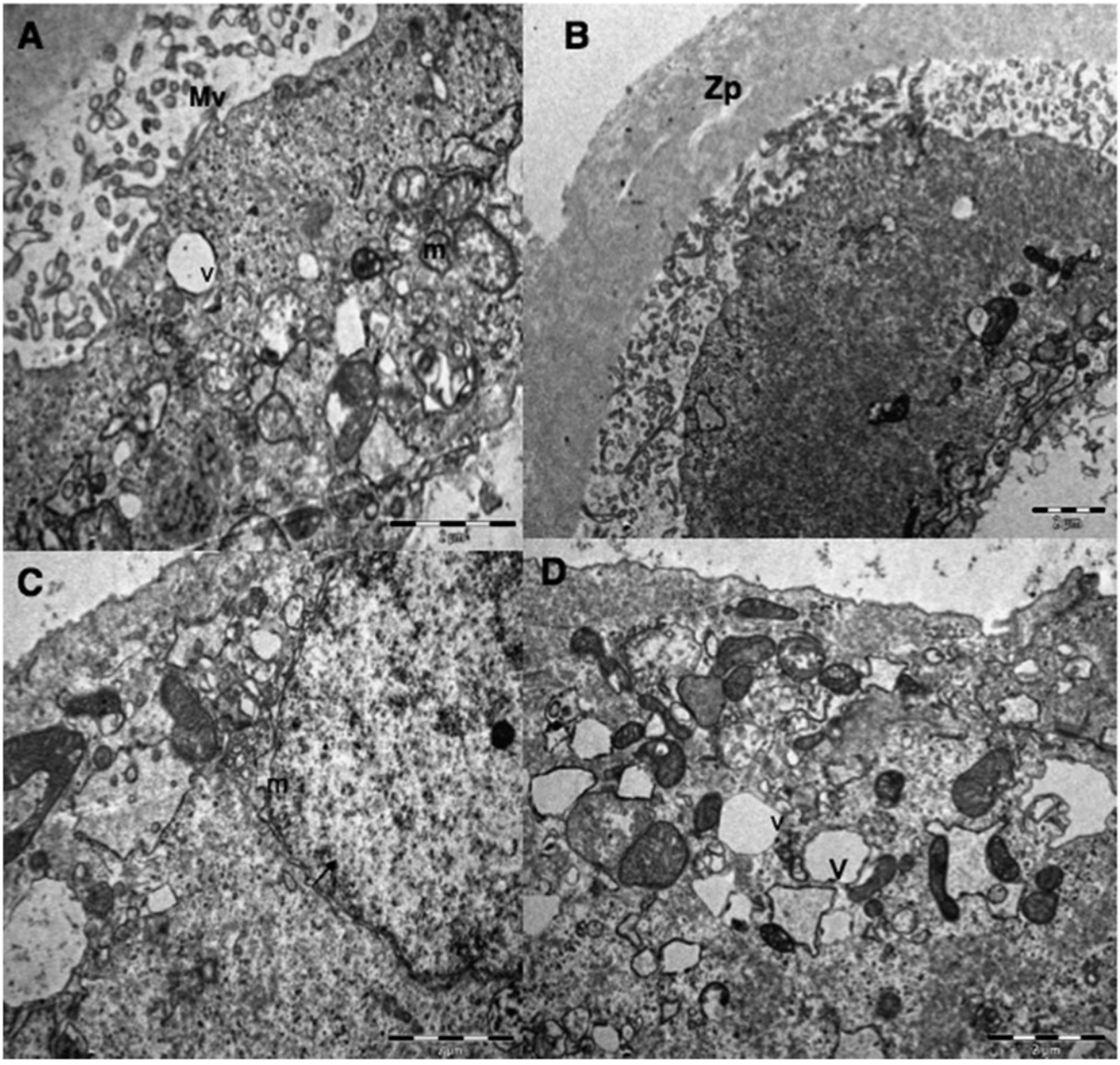

Figure 2 Caprine embryos cryopreserved by conventional freezing. (*A*) Swollen and ruptured mitochondria (m) and reduced microvilli (mv). (*B*) Intact zona pellucida (zp) and presence of microvilli (mv). (*C*) Loss of cellular junctions (arrow). (*D*) Vacuoles (v).

embryos were analysed by transmission electronic microscopy. The ultrastructural study revealed that conventional freezing was more detrimental to cell viability than both the vitrification protocols, with extensive damage to membranes, nucleus and mitochondria. DMF/EG vitrification displayed similar effects on cell structure to conventional freezing, but less frequently and with milder intensity. In contrast, DMSO/EG vitrification efficiently preserved cell structure including the zona pelucida. DMSO has been described as an efficient cryoprotectant for ovarian tissue cryopreservation by conventional freezing (Castro *et al.*, 2011). To our knowledge, this report is the first that describes the effects of goat embryo cryopreservation at the ultrastructural level.

In order to functionally evaluate the efficiency of cryopreservation methods, blastocysts were thawed/warmed and cultured *in vitro* to determine embryo survival and re-expansion rates. For embryo viability *in vitro*, conventional freezing and DMF/EG

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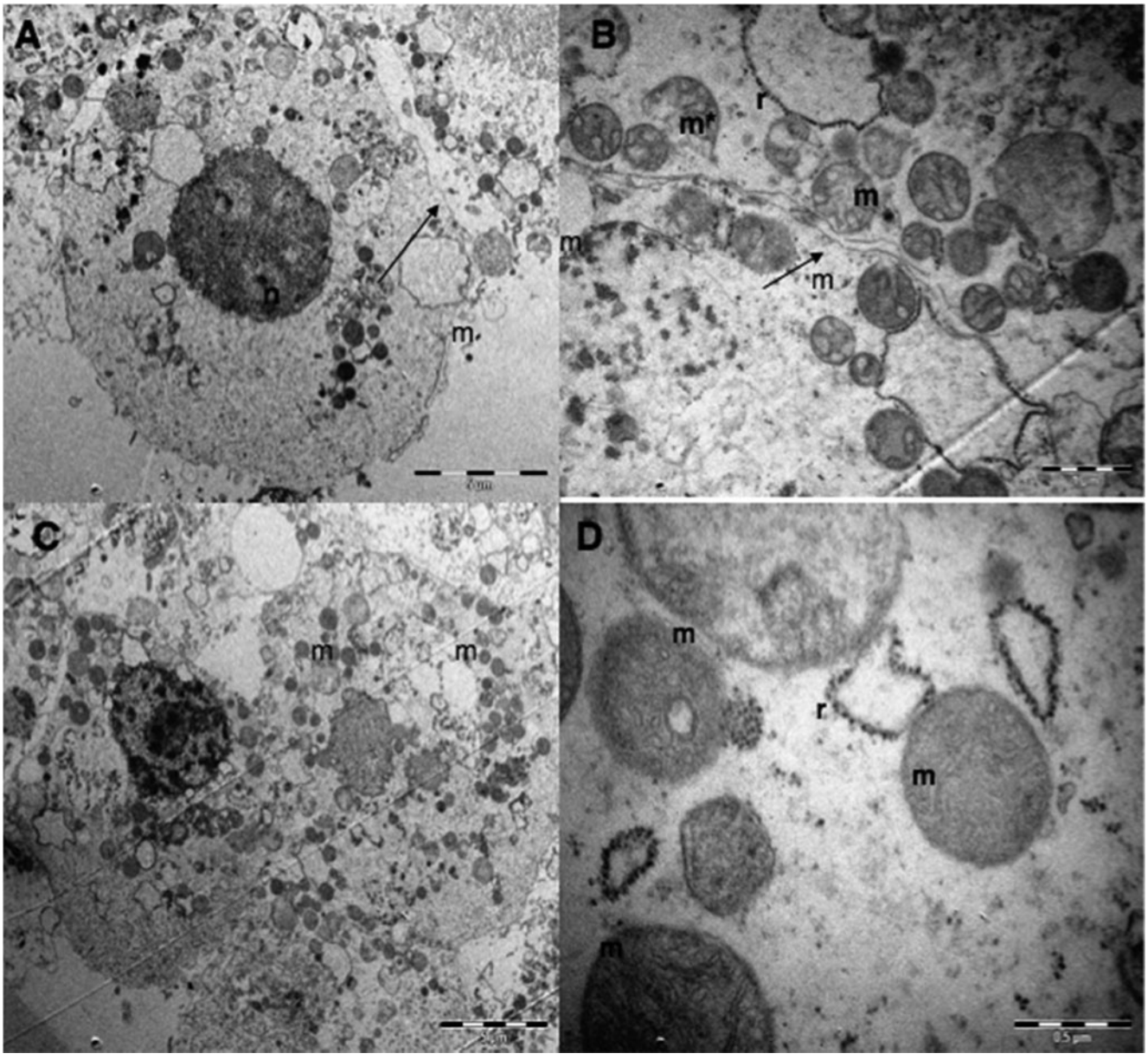

Figure 3 Caprine embryos cryopreserved by vitrification using DMF/EG vitrification. (*A*) Cells lacking uniformity, multiple mitochondria, loss of cellular junctions (fine arrow), nucleus (n). (*B*) Swollen and ruptured mitochondria (m*) and loss of cellular junctions (fine arrow). (*C*) Non-organized cells with multiple mitochondria. (*D*) Presence of ribosomes (r) and mitochondria with distinguishable cristae. DMF: dimethylformamide. EG: ethylene glycol.

vitrification were indistinguishable and less efficient than DMSO.

The results outlined here are discouraging towards the use of DMF for vitrification of goat embryos. DMF was initially used as a cryoprotectant for slow freezing of mammalian embryos and vitrification of fish embryos without success (Kasai *et al.*, 1981; Chen & Tian, 2005). Several reports tested DMF for semen cryopreservation (Chalah *et al.*, 1999; Lukaszewicz, 2002; Moustacas *et al.*, 2011) but, despite the notable species-specific variation of DMF potential for semen cryopreservation, horse semen can be efficiently cryopreserved with DMF (Alvarenga *et al.*, 2005; Gibb *et al.*, 2013).

The differences between DMF/EG and DMSO/EG vitrification on embryo quality suggest that, at the mechanistic level, their difference comes from their potential to avoid osmotic damage. DMSO/EG yielded higher cell survival and overall embryo viability. The use of DMF/EG or DMSO/EG preserved cell structure

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Table 1 Re-expansion rates of goat embryos cryopreserved by conventional freezing and vitrification

Group	Embryos	Re-expanded embryos after cryopreservation (%)		
		6 h	12 h	24 h
Conventional freezing	20	0 (00.00)	2 (10.00)	5 (25.00)[a]
DMSO/EG vitrification	19	3 (15.79)	6 (31.58)	9 (47.36)[b]
DMF/EG vitrification	19	1 (05.26)	4 (21.05)	6 (31.58)[a]

[a,b]Percentages with different superscripts differ significantly ($P < 0.05$).
DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EG, ethylene glycol.

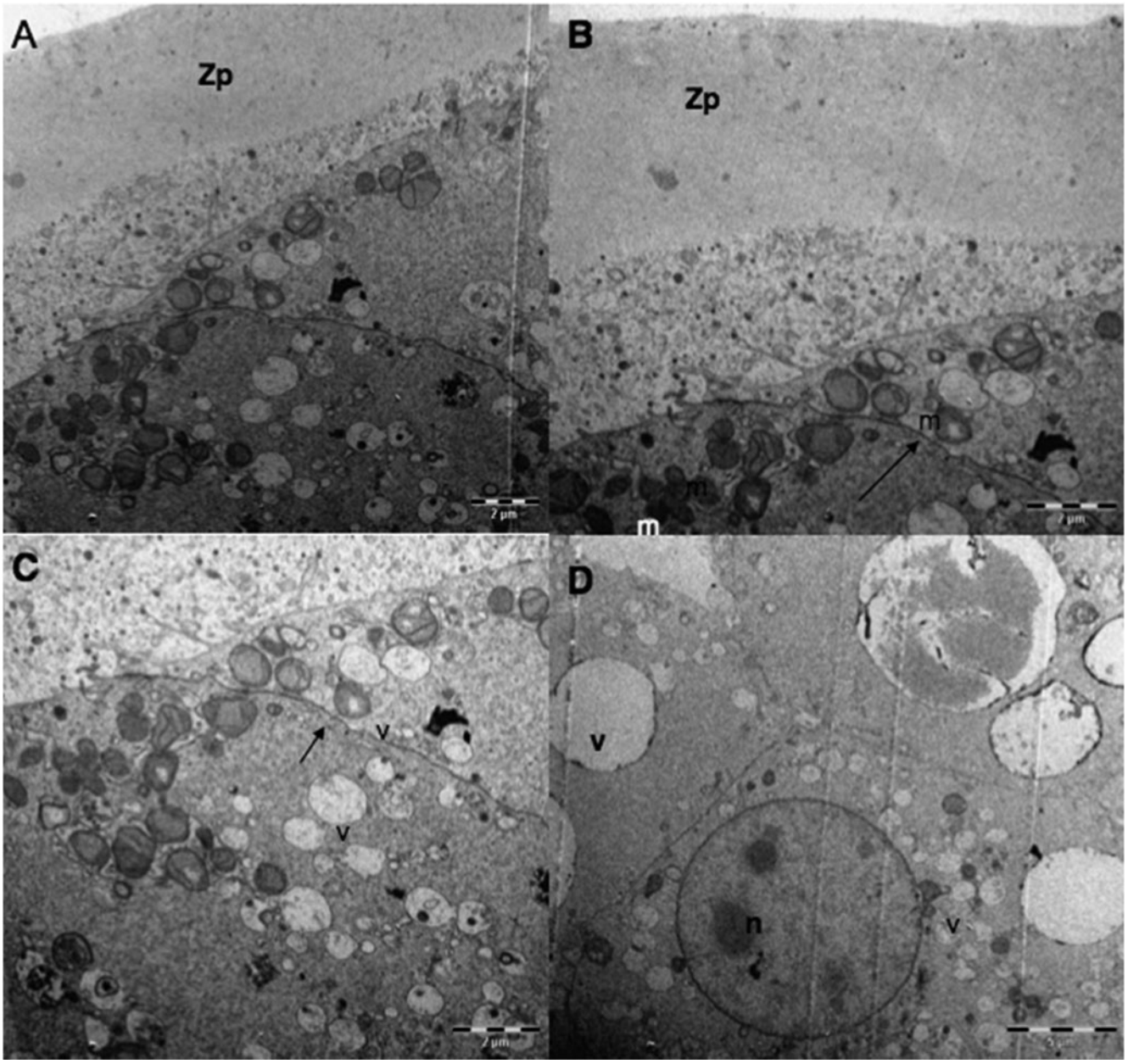

Figure 4 Caprine embryos cryopreserved by vitrification using DMSO/EG vitrification. (*A*) Severe cellular damage, zona pellucida (zp). (*B*) Nucleus (n) with lysed membrane (arrow). (*C*) Mitochondria (m), and vacuoles (v). (*D*) Cellular junctions (arrow). DMSO: dimethyl sulfoxide. EG: ethylene glycol.

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and organelle viability with similar efficiency, thereby ruling out other toxicity events observed such as damage to membranes and cytoskeleton (Alvarenga *et al.*, 2005).

In conclusion, the data described here show that DMSO/EG vitrification is the most efficient cryopreservation protocol for goat embryos and reinforces the species-effect variation on gamete and embryo cryopreservation outcome.

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Conflicts of interest

The authors declare no conflicts of interest.

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