Ultrastructural changes of sheep cumulus–oocyte complexes following different methods of vitrification

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Summary

To determine the ultrastructural changes of sheep cumulus–oocyte complexes (COCs) following different methods of vitrification, good quality isolated COCs (GV stage) were randomly divided into the non-vitrified control, conventional straw, cryotop and solid surface vitrification groups. In both conventional and cryotop methods, vitrified COCs were respectively loaded by conventional straws and cryotops, and then plunged directly into liquid nitrogen (LN_2); whereas in the solid surface group, vitrified COCs were first loaded by cryotops and then cooled before plunging into LN_2. Post-warming survivability and ultrastructural changes of healthy COCs in the cryotop group especially in comparison with the conventional group revealed better viability rate and good preservation of the ooplasm organization. However in all vitrification groups except the cryotop group, mitochondria were clumped. Solely in the conventional straw group, the mitochondria showed different densities and were extremely distended. Moreover in the latter group, plenty of large irregular connected vesicles in the ooplasm were observed and in some parts their membrane ruptured. Also, in the conventional and solid surface vitrification groups, cumulus cells projections became retracted from the zona pellucida in some parts. In conclusion, the cryotop vitrification method as compared with other methods seems to have a good post-warming survivability and shows less deleterious effects on the ultrastructure of healthy vitrified–warmed sheep COCs.

Keywords: Cumulus–oocyte complexes, Sheep, Ultrastructure, Vitrification

Introduction

Successful cryopreservation of mammalian oocytes is quite difficult because of the high volume to surface ratio, chilling injury, alterations of the zona pellucida and cumulus cell interactions (Vajta, 1997). In addition to the large size of the oocyte, water content and high degree of cytoplasmic specialization are considered as obstacles in the way of safe oocyte vitrification (Nottola *et al.*, 2009).

Different freezing methods, cryoprotectant concentrations, oocyte maturity and the specific biological structure of mammalian oocytes make their cryopreservation difficult (Arav *et al.*, 1993; Asada *et al.*, 2000). Moreover, the cell cycle stage during meiosis may influence both survival and cryopreservation results due to a varying sensitivity to the cooling procedure. Accordingly, oocyte cryopreservation could be performed at two meiotic stages: germinal vesicle (GV) and metaphase II (MII). As the genetic material in GV oocytes remains confined within the nucleus and protected by a membrane, it is probably less sensitive to cooling when compared with MII (Tucker *et al.*, 1998; Ghelter *et al.*, 2006; Bogliolo *et al.*, 2007). Unfortunately the broad difference in size between the oocyte and its associated cumulus cells in COCs yields distinct

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responses to stress during cryopreservation (Ruppert-Lingham *et al.*, 2003). However, the survivability of immature oocytes, as well as their latter maturation and development are significantly impaired after cryopreservation in comparison with fresh ones (Bogliolo *et al.*, 2007).

Different lines of evidence have demonstrated that GV-stage oocytes that have been stripped of cumulus cells show a lack of coordination between nuclear and cytoplasmic maturation (Goud *et al.*, 2000; Combelles *et al.*, 2002) and undergo a lower development compared with cumulus-enclosed oocytes (Wongsrikeao *et al.*, 2005), thus gap functional coupling between oocytes and cumulus cells plays an important role in the maturation process (Gilchrist *et al.*, 2004; Li *et al.*, 2006). Accordingly, the success of immature oocyte cryopreservation could depend on the ability to preserve the structural and functional integrity of a COC as a whole.

It is obvious that oocyte quality determination by phase contrast microscopy is not an adequate measure of oocyte fertilization potential and developmental competence (Swain & Pool, 2008), so evaluation of the cryopreservation effects on oocyte quality by using transmission electron microscopy (TEM) seems essential and could be effective. TEM allows observation of the fine details of cell microanatomy and suprisingly it has not been used in the cryopreservation field very much. This inattention exists even though structural damage is one of the most likely adverse events associated with cryopreservation and its concomitant osmotic stress (Coticchio *et al.*, 2010).

For the ultrastructural aspect, a constant association between mitochondria and smooth endoplasmic reticulum (SER) is observed at all stages of follicular development in most mammalian oocytes (Cran *et al.*, 1980) and this aggregation serves different functions such as: providing a rich pool of energy reserves, the production/secretion of substances (nutrients, growth factors) useful for fertilization, and rapid neogenesis of plasma and nuclear membranes during the first embryo cleavages (Motta *et al.*, 1995, 2000, 2003). Also, mitochondria–SER aggregates may regulate local concentrations of free calcium and ATP production, thus acting on different cellular calcium including mediation of calcium-dependent signal transduction pathways at fertilization (Makabe *et al.*, 2006). In sheep, hooded mitochondria appear during late estrous (Russe, 1975) and clearly increase their surface area. They may also provide a specific microenvironment to facilitate the exchange of metabolic intermediates with the endoplasmic reticulum (Fleming & Saake, 1972; Cran *et al.*, 1980). It has been demonstrated that cryopreservation affects the pericortical distribution of mitochondria and smooth endoplasmic reticulum. These organelles are associated with the cytoskeleton

and cryopreservation might cause their displacement, by directly affecting the associated cytoskeleton elements or merely through physiological forces that break their links with the cytoskeleton (Gualtieri *et al.*, 2009). Swelling of the smooth endoplasmic reticulum elements might also be observed after cryopreservation. Such damage is markedly higher if mitochondria are also injured (Kline & Kline, 1992; Whitaker, 2006).

Another ultrastructural characteristic of ovine oocytes is the presence of numerous large vesicles. In sheep, the number of vesicles increases during the early stage of follicular development. Information regarding their chemical nature or their derivation is lacking. Small vesicular elements of endoplasmic reticulum are often observed among them and profiles suggestive of fusion or budding of the large vesicles are also noted. However, no clear intermediates between endoplasmic reticulum and the vesicles have been observed (Cran *et al.*, 1980). The presence of vacuoles after vitrification can be considered as a non-specific feature commonly found in cells that are responding to an injury (Ghadially, 1982) and is probably the most apparent characteristic in human oocytes when routinely examined by microscopy (Ebner *et al.*, 2005). Oocyte vacuolization causes fertilization failure (both IVF and ICSI) and rare cleavage (Ebner *et al.*, 2005).

Generally, limited numbers of studies have focused on the effects of low temperatures on the oocyte ultrastructure, and little is known about ultrastructural modifications of sheep oocytes following different methods of vitrification. As a result, the object of this study was to evaluate the effects of three different vitrification methods on the ultrastructural changes of sheep COCs.

Materials and methods

Collection of immature oocytes

Sheep ovaries were transported in phosphate-buffered saline (PBS) supplemented with gentamycin (0.5%), streptomycin (0.05 mg/ml) and penicillin (0.06 mg/ml) at 35–37 °C within 2–4 h from the slaughter house. COCs were aspirated from antral follicles (2–6 mm in diameter) with a 19-gauge needle fitted to a 5 ml syringe. After aspiration, COCs were washed three times in HEPES-buffered TCM199 (Tissue Culture Medium 199, Sigma) supplemented with 10% fetal bovine serum (FBS, HyClone). The mean diameter of good isolated COCs (fine homogenous granular ooplasm surrounded with compact layers of cumulus cells) was evaluated (Ebrahimi *et al.*, 2010). These COCs were chosen for the experiment and randomly

divided into four groups: control, conventional straw vitrification, cryotop vitrification, and solid surface vitrification.

Vitrification procedure

COCs in each of the vitrification groups were vitrified by one of three different methods: conventional straws, cryotop and solid surface. Equilibration and vitrification were performed at room temperature (25–27 °C). COCs were first equilibrated for 15 min in a solution of 7.5% ethylene glycol (EG, Sigma), 7.5% dimethyl sulphoxide (DMSO, Sigma) and 20% FBS in α-minimal essential medium (MEM) (Gibco), then immersed in a vitrification medium for 1 min. The vitrification medium consisted of 15% EG, 15% DMSO, 0.5 M sucrose and 20% FBS in α-MEM. Thereafter, in the conventional group, 10 COCs were loaded into conventional straws (0.25 ml) with about 2 cm of vitrification medium around the samples as described by Chen *et al.* (2001); whereas in the cryotop groups, five COCs were loaded on a cryotop tip (Kitazato, Japan; Kuwayama & Kato, 2000) with the minimum amount of vitrification medium as possible (about 1 μl). In the conventional and cryotop groups, devices were plunged directly in liquid nitrogen (LN_2). In the last group, a modified method of solid surface vitrification was applied by using a cryotop as a carrier. Cryotops were used according to the minimum essential volume rule and due to their success in the vitrification procedure in comparison with the other methods. In the solid surface group, after loading the COCs on a cryotop tip, they were first placed for 15 s on a cold steel surface that was previously cooled with LN_2 and then plunged in LN_2. Samples in all experimental groups were preserved in LN_2 for 1 week (Ebrahimi *et al.*, 2010).

Warming procedure

After storage in LN_2 for 1 week, the conventional straws were warmed by holding the frozen straw for 30 s in room air. The content of the straw was emptied into a central well dish containing α-MEM, 20% FBS and 1 M sucrose solution for 1 min. In the cryotop and solid surface groups, cryotop tips were placed in a central well dish containing the abovementioned solution. COCs were sequentially rehydrated in α-MEM and 20% FBS containing 0.5 and 0.25 M sucrose for 3–5 min in each, respectively, at room temperature and rinsed 5–10 times in α-MEM and 20% FBS (Ebrahimi *et al.*, 2010).

Viability assessment

Two hours after warming, COCs within all three vitrification groups were examined under the stereo-microscopy with ×20 magnification for survival determination. Viable COCs showed a fine homogenous granular ooplasm, no space was observed between the oolemma and ooplasm with healthy compact cumulus layers surrounding the oocyte. Degenerated COCs showed a dark ooplasm, which shrank in some cases, and a space was observed between the oolemma and ooplasm (Ebrahimi *et al.*, 2010). Experiments in each vitrified groups, were repeated eight times and compared with the non-vitrified control group.

Electron microscopy procedure

In all experiments, at least three healthy vitrified–warmed and non-vitrified COCs were randomly selected and washed three times in phosphate-buffered solution, then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. COCs were subsequently rinsed in the same buffer for 10 min and post-fixed with 1% osmium tetroxide in PBS for 3 h. Samples were washed again in PBS and dehydrated through ascending concentrations of ethanol. COCs were immersed overnight in propylene oxide and embedded in araldite resin (Taab). Resin blocks were sectioned using an ultra microtome (Leica UC6). Semithin sections (500 nm thick) were stained with toluidine blue and examined by light microscopy (Zeiss) and ultrathin sections (70 nm thick) were cut with a diamond knife, mounted on copper grids and stained with alcoholic uranyl acetate and aqueous lead citrate. They were examined and photographed using a TEM-900 electron microscopy (Zeiss).

Statistical analysis

Percentages of healthy COC were confirmed by the Kolmogorov–Smirnov normalization test and statistical analysis was done using one-way analysis of variance (ANOVA) and post Tukey's test. Data were expressed as mean ± SE. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Post warming viability rate

Two hours post warming the mean percentage of viable COCs in the cryotop ($77.97 \pm 2.43\%$) and solid surface ($71.13 \pm 1.79\%$) groups were significantly higher than the conventional straw ($56.40 \pm 4.88\%$) group ($p < 0.05$). Better survivability was seen in the cryotop in comparison with the solid surface group but no significant difference was detected.
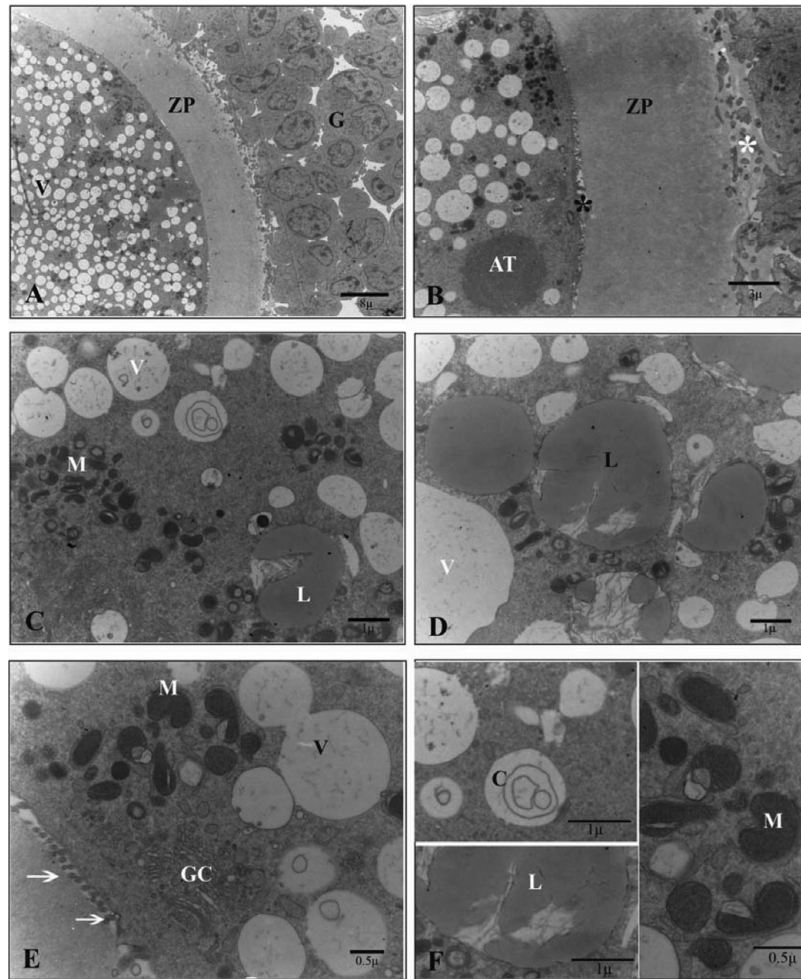
Figure 1 Ultrastructure of the oocyte of the sheep COCs in the control group. (*A*) G, cumulus cell; ZP, zona pellucida; V, vesicle. (*B*) AT, aggregation of tubules; *, cumulus cells projections (white) and microvilli (black); ZP, zona pellucida. (*C*) M, mitochondrion; L, lipid droplet; V, vesicle. (*D*) L, lipid droplet; V, vesicle. (*E*) GC, Golgi complex; M, mitochondrion; V, vesicle; arrow, microvilli. (*F*) C, concentric cristae M, mitochondrion; L, lipid droplet.

Ultrastructural changes of healthy COCs

Control group

Ultrathin sections of the oocytes in the control group showed numerous small vesicles that were distributed throughout the ooplasm. Vesicles were regular and membrane bound. Some lipid droplets were found in the vesicles that had microfilamentous structures within. Large amounts of round, ellipsoid and hooded mitochondria and well developed Golgi complexes were also observed between the vesicles. In the periphery of the ooplasm, a round structure that seemed to be the aggregation of smooth endoplasmic reticulum tubules was occasionally seen. Oolemma had noticeable microvilli in the perivitelline space (Fig. 1).

Moreover, compact cumulus layers around the oocyte revealed different types of junctional complexes. There was a clear small space between its inner layer and zona pellucida. This space was occupied by some pedicle-like process of cumulus cells. However, these processes extended, in some part, into the homogenous zona pellucida. Cumulus cells also had a large nucleus with heterochromatin periphery and a cytoplasm that included lipid droplets, endoplasmic reticulum, centrioles and mitochondria (Fig. 2).

Conventional straw group

Ultrathin sections of the oocytes in the conventional straw as compared with the control group showed the extreme space between their oolemma and zona pellucida. Microvilli decreased in the perivitelline space and disorganized ooplasm contained clumped mitochondria that possessed matrixes with different electrodensities that, in some cases, were swollen. Lipid droplets and constructed lipid droplets in the vesicles were obvious in the ooplasm. Large irregular connected vesicles were apparent, some of them seemed to be ruptured and invaded by other
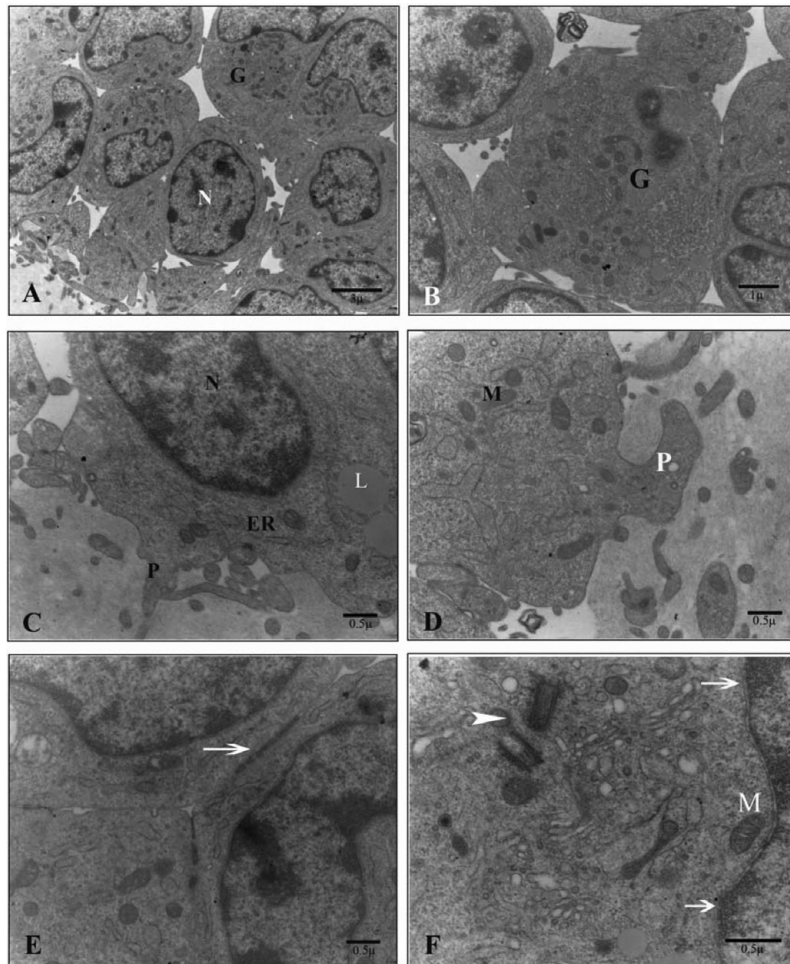
Figure 2 Ultrastructure of the cumulus cells of sheep COCs in the control group. (*A*) G, cumulus cell; N, nucleus. (*B*) G, cumulus cell. (*C*) P, pedicle like projection; ER, endoplasmic reticulum; L, lipid droplet; N, nucleus. (*D*) M, mitochondrion; P, pedicle like projection. (*E*) arrow, cell junctions; arrowhead, perpendicular centrioles. (*F*) arrow, cell junctions; arrowhead, perpendicular centrioles; M, mitochondrion.

cytoplasmic organelles. In some areas the oolemma appeared to be ruptured after this kind of vitrification (Fig. 3).

Although junctional complexes were preserved between cumulus cells, however there was an increase in the spaces between them, particularly between their inner layers and the zona pellucida. Moreover, the zona pellucida lost its homogeneity and cumulus cell projections decreased and in most cases, retracted from the zona pellucida. The cytoplasm of surrounding cumulus cells had different kinds of organelles with large amounts of vacuoles (Fig. 4).

Cryotop group
Ultrathin sections of the oocytes in the cryotop group as compared with the control group showed an extended perivitelline space with numerous microvilli. However, in the well preserved ooplasm, some different sized regular and membranous vesicles,

numerous lipid droplets and large amounts of round, ellipsoid and hooded mitochondria, as seen in the control group, were detected (Fig. 5).

Moreover, similar to the control group, different types of junctional complexes were located between compact cumulus layers, and numerous cell projections in the space between their inner layer and homogenous zona pellucida without any cracks were apparent. In the cytoplasm of cumulus cells, different types of organelles, such as mitochondria, centrioles, endoplasmic reticulum, some vacuoles and lipid droplets were observed. Occasionally, degenerated cells were detected among the healthy cumulus cells (Fig. 6).

Solid surface group

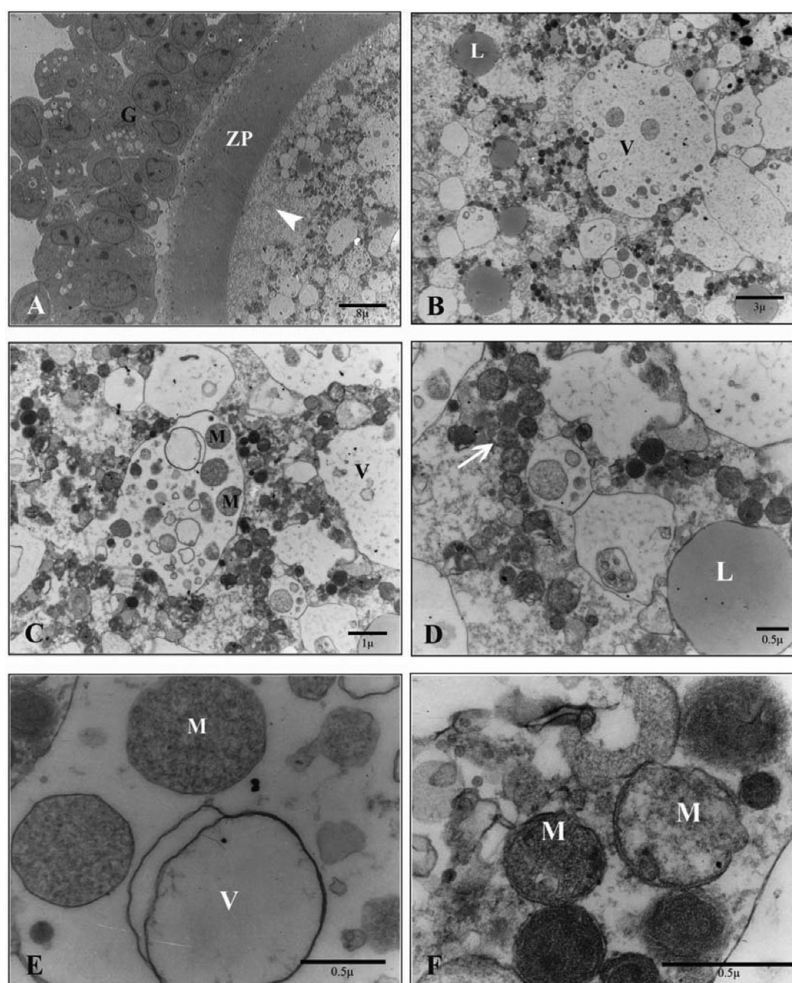Ultrathin sections of the oocytes in the solid surface group as compared with the control group revealed a

Figure 3 Ultrastructure of the oocytes of vitrified–warmed sheep COCs in the conventional straw group. (*A*) G, cumulus cell; ZP, zona pellucida; arrowhead, ruptured oolemma. (*B*) L, lipid droplet; V, irregular large vesicle. (*C*) Completely disorganized ooplasm: M, mitochondrion; V, irregular large vesicle. (*D*) Arrow, distended or ruptured mitochondrion; L, lipid droplet. (*E*) Disorganized ooplasm: M, mitochondrion; V, irregular large vesicle. (*F*) M, distended or ruptured mitochondrion.

similar perivitelline space with numerous microvilli. In the ooplasm, however, instead of the normal distribution of mitochondria, batched groups of round, ellipsoid and hooded mitochondria were seen. These mitochondria expanded in some cases. In this group, as found in the cryotop vitrification group, the number of small membranous regular well-preserved vesicles increased but they remained independent and did not show any connections. However, no homogenous distribution of numerous lipid droplets was obvious (Fig. 7).

Moreover, different types of junctional complexes between cumulus cells and decreased cumulus cell projections in the space between their inner layer and zona pellucida were seen. Cracks were seen in some parts of the zona pellucida. Within the cumulus cells' cytoplasm, some vacuoles, lipid droplets and other organelles such as mitochondria, centrioles and

endoplasmic reticulum were observed. Degenerated cells were also noted between the healthy cumulus cells. Also, it must be noted that the outer surface of the zona pellucida became rough in some places (Fig. 8).

Discussion

The most important characteristics of fresh sheep immature oocytes are large amounts of mitochondria, vesicles and lipid droplets distributed throughout the ooplasm (Reader, 2007). In immature oocytes, the Golgi complexes are more developed and consist of many dilated lamellae associated with membranous vesicles of variable electrodensity (Hyttel *et al.*, 1989; Grondahl *et al.*, 1995). In this study, we examined
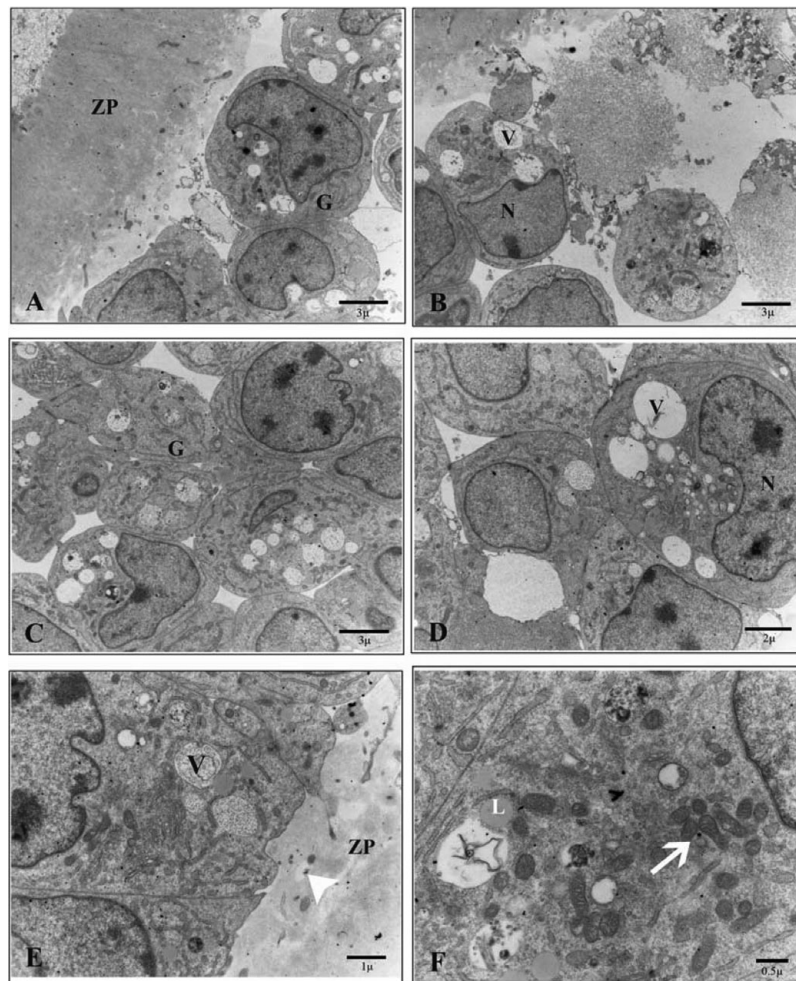
Figure 4 Ultrastructure of the cumulus cells of vitrified–warmed sheep COCs in the conventional straw group. (*A*) G, cumulus cells; ZP, zona pellucida. (*B*) V, vacuoles; N, nucleus. (*C*) G, cumulus cells. (*D*) N, nucleus; V, vacuoles. (*E*) Arrowhead, non-uniformity in zona pellucida; V, vacuoles; ZP, zona pellucida. (*F*) L, lipid droplet; arrow, mitochondrion.

the ultrastructural changes of sheep COCs following three different methods of vitrification. This research was limited to a population of obviously good quality COCs by phase contrast microscopy. In fact only cryopreserved oocytes that appeared normal and viable after thawing were routinely selected for further analysis.

It has been demonstrated that osmotic damage to cytoplasmic membranes and membranes enveloping intracellular organelles cause various ultrastructural changes after pronuclear and MII oocyte freezing (Hyttel *et al.*, 2000; Isachenko *et al.*, 2001, 2003, 2004; Rho *et al.*, 2002). These ultrastructural changes in MII stage oocytes include the appearance of cracks in the zona pellucida (Fuku *et al.*, 1995a), zona hardening by premature release of cortical granules (Pickering *et al.*, 1990; Vincent *et al.*, 1990), disruption of the plasma membrane, destruction of the meiotic spindle (Park *et al.*, 1997), peripheral migration of

cortical granules (Schalkoff *et al.*, 1989; Fuku *et al.*, 1995b), extensive disorganization of the ooplasm, mitochondria and cytoskeleton (Fuku *et al.*, 1995b; Diez *et al.*, 2005), loss of microvilli (Hyttel *et al.*, 1989; Asada *et al.*, 2000) and loss of oocyte–corona cell attachments (Hyttel *et al.*, 1986; Hochi *et al.*, 1996; Asada *et al.*, 2000). Some of these alterations such as zona hardening (Johnson *et al.*, 1988), plasma membrane destruction (Eroglu *et al.*, 1998), cracks in the zona pellucida (Fuku *et al.*, 1995a) and ooplasm disarrangement (Diez *et al.*, 2005) are also seen in the GV stage of cryopreserved oocytes. Moreover, the sight of numerous cytoplasmic vacuoles in most of the surrounding cumulus cells, disorganization of actin filaments within the transzonal process of the cumulus cells through which they establish physical contact with the oocyte and interruption of the cumulus cells´ projections is also common after vitrification of COCs (Younis *et al.*, 1996).
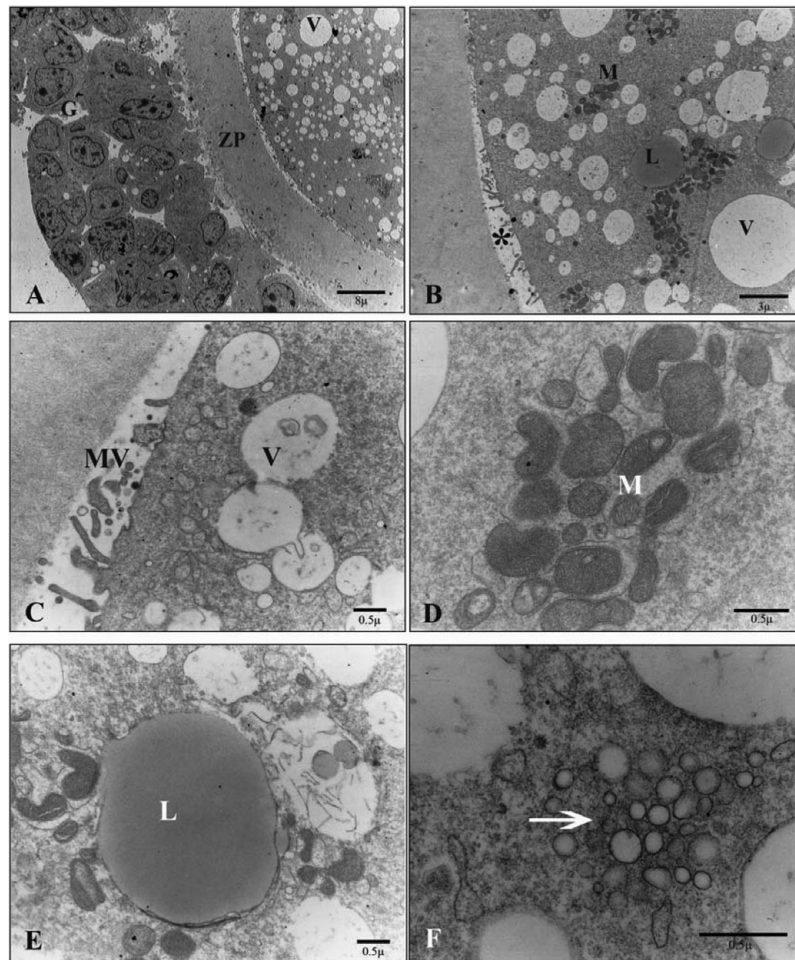
Figure 5 Ultrastructure of the oocyte of vitrified–warmed sheep COCs in the cryotop group. (*A*) G, cumulus cells; V, vesicles; ZP, zona pellucida. (*B*) M, mitochondrion; L, lipid droplet; *, perivitelline space; V, vesicles. (*C*) MV, microvilli; V, vesicles. (*D*) M, aggregation of mitochondrion. (*E*) L, lipid droplet. (*F*) Arrow, aggregation of vesicles.

Additionally, Yang *et al.* (1994) have suggested that the developmental capacity of cryopreserved immature bovine oocytes is impaired because of severe ultrastructural damage.

According to some research, there are small membrane-bound vesicles and in some cases, a rim of both small and large vesicles located underneath the oolemma after vitrification with the solid surface and open pulled straw (OPS) methods (Hyttel *et al.*, 2000). Vacuolization is a non-specific response to cryopreservation that threatens latter maturation and development of the oocyte (Ebner *et al.*, 2005). However, we observed vacuolization phenomena after conventional straw vitrification. In the latter group, the amount of large irregular vesicles was considerable and they completely connected to each other to form new large vacuoles. Not only in this group, but also in the two other vitrification groups, surrounding cumulus cells showed some vacuoles in their cytoplasm. Cryotop vitrification had the best condition amongst the groups regarding this variant.

According to the origin of the vesicles, Van Blerkom (1990) has proposed that vesicles in ooplasm may arise either spontaneously or by fusion of pre-existing vesicles derived from the smooth endoplasmic reticulum and are usually filled with a fluid that is virtually similar to perivitelline fluid. In agreement with his proposal, in our study, some vesicles were filled with structures resembling intermediate filaments and in some of them, lipid droplets were constituted on these filaments. Therefore, we assumed that they were the sites for lipid droplet formation. Another supposition is that these vesicles are the remnants of lipid droplets that washed out during the transmission electron microscopy preparation procedure. However more investigation regarding the nature of the vesicles or the filaments within them is still needed.
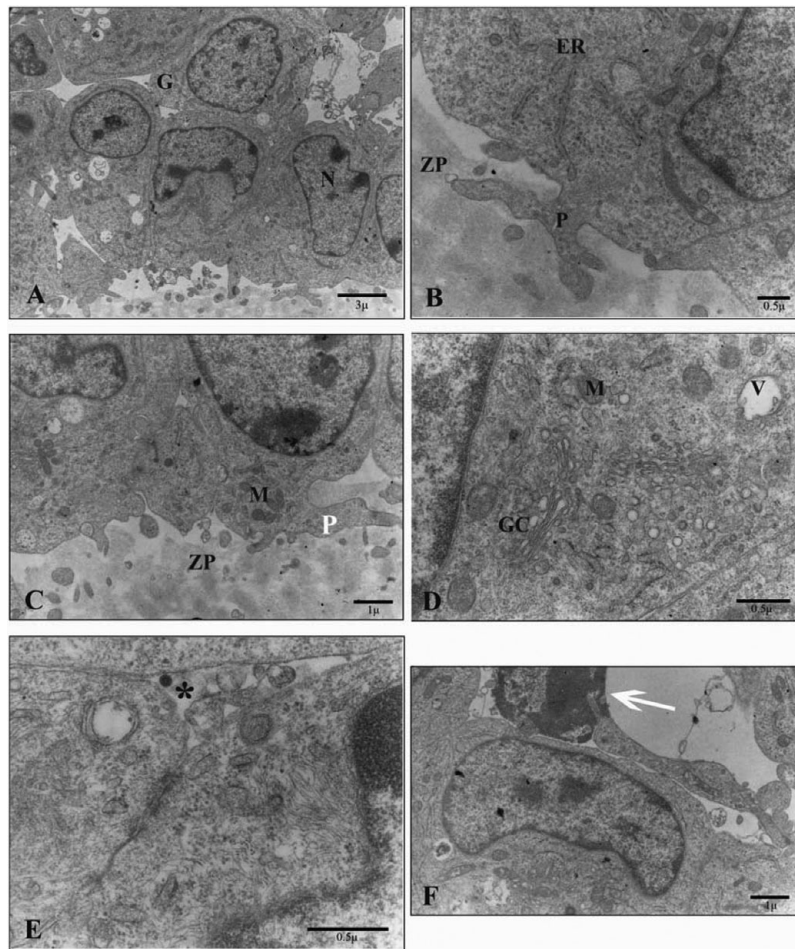
Figure 6 Ultrastructure of cumulus cells of vitrified–warmed sheep COCs in the cryotop group. (*A*) G, cumulus cells; N, nucleus. (*B*) ZP, zona pellucida; ER, endoplasmic reticulum; P, pedicle-like process. (*C*) M, mitochondrion; P, pedicle-like process; ZP, zona pellucida. (*D*) GC, Golgi complex; M, mitochondrion; V, vacuoles. (*E*) *, cumulus cell membrane expansions. (*F*) arrow, degenerated cumulus cell.

Moreover, previous research has shown that the viability of immature ovine oocytes reduced after cryotop vitrification. This finding could result from an extensive loss of cumulus cell plasma membrane integrity as well as from a drastic reduction in gap junction communication between the oocytes and surrounding cells during cryopreservation procedures (Bogliolo *et al.*, 2007). Our results showed that cryotop vitrification did not affect plasma membrane integrity of cumulus cells or cause severe changes in the gap junction communications between oocytes and surrounding cumulus cells. However, conventional straw vitrification brought about severe changes in the gap junction communications between cells and caused retraction of the cumulus cells' process from the zona pellucida in a way that the space between the inner layers of cumulus cells and zona pellucida increased. The latter event was seen to a certain degree in the solid surface vitrification group, too.

On top of that, Hochi *et al.* (1996) showed that vitrification induced some ultrastructural changes such as: swelling of the mitochondria associated with reduced matrix density and the presence of vacuoles in the periphery of the ooplasm. Also, induction of ultrastructural modifications in microvilli and mitochondria as well as vacuole formation in the ooplasm of cattle germinal vesicle oocytes were reported by Fuku and colleagues (1995b). In addition, some researchers have shown that elongated mitochondria with coarse surfaces and small drops of cracked lipid droplets appeared in the oocytes after vitrification (Fu *et al.*, 2009). Cryodamage targets mitochondria functionality and it has been shown that the rate of oocytes with an abnormal mitochondrial distribution increased after vitrification (Rho *et al.*, 2002; Dumollard *et al.*, 2007; Shi *et al.*, 2007; Fu *et al.*, 2009; Gualtieri *et al.*, 2009).

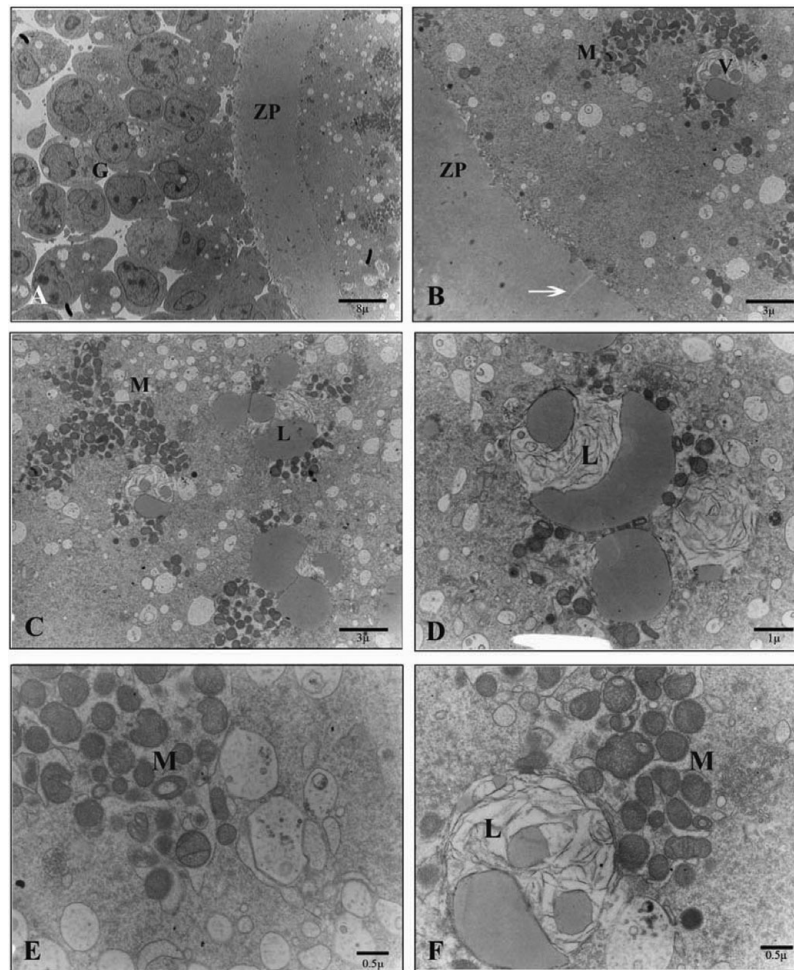In this study, mitochondria batched around lipid droplets after vitrification except in the cryotop

Figure 7 Ultrastructure of the oocyte of vitrified–warmed sheep COCs in the solid surface group. (*A*) G, cumulus cells; ZP, zona pellucida. (*B*) V, vesicles; M, mitochondrion; arrow, crack in zona pellucid; ZP, zona pellucida. (*C*) L, lipid droplet; M, mitochondrion. (*D*) L, lipid droplet. (*E*) M, mitochondrion. (*F*) L, washed or constructing lipid droplet in a vesicles; M, mitochondrion.

group. A reduction in mitochondrial matrix density, mitochondrial swelling and rupture of some of the mitochondrial membranes were only seen in the conventional straw vitrification group.

On the other hand, Valojerdi & Salehnia (2005) demonstrated the sensitivity of core actin filaments of microvilli to cryopreservation and a reduction in microvilli abundance after vitrification. Correspondingly, it was shown that sheep oocyte microvilli decreased in some areas after solid surface and conventional straw vitrification methods.

In agreement with previously mentioned results, the cryotop vitrification method approximately preserved the integrity of sheep COCs as found in the non-vitrified control group, but the conventional straw vitrification method completely destroyed the organization of the ooplasm, the surrounding cumulus cells and the gap junction connection between them. According to the survivability assays, the viability

rates of vitrified–warmed COCs in the cryotop and solid surface groups were higher than the conventional straw group. When considering the resemblance of the vitrification procedure in the conventional and cryotop groups, this extreme difference could be explained as a result of the small amount of the vitrification medium that was used in the cryotop group that accelerated the cooling speed. Additionally, as in the solid surface vitrification method, the same carrier and the same volume of vitrification medium as in the cryotop vitrification group was employed, therefore some deficiencies observed in the solid surface group could be related to the delayed cooling speed of this procedure due to the use of nitrogen vapour instead of the liquid nitrogen.

In conclusion the cryotop vitrification method did not modify the ultrastructure of healthy vitrified–warmed oocytes and can be used for the
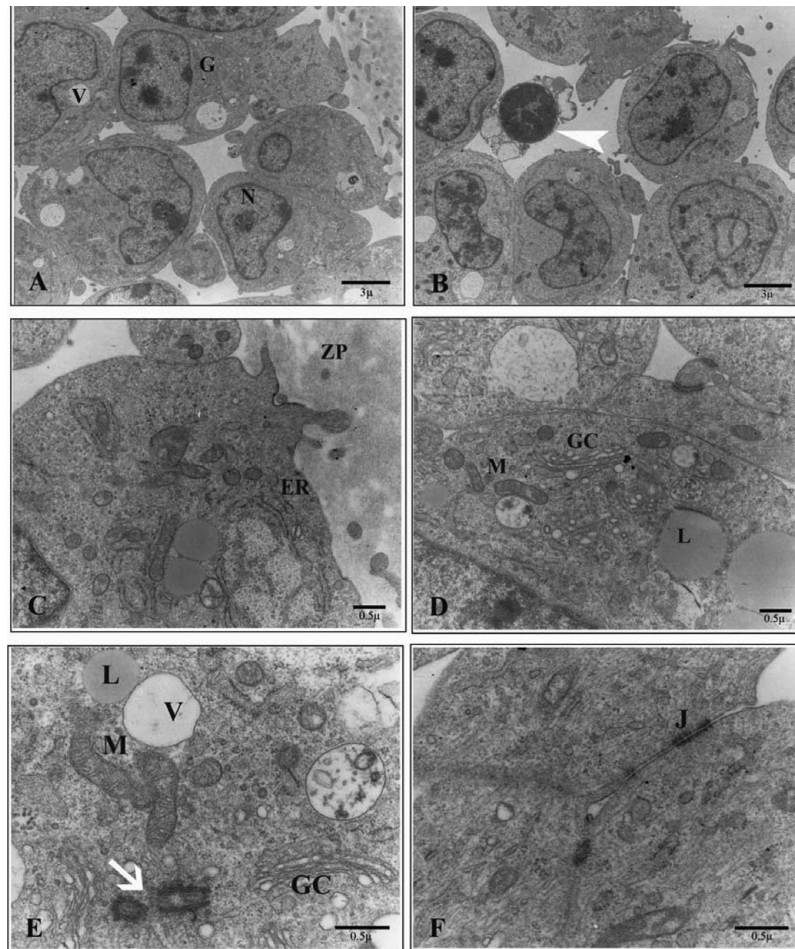
Figure 8 Ultrastructure of the cumulus cells of vitrified–warmed sheep COCs in the solid surface group. (*A*) G, cumulus cells; V, vacuoles; N, nucleus. (*B*) Arrowhead, degenerated cumulus cell. (*C*) ZP, zona pellucida; ER, endoplasmic reticulum. (*D*) M, mitochondrion; L, lipid droplet; GC, Golgi complex. (*E*) Arrow, centrioles; GC, Golgi complex; L, lipid droplet; M, mitochondrion; V, vacuoles. (*F*) J, Junctional complex.

cryopreservation of sheep COCs. However more research about the maturation and developmental capacity of these oocytes is necessary in order to determine the best procedure.

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