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Cytoskeleton structure, pattern of mitochondrial activity and ultrastructure of frozen or vitrified sheep embryos ☆

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ABSTRACT

Even though sheep embryo cryopreservation is a commonly used procedure the survival and pregnancy outcomes can vary greatly. This study investigated whether cryopreservation was causing subtle changes in ultrastructure, mitochondrial activity or cytoskeletal integrity. Sheep embryos were either slow cooled in 1.5 M EG (n = 22), or vitrified in 20% EG + 20% DMSO with 0.5 M sucrose in Open Pulled Straws (OPS) (n = 24). One hour after warming the cryopreserved embryos differed from control embryos in that they had no mitochondrial activity combined with cytoskeletal disorganization and large vesicles. Vitrified embryos also showed many points of cytoskeleton disruption. Ultrastructural alterations resulting from actin filaments disorganization were observed in both cryopreserved groups. This includes areas presenting no cytoplasmic organelles, Golgi complex located far from the nucleus and a decrease of specialized intercellular junctions. Additionally, large vesicles were observed in vitrified morulae and early blastocysts. The alterations after cryopreservation were proportional to embryo quality as assessed using the stereomicroscope. Even in the absence of mitochondrial activity, grade I and II cryopreserved embryos contained mitochondria with normal ultrastructure. Embryos classified as grade I or II in the stereomicroscope revealed mild ultrastructural alterations, meaning that this tool is efficient to evaluate embryos after cryopreservation.

Introduction

In order to improve sanitary management and preservation of germplasm, cryopreservation has become essential in embryo transfer programs [11]. The disadvantage is that cryopreservation causes morphological and functional cell damage. However, it is widely accepted that the extension of cryodamage depends on many factors, such as cryopreservation protocols, species, developmental stage and if embryos were *in vivo* or *in vitro* produced [31].

An inevitable consequence of the cryopreservation is the cold-shock, which may affect intracellular organization or the inactivation of enzyme systems [35]. Cryopreservation can be extremely disruptive to the cellular organization of embryos, and it has been showed by different authors that depolimerization of microtubules and microfilaments occur after cryopreservation (reviewed in [12]). Moreover, mitochondria are essential for aerobic metabolism and ATP production in the cell, and mitochondrial functionality has been considered a hallmark of quality and developmental potential [15]. Although some works have focused attention on the functional capabilities of mitochondria after freezing [17,25,29,40], most of them were performed on isolated mitochondria. So, further investigations are necessary to understand how mitochondria are affected when whole embryos are cryopreserved.

Cryopreserved sheep embryo transfer is not as widely practiced as in the cow; however it has become important with sheep breeding modernization [24]. The cost of this technology is high compared to the economic value of the animals [1], but breeders continue to search for ways to reduce the cost and improve the efficiency [3]. Slow-freezing and vitrification have both been used for the cryopreservation of sheep embryos, with variable survival rates. These rates vary from 53% to 70% after slow freezing of morulae in glycerol and EG [5] to 83.7% after slow freezing of blastocysts in EG [19,20]. Significant variability has also been observed in sheep embryo vitrification results. While some studies found embryo survival rates of 60–85% after warming [1,8,21,24,33], others only reached rates close to 30% or 50% [3,22,30].

Although there are effective protocols to cryopreserve sheep embryos, and even though the survival rates are good, it is possible

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that embryos are suffering damages that not lead them to death. However, describing the changes in organelles is not a usual approach.

Recently, Bettencourt et al. [2] compared slow freezing and vitrification methods of ovine embryos and described ultrastructural findings. Still, no reports on the cytoskeleton structure and mitochondrial activity were discussed after these procedures. In addition, some authors [5–7] doubted the effectiveness of the stereomicroscope to evaluate cellular damage during embryo cryopreservation, a phenomenon more commonly observed using other methods.

This paper aims to describe and associate the ultrastructural characteristics, cytoskeleton structure and mitochondrial activity of *in vivo* produced sheep embryos after slow freezing and vitrification techniques, and to verify whether the stereomicroscope is efficient to evaluate cryopreserved embryos.

Materials and methods

Embryo collection

Embryos from 30 superovulated Santa Ines ewes were collected 5–7 days after laparoscopic artificial insemination. Embryos were recovered by surgical procedure (laparotomy followed by flushing of the uterus horns). The obtained morulae and blastocysts were selected and classified according to the International Society of Embryo Transfer (IETS) [32]. Grade I and II embryos were washed in Phosphate Buffered Saline (PBS) plus 20% fetal calf serum (PBSS), maintained in holding medium (Holding Plus®, Vitrocell, São Paulo, Brazil) at 36 °C and protected from light until cryopreservation or fixation. Grade I and II embryos were divided into three groups: slow freezing ($n = 22$), vitrification ($n = 24$) and control ($n = 33$). Embryos were randomly distributed, but always maintaining similar numbers of blastocysts and morulae, and Grade I and II embryos in every group. Fresh embryos (control group) were immediately evaluated for mitochondrial activity and cytoskeleton structure by confocal microscopy and for ultrastructure by transmission electron microscopy (TEM). Grade III embryos were not cryopreserved. Some were processed only as controls, both for mitochondrial activity and cytoskeleton structure ($n = 3$) and for transmission electron microscopy ($n = 2$).

Slow freezing and thawing

Slow freezing was performed using the protocol of Garcia-Garcia et al., [19] with a slight modification on the freezing program, which missed the third cooling ramp (0.1 °C/min from −30 to −35 °C). All cryoprotectant solutions were prepared in PBSS. Initially, embryos were equilibrated in 0.75 M EG for 10 min and then placed for a further 10 min in 1.5 M EG at 32 °C. One to four embryos were loaded into each 0.25 mL straw. Afterwards, the straws were placed in a controlled-rate freezer (Dominium K, Biocom, MG, Brazil) at 10 °C and immediately cooled at 1 °C/min to −7 °C and then manually seeded. After 5 min at −7 °C, embryos were cooled at 0.3 °C/min to −35 °C. After 10 min at −35 °C, the straws were immersed into liquid nitrogen and stored for 2–9 months.

Straws were thawed by immersion in distilled water at 32 °C for 30 s. Embryos were then transferred to a 0.25 M sucrose solution in PBSS for 10 min, and washed three times in PBSS for 5 min each.

Vitrification in OPS and warming

Vitrification was performed using the protocol of Dattena et al. [9] with the equilibrium time modified (1.5 min instead of 3 min). All vitrification solutions were prepared using PBSS. Embryos were exposed to 10% EG and 10% DMSO for 1 min and 30 s, and then to 20% EG, 20% DMSO and 0.5 M sucrose for 30 s, always at room temperature. The embryos were loaded into OPS according to Vajta et al. [38], by capillarity together with ~2 μl of medium and directly immersed into liquid nitrogen and stored for 2–9 months.

Embryos were warmed according to Vajta et al. [38] by immersion of the OPS tip in PBSS with 0.25 M sucrose at 37 °C for 6 s. Afterwards, embryos were washed in the same solution for 5 min, then in PBSS with 0.15 M sucrose for 5 min and finally three times in PBSS for 5 min each.

In vitro culture

After thawing or warming, embryos were placed into *In Vitro* Culture (IVC) [19]. The culture medium used was TCM 199 (TCM medium 199 Earle's salts, Sodium bicarbonate, Gibco, Paisley, Scotland, UK) supplemented with 10% Fetal Calf Serum (FCS) (Gibco, Paisley, Scotland, UK), 1% L-glutamine and antibiotics. The culture plates were incubated at 38 °C with an atmosphere of 5% CO_2 in air and saturated humidity for 1 h. The aim of this short-term embryo incubation was only to allow embryonic cells to return to its normal temperature. After IVC, embryo quality was assessed under stereomicroscope and embryos were destined to mitochondrial activity and cytoskeleton structure evaluations or TEM.

Mitochondrial activity and cytoskeleton structure analysis

All fluorescent dyes were obtained from Molecular Probes Inc. (Eugene, OR, USA). For mitochondrial activity, embryos were incubated with 33.12 mg/mL Mitotracker® Red CMXRos in TCM199 with L-glutamine and antibiotics for 15 min under IVC conditions, and then fixed in 2.5% paraformaldehyde for 40 min. For evaluation of cytoskeleton actin filaments organization embryos were labeled with 0.145 mg/mL of Alexa Fluor® 488 Phalloidin in PBS for 1 h. For nuclei identification, embryos were labeled with 0.2 mg/mL of 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI® Nucleic Acid Stain) for 20 min. Embryos were evaluated using a Leica laser scanning confocal microscope TCS SP5 (Leica, New York. USA). DAPI-stained nuclear material was excited using a Diode laser (excitation and emission wavelengths of 405 and 460 nm, respectively). An argon-ion laser was used to excite and produce optical scans of the Alexa Fluor 488-Phalloidin-labelled actin filaments (excitation and emission wavelengths of 499 and 520 nm, respectively). Similarly, for the visualization of Mitotracker Red CMXRos a 594-Helium neon laser was used in the excitation of 578 nm and emission 600 nm wavelengths. The images produced by sequential scans via different color channels were then merged and recorded in digital format. Fresh ($n = 21$), frozen ($n = 9$) and vitrified ($n = 12$) embryos were evaluated.

Light and transmission electron microscopy evaluation

Fresh ($n = 12$), frozen ($n = 9$) and vitrified embryos ($n = 9$) were fixed in Karnovsky (2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2) for 4 h at room temperature. Then, they were washed with sodium cacodylate buffer, post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer. Subsequently, the samples were dehydrated in acetone and embedded in Spurr resin.

Semi-thin sections (3 μm) were stained with toluidine blue and examined under a light microscope. Ultra-thin sections (70 nm) were examined using a Jeol 1011 transmission electron microscope

Table 1
Percentage of embryos that maintained their quality, decreased to grade II or III, or degenerated after cryopreservation/warming and 1 h IVC.

	Vitrified	Frozen	Total
Quality maintained	45.8% (11/24)	22.7% (5/22)	34.8% (16/46)
Decreased to grade II or III	20.8% (5/24)[a]	63.6% (14/22)[b]	41.3% (19/46)
Degenerated	33.4% (8/24)	13.7% (3/22)	23.9% (11/46)
Total	24	22	46

[a,b] Numbers in the same row with different superscripts differ significantly ($P < 0.05$).

(Jeol, Tokyo, Japan) to evaluate the ultrastructure of embryonic cells.

Statistical analysis

The percentages of viable and degenerated embryos were compared between treatments by Chi-square test ($P < 0.05$).

Results

Evaluation at the stereomicroscope

A total of 79 grade I and II embryos, 58 grade III embryos and 57 degenerated, delayed or unfertilized oocytes were recovered. Of the 79 grade I or II classified embryos, 22 were frozen and 24 were

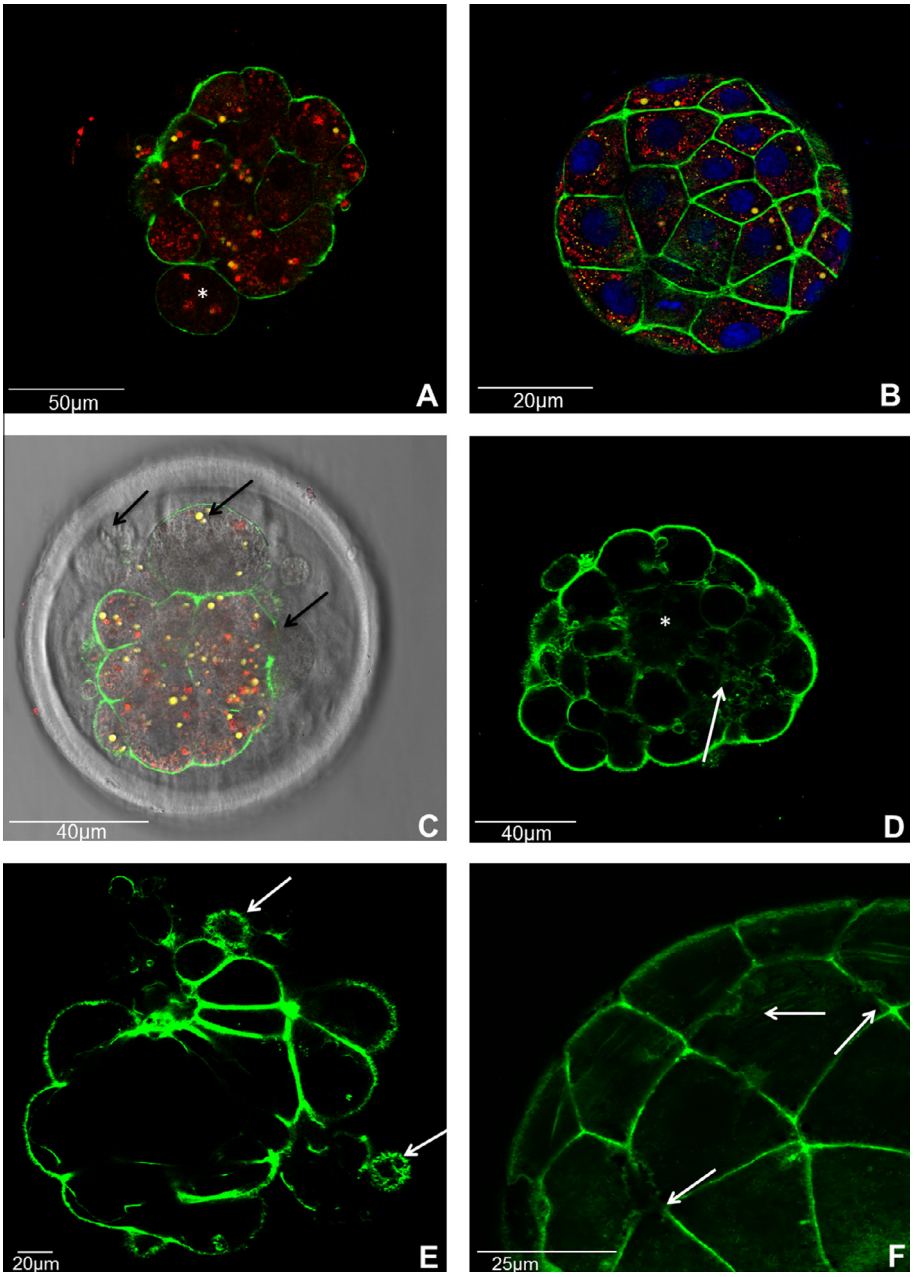

Fig. 1. Confocal microscopy evaluation of actin filaments (green) and mitochondrial activity pattern (red) of embryos. (A) Grade I fresh morulae presenting good cytoskeleton organization and mitochondrial activity and extruded cells (*). (B) Trophoblastic cells of a Grade I fresh blastocyst. (C) Grade III fresh morulae presenting good cytoskeleton organization but low mitochondrial activity stain in viable cells, and no cytoskeleton or mitochondrial stain in extruded cells (arrows). (D) Grade I fresh initial blastocyst showing special organization of actin filaments (arrow). * Initial blastocele. (E) Grade III blastocyst after slow freezing and thawing with complete cytoskeleton disorganization. Note the rough appearance of actin filaments in some regions (arrows). (F) Grade I expanded blastocyst after vitrification and rewarming presenting many points of cytoskeleton disruption (arrows).

vitrified. Table 1 shows the percentage of embryos that maintained their quality, decreased to grade II or III, or degenerated after warming. Statistic difference was observed only in the number of embryos that decreased in quality (from grade I to II, or from Grade II to III), with higher rates in slow freezing (63.6%) than in vitrification (20.8%) groups ($P < 0.05$).

Mitochondrial activity and cytoskeleton structure

The cytoskeleton of the fresh embryos was characterized by typical architecture, as previously described [34,36,41]. Fresh embryos grade I and II showed actin filaments with characteristic organization as well as intense fluorescence indicative of mitochondrial activity regardless of their developmental stage (Fig. 1A and B). In grade III embryos, only the small group of viable cells presented an organized cytoskeleton, however mitochondrial activity was lower in all cells (Fig. 1C) as well as in portions of extruded cells of grade I and II embryo (Fig. 1A).

Some cytoskeleton differentiation was observed in early blastocysts. These embryos presented peculiar round blebs in some regions (Fig. 1D).

Frozen and vitrified embryos showed disorganization of actin filaments (Fig. 1E and F). Besides this, cytoskeleton appeared rough in some regions (Fig. 1E). Moreover, vitrified embryos showed

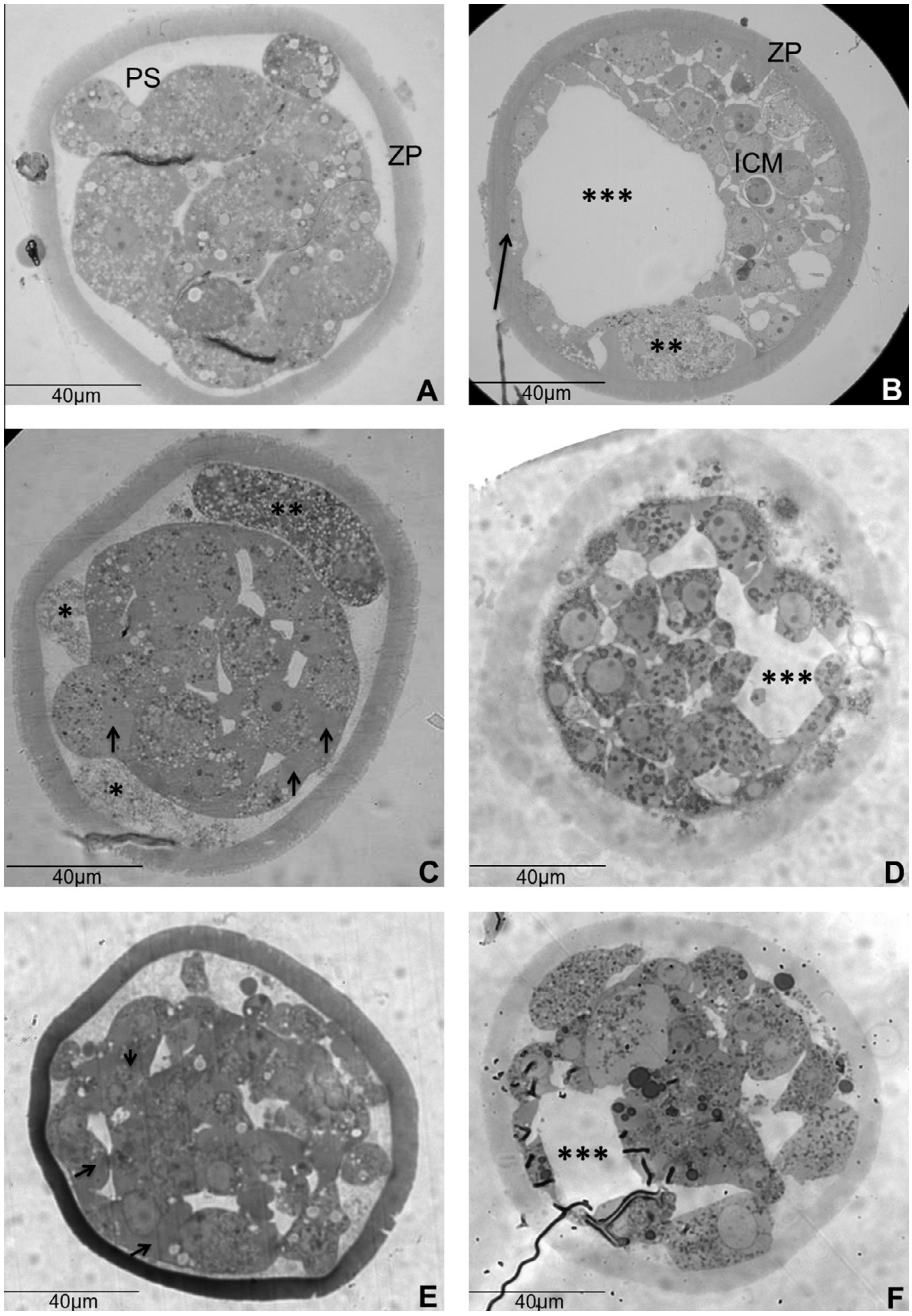

Fig. 2. Light microscopy of embryos. (A) Grade I fresh morulae presenting a close contact between blastomeres, many vesicles in cytoplasm and a large perivitelline space (PS). (B) Grade I fresh expanded blastocyst presenting thin zona pellucida, very small perivitelline space, elongated trophoblast cells and extruded cells (∗∗). PS: perivitelline space; ZP: zona pellucida; ∗∗∗: blastocele; ICM: inner cell mass; arrow: trophoblast cells. (C) Grade II morulae after slow freezing presenting a close contact between blastomeres, debris (∗) and extruded cells (∗∗) in perivitelline space. Cytoplasm with organelle-free area (short arrows). (D) Grade II blastocyst after slow freezing with defined blastocele (∗∗∗), but with some retraction. (E) Grade I morulae after vitrification presenting a greater distance between blastomeres and organelle-free area (short arrows). (F) Grade III blastocyst after vitrification with disorganized blastocele (∗∗∗).

many points of cytoskeleton disruption, even the ones that presented good blastocelic cavity re-expansion (Fig. 1F). This feature was not observed in frozen embryos. Mitochondrial activity was not observed in cryopreserved embryos, either frozen or vitrified, independent of embryo quality.

Structural evaluation

Light microscopy of the control group revealed morulae with a close contact between blastomeres and a large perivitelline space (Fig. 2A). Many vesicles were seen in both viable and extruded blastomeres. In early blastocysts, as the blastocele forms, trophoblastic cells lengthened and the Inner Cell Mass (ICM) cells distanced from each other forming projections. Blastocyst presented very elongated trophoblastic cells close to each other and to the zona pellucida (ZP). Perivitelline space was very small and became smaller as embryos expanded. Contact between ICM cells was mediated by long projections. Embryo cells have fewer and smaller vesicles, presenting a more homogeneous cytoplasm, except extruded cell, which still presented a vesicular cytoplasm (Fig. 2B).

Cryopreserved embryos, both by slow freezing and vitrification, presented some structural changes (Fig. 2C–F): cells cytoplasm became more heterogeneous, organelles and vesicles were agglomerated, leaving an organelle-free area; perivitelline space increased and contained a higher amount of debris. Cryopreserved blastocysts did not achieve complete restructuring of the blastocele after warming (Fig. 2D and F). In general, vitrified embryos (Fig. 2E and F) showed greater cellular disorganization than frozen ones (Fig. 2C and D).

Ultrastructural evaluation

In fresh grade I and II morulae, blastomeres showed large and spherical nucleus, usually with one or two nucleoli. The cytoplasm presented a large amount of vesicles, many of them coalescing, large lipid droplets, smooth endoplasmic reticulum (SER), Golgi complex close to the nucleus and a large number of mitochondria, predominantly hooded (Fig. 3A). Large round or oval electron-dense mitochondria with peripheral cristae were also common, many of them in apparent division and always associated with SER. Junctions were observed between cells. Grade III morulae presented a more granular and disorganized cytoplasm, with organelle-free areas, a large number of small vesicles (Fig. 3B), and some degenerated mitochondria.

In grade I blastocysts, ICM cells maintained contact through cytoplasmic projections. Trophoblast cells were elongated, close to the ZP, and developed many microvilli (Fig. 3C). These two cell types presented large lipid droplets, polyribosomes and a perinuclear Golgi complex. A huge variation in mitochondrial shape was observed, and they were still in close contact with SER (Fig. 3D). Desmosomes were seen attaching adjacent cells (insert in Fig. 3C). The number of desmosomes increased as the blastocyst expanded, as well as the density of polyribosomes and the granulation of cytoplasm and nucleus.

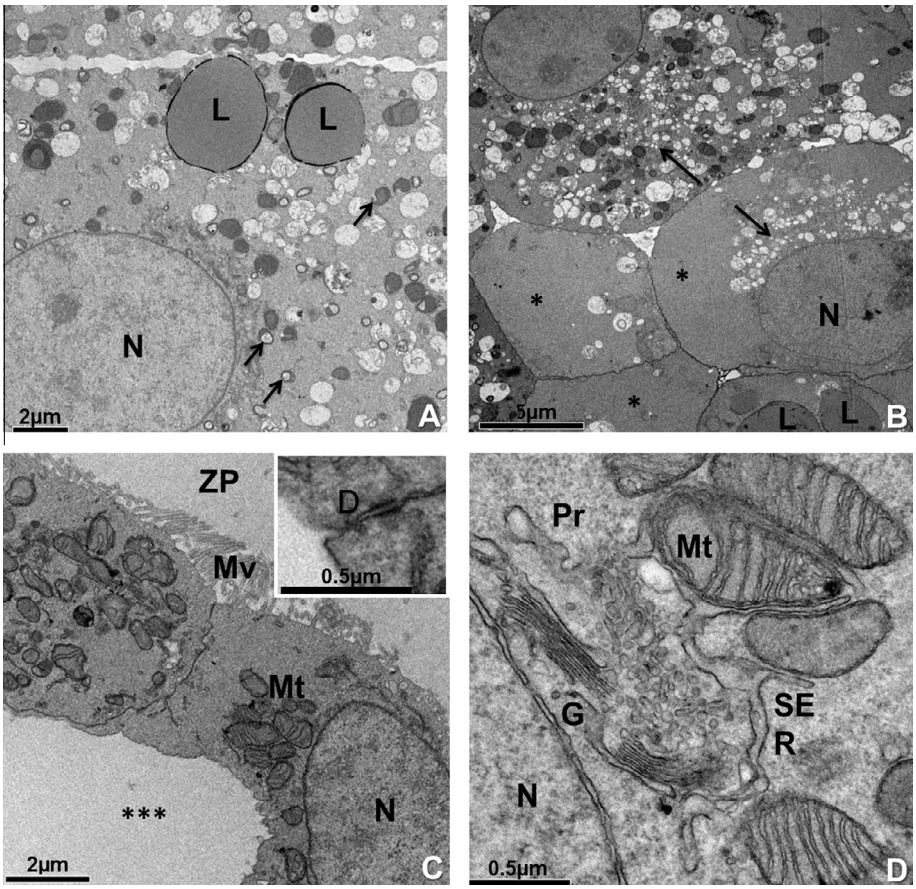

Fig. 3. Transmission electron micrographs of fresh embryos. (A) Blastomeres of a Grade I morulae presenting a large number of *hooded* mitochondria (short arrows), large lipid droplets and coalescing vesicles. (B) Blastomeres of a Grade III morulae with organelle-free areas (∗) and a large number of small vesicles (long arrows). (C) Trophoblastic region of a Grade I blastocyst showing the contact between microvilli (Mv) and zona pellucida (ZP). (∗∗∗) blastocele. Insert shows desmossomes (D) attaching adjacent cells. (D) Cytoplasmic detail of a Grade I blastocyst cell. Note the cytoplasm granulation. N: nucleus, L: lipid droplet, Mt: mitochondria, Mv: microvilli, ZP: zona pellucida, Golgi complex, SER: smooth endoplasmic reticulum, Pr: polyribosome.

Ultrastructural alterations resulting from actin filaments disorganization were observed in both cryopreserved groups. This includes areas presenting no cytoplasmic organelles, Golgi complex located far from the nucleus and a decrease of specialized intercellular junctions.

Frozen embryos that maintained their quality as grade I or II were similar to the control group (Fig. 4A and B). However, some ultrastructural changes were observed, such as: reduced contact between microvilli and ZP in blastocysts, fewer visible desmosomes, organelle-free cytoplasmic areas, and SER and mitochondria swelling. No rupture of mitochondrial membranes was seen. Golgi complex was seen in different locations of the cell, not always close to the nucleus. Moreover, cytoplasm discontinuities were sometimes observed, especially at lipid droplets periphery. Frozen grade III embryos showed SER swelling, heterogeneous cytoplasm, many degenerated mitochondria (Fig. 4C) and large vesicles (Fig. 4D). Despite that fresh grade III embryos also presented vesicles and degenerated mitochondria, in the control group the cytoplasm was homogenous and there was no swollen SER.

Vitrified embryos that maintained their quality as grade I or II were similar to those in frozen group, showing cytoplasmic granulation, many vesicles, elongated mitochondria with transversal cristae associated with SER, and microvilli. However, large vesicles were observed in vitrified grade II morulae and early blastocysts (Fig. 5A). These vesicles were not seen in fresh or frozen morulaes of same quality. Cytoplasm discontinuities, as well as organelle-free areas were common after vitrification (Fig. 5B), as in frozen embryos. Vitrified grade III and also frozen embryos had heterogeneous cytoplasm in addition to mitochondrial and SER swelling (Fig. 5C). Large vesicles occupying great areas of the cytoplasm (Fig. 5D) and degenerated cells among viable embryonic cells (not shown) were characteristics found only in the vitrified group.

Discussion

In this study, fresh embryos revealed intense mitochondrial activity. Active mitochondria were distributed throughout the cytoplasm, regardless of the embryonic developmental stage. However, no mitochondrial activity could be observed in cryopreserved embryos, either frozen or vitrified. The evaluation of mitochondrial activity after cryopreservation are unpublished in this species and it is known that mitochondrial dysfunctions or abnormalities may compromise developmental processes by inducing chromosomal segregation disorders, maturation and fertilization failures or even embryo fragmentation [4]. Sohn et al. [35] studied the effect of two frequencies of liquid N_2 infusion on the cryopreservation of mice two-cell embryos on the mitochondrial activity and actin filaments distribution using fluorescent markers similar to those used on the present work. Very similar to what this study revealed, those authors [35] showed that the number of mitochondria with high membrane potential decreased on cryopreserved embryos, and

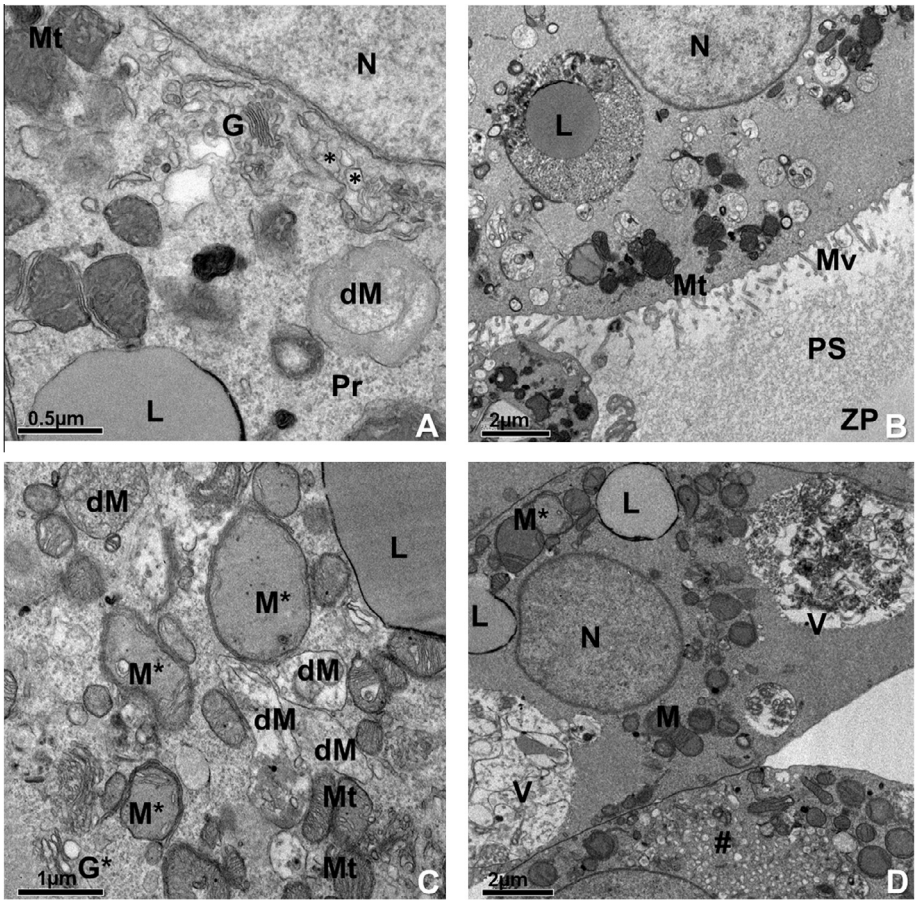

Fig. 4. Electron micrographs of frozen/thawed embryos. (A) Grade II blastocyst with normal and degenerated mitochondria, Golgi complex, normal and turgid endoplasmic reticulum (∗), and polyribosomes. (B) Grade II blastocyst presenting ultrastructural similarity to the control group, with peculiar appearance of the cytoplasm around lipid droplets. (C) Grade III morulae presenting highly swollen and degenerated mitochondria. (D) Grade III blastocyst with great vesicles and areas of highly vesiculated cytoplasm (#). Mt: normal mitochondria, dM: degenerated mitochondria, M*: swollen mitochondria, N: nucleus, G: golgi complex, L: lipid droplet, Pr: polyribosome, Mv: microvilli, PS: perivitelline space, ZP: zona pellucida, V: vesicle.

described gaps or discontinuities in the peripheral actin fibers (those in close association with the cell membrane), especially on the low frequency N_2 infusion treatment. Disturbances in function and distribution of mitochondria, as well as changes in the organization of cytoskeleton related to insufficient culture conditions or cryopreservation are expected to occur and may reduce developmental capacity [12,15].

Previous studies have demonstrated succesfull cryopreservation of mitochondria isolated from rat liver [17], muscles [25] and brain [29]. In brain tissue, mitochondria showed a reduction in respiratory activity after cryopreservation. However, this effect was not due to mitochondrial membranes rupture [29]. Penetration of the fluorochrome used in this experiment is proportional to the inner mitochondrial membrane activity and equilibrium [28], which was surely altered. However, in the present work no rupture of mitochondrial membranes was seen on the ultrastructural analysis. Nukala et al. [29] also found that freezing mitochondria without any cryoprotective agent destroyed their structural integrity and functional viability, and that the use of a cryopretective agent prevents most but not all damages.

Moreover, the ability to restore a satisfactory metabolic activity or regenerate damaged structures after exposure to low temperatures requires time. For example, Leoni et al. [26] sowed that vitrified/warmed ovine embryos require 9–12 h of culture to complete resumption of DNA synthesis, and 29–35 h to re-acquire their full capacity of protein secretion. Likewise, Vajta et al. [37] demonstrated severe degenerative changes in cells of *in vitro* produced bovine embryos immediately after warming. But during the subsequent 4 h culture evident signs of regeneration were observed, and after 24 h only slight signs of injury could still be seen. In preantral follicle oocytes, vitrification significantly affected mitochondrial inner membrane potential [10], but mitochondrial activity was recovered after 12 days in culture. Similarly, human blastocysts had their respiratory rate lowered or even absent after vitrification/warming, only detected again after 24 h [40]. Undoubtedly, one hour of IVC was not enough to allow metabolic recovery in the present study. How long would it take to mitochondrial activity to be restored in these cryopreserved embryos remains a question.

Mitochondrial malfunction may be caused by decline in the mitochondrial membrane potential and disruption of mitochondrial membrane. While the first is often reversible [10,29,40], membrane disruption is a more critical damage. Comparing mitochondrial ultrastructure of fresh and cryopreserved embryos, swollen mitochondria were more frequent in cryopreserved embryos. However, most mitochondria from embryos grade I and II post-cryopreservation presented typical ultrastructure. No rupture of mitochondrial membranes was seen on grade I and II embryos in this study. Higher degrees of mitochondrial swelling were observed in previous studies on cryopreserved grade I and II sheep embryos [2,5]. Mitochondrial swelling is also commonly described in cryopreserved oocytes [14,16,23]. Using *in vitro* produced embryos and similar procedures of slow freezing and vitrification Bettencourt et al. [3] achieved satisfactory pregnancy rates of 68.4% and 54.6% on day 45, respectively. This shows that some ultrastructural changes observed on transferable embryos after cryopreservation are reversible, and embryos can fully recover.

Besides playing a role in organelle organization the primary function of actin filaments is acting on intercellular junctions during the compaction process and to maintain structural integrity during the initial embryo stages [18]. The layout of actin filaments

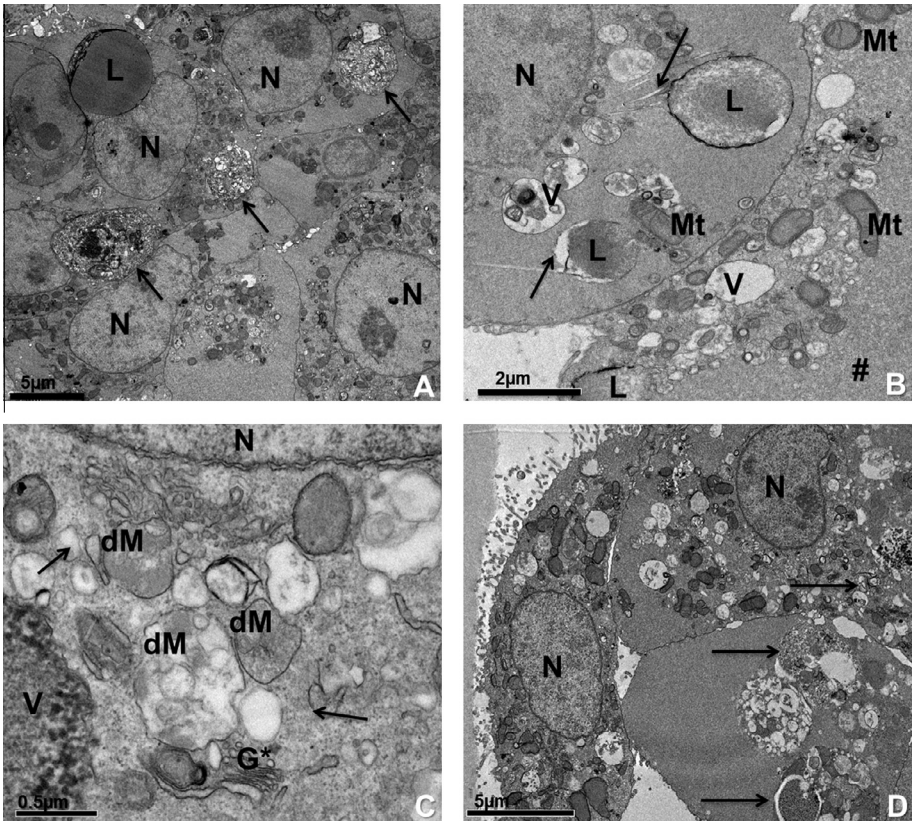

Fig. 5. Electron micrographs of vitrified/warmed embryos. (A) Grade II morulae with large vesicles in many blastomeres (arrows). (B) Grade I morulae with organelle-free areas (#) and cytoplasm discontinuities (arrows) next to lipid droplets (L). (C) Grade III blastocyst with degenerated mitochondria (dM) and swollen smooth endoplasmic reticulum (arrows). (D) Grade III blastocyst with vesicles (arrows) occupying much of the blastomeres cytoplasmic area. N: nucleus, L: lipid droplet, Mt: mitochondria, dM: degenerated mitochondria, V: vesicle, G: golgi complex.

during the transition stage from morulae to initial blastocyst is justified by asymmetric division, polarization and differentiation of ICM and trophoblastic cells [27].

Cryopreserved embryos were characterized by mild to severe disorganization of actin filaments. Better quality embryos (grade I and II) presented small cytoskeleton damage. Cryopreserved grade III embryos showed a high level of cytoskeleton disorganization, independent of the cryopreservation treatment. However, evaluating grade I blastocysts with recovered blastocele after vitrification/warming and culture, there were many discontinuous actin filaments. This was not observed in the slowly frozen group. According to Skidmore et al. [34], the slow freezing procedure allows better cytoskeleton preservation when compared to vitrification. As mentioned above, Sohn et al [35] also described gaps or discontinuities in the peripheral actin fibers in mouse two-cell embryos slowly frozen. Microfilaments and microtubules are a fragile network, and it is already proved that the cytoskeleton of mammalian embryos change in response to cooling and during cryopreservation and reform on return to culture [13]. Thus, embryos must be able to recover the cytoskeleton structure after cryopreservation because cytoskeleton damage may affect cell division and many other crucial functions for embryo survival [39]. On the ultrastructural analysis, organelle-free areas were observed in some cells of cryopreserved embryos. This may be a result of changes to the cytoskeleton.

In the vitrified group it was possible to observe large vesicles throughout all the cytoplasm and a higher incidence of degenerated cells in the middle of the viable embryonic portion. The presence of large vesicles in vitrified embryos may indicate that this technique caused greater embryo damage. Studying the recovery of vitrified bovine embryos after 0, 4 and 24 h of IVC Vajta et al. [37] also observed degenerated cells within the viable embryonic portion. However, in their study the nonviable cells were expelled to the perivitelline region and after 24 h the embryos had recovered their normal morphology, except for the debris found in the perivitelline space. Evaluation of semi-thin sections under the light microscope often reveals structural damage that is not detected by stereomicroscope [2,7]. Light microscopic analysis of grade I and II embryos in this experiment revealed only small differences between cryopreserved and fresh embryos. Typical characteristics of all grade I and II embryos after cryopreservation were irregular distribution of organelles and vesicles, larger perivitelline space, greater amount of debris and blastocele collapse. As in previous studies [2,7], grade III embryos in both groups presented complete blastocele disarray, great amount of extruded cells and irregular shape.

This study presented some aspects of the cytoskeleton structure, mitochondrial activity patterns and the ultrastructure of ovine morulaes and blastocysts. Cytoskeletal alterations after cryopreservation were proportional to embryo quality as assessed using the stereomicroscope, revealing an association with the ultrastructure after cryopreservation. Even in the absence of mitochondrial activity, grade I and II cryopreserved embryos contained more ultrastructuraly normal mitochondria and better preservation of nuclear and plasma membrane. Vitrified embryos were marked by their ultrastructure with large vesicles within the first hour after warming. Embryos classified as grade I or II in the stereomicroscope revealed mild ultrastructural alterations, meaning that this tool is efficient to evaluate embryos after cryopreservation.

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