Effect of roscovitine and cycloheximide on ultrastructure of sheep oocytes

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ABSTRACT

It is believed the temporary meiosis arrest with roscovitine or cycloheximide may improve the *in vitro* developmental competence of oocytes in different animal species. However, little is known about the effects of these inhibitors on ultrastructure of ovines *cumulus*–oocyte complexes (COCs). The aim of this study was to evaluate the progression of cytoplasmic maturation and the ultrastructural changes in sheep COCs exposed to roscovitine or cycloheximide, at acceptable concentrations. COCs were *in vitro* cultured for 24 h in maturation medium (control group) containing 100 μM roscovitine or 1 μg/mL cycloheximide (treatment groups). After this time, some COCs were cultured for further 22 h in inhibitor-free medium. The ultrastructure organization of COCs was evaluated by transmission electron microscopy before (immature group) and after *in vitro* culture for 24 and 46 h. As expected, signs of immaturity and maturity were observed in immature and control groups, respectively. In treatment with roscovitine, there were *cumulus* cells degeneration, swelling of mitochondrias, few cortical granules and many vesicles with electron-dense material. However, in cycloheximide treatment there were not signs of degeneration or cellular senescence. Metabolic units and mitochondrial pleomorphism were found in all experimental groups. These evidences demonstrate that roscovitine promoted irreversible ultrastructural changes while cycloheximide did not affect the cytoplasmic maturation. However, the implications on embryo development are still unclear.

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1. Introduction

The efficiency of *in vitro* embryo production (IVP), the development and survival of embryo, the establishment and maintenance of pregnancy and the fetal development depend on the oocyte quality. This oocyte developmental competence is acquired during folliculogenesis and is directly related to nuclear and cytoplasmic maturation (Krisher, 2004).

The process by which the oocyte completes the first meiotic division and progresses to metaphase II is known as nuclear maturation (Mehlmann, 2005). However, the correct dynamic of chromosome segregation in itself is not enough to ensure subsequent embryo development. The oocyte also needs to undergo ultrastructural modifications to attain the full developmental competence. These cytoplasmic changes include mRNA transcription, protein translation and organelles redistribution (Meirelles et al., 2004). In this process of oocyte maturation, the bidirectional communication between the oocyte and its surrounding *cumulus* cells is of vital importance. Through the gap junctions, *cumulus* cells keep the oocyte under meiotic arrest, inducing the meiosis resumption and supporting

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the cytoplasmic maturation (Dadashpour Davachi et al., 2012).

In most mammals, oocytes are kept at the germinal vesicle stage by follicular factors until the preovulatory LH surge. During this meiotic arrest, oocytes undergo morphological and biochemical changes to achieve developmental competence (Hyttel et al., 1997). *In vitro*, the resumption of meiosis is spontaneously induced by the removal of *cumulus*–oocyte complexes (COCs) from follicular environment (Pincus and Enzmann, 1935), impairing the oocyte capacitation and resulting in lower rates of embryo development (Rizos et al., 2002).

The temporary arrest of meiosis with pharmacological or physiological inhibitors has been proposed in order to allow adequate progression of cytoplasmic maturation and consequently to improve the quality of *in vitro* matured oocytes (Le Beux et al., 2003). Roscovitine, a specific inhibitor of cyclin-dependent kinases and cycloheximide, a protein synthesis inhibitor have been extensively studied in different animal species (bovine: Albarracin et al., 2005; Takayama et al., 2006; pig: Coy et al., 2005; Ye et al., 2005; goat: Han et al., 2006; cat: Sananmuang et al., 2010). However, there is no information about the effects of these inhibitors on nuclear maturation and ultrastructure of ovines COCs.

In addition, despite of the reports of efficiency and reversibility of meiosis arrest, there is discrepancy between authors regarding the subsequent embryonic developmental potential and little is known about the morphological changes in COCs exposed to these meiosis inhibitors. Thus, in the present study, we evaluated the characteristics, distribution and ultrastructure changes of cytoplasmic organelles and *cumulus* cells in ovines COCs exposed, for 24 h, to either roscovitine or cycloheximide, followed by *in vitro* culture for additional 22 h in inhibitor free medium.

2. Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma (Sigma–Aldrich Corp., St. Louis, MO, USA).

2.1. COCs collection and meiotic inhibition

Sheep ovaries were collected at slaughter and transported to the laboratory in sterile saline solution (0.9% NaCl) at 35 °C. All visible follicles were aspirated with a 20-gauge needle attached to a 10 mL syringe containing 0.5 mL pre-incubated TCM 199 HEPES supplemented with 50 IU/mL heparin (Shirazi and Sadeghi, 2007). Groups of 20 COCs grades I and II, with more than two layers of compact *cumulus* cells and homogeneous ooplasm (Morton et al., 2005), were cultured in 90 μL droplets of maturation medium (control group) containing 100 μM roscovitine or 1 μg/mL cycloheximide (treatment groups), under mineral oil, for 24 h at 38.5 °C in a 5% CO_2 saturated humidity air atmosphere. The maturation medium was comprised of TCM 199 with Earle's salts supplemented with 0.1 IU/mL FSH (Folltropin®, Bioniche Co.); 0.1 IU/mL LH (Lutropin-V®, Bioniche Co., Belleville, ON, Canada), 0.3 mM sodium pyruvate, 75 μg/mL penicillin, 10% fetal calf serum and 100 μM cysteamine. Stock solutions of 1 g/L roscovitine and 20 mg/L cyclohexemide were prepared in dimethylsulphoxide and in TCM 199, respectively, aliquoted and stored at −20 °C until use.

2.2. Reversion of meiotic inhibition

After meiotic inhibition for 24 h, COCs were washed several times in TCM 199 HEPES for removal of inhibitors and cultured, for a further 22 h,

in maturation medium without inhibitors at 38.5 °C in a 5% CO_2 saturated humidity air atmosphere.

2.3. Transmission electron microscopy (TEM)

The COCs were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline solution (pH 7.4) for 24 h, and post-fixed in 1% osmium tetroxide. After dehydration in acetone, COCs were embedded in blocks of Epon resin and were kept for 3–4 days in an incubator at 60 °C for polymerization. The blocks of Epon were serially semithin-sectioned (1–2 μm) and the semi-thin sections were stained with toluidine blue and evaluated under light microscope. After evaluation, the selected semi-thin sections were ultra-thin sectioned with diamond blade, mounted on cooper grids and contrasted with uranyl acetate and lead citrate for analysis in a transmission electron microscope. The COCs were evaluated according the distribution and characteristics of organelles and *cumulus* cells.

2.4. Experimental design

Sheep COCs, grades I and II, were randomly divided into seven groups:

(1) *Immature*: immediately after the harvest, COCs were fixed for TEM.
(2) *Control 24 h*: COCs fixed after 24 h of *in vitro* culture in inhibitor-free medium.
(3) *Roscovitine*: COCs fixed after 24 h of *in vitro* culture with 100 μM roscovitine.
(4) *Cycloheximide*: COCs fixed after 24 h of *in vitro* culture with 1 μg/mL cycloheximide.
(5) *Control (46 h)*: COCs fixed after 46 h of *in vitro* culture in inhibitor-free medium.
(6) *Roscovitine reversibility*: COCs treated with roscovitine for 24 h were fixed after *in vitro* culture for additional 22 h in inhibitor-free medium.
(7) *Cycloheximide reversibility*: COCs treated with cycloheximide for 24 h were fixed after *in vitro* culture for additional 22 h in inhibitor-free medium.

Each experimental group consisted of 100 COCs, approximately, which were obtained in 5 replicates for each group performed on different days. All COCs were prepared as described in Section 2.3. The ultra-thin sections were randomly selected and about 10 COCs per group were analyzed in transmission electron microscope.

3. Results

3.1. Group (1): immature COCs

COCs fixed immediately after harvest showed immaturity signs characterized by the presence of several layers of compact *cumulus* cells (Fig. 1A); cytoplasmic projections emitted by *cumulus* cells which cross the zona pellucid and form communications with the oocyte through gap junctions (Fig. 1B); narrow perivitelline space and cytoplasmic membrane with few and small microvilli (Fig. 1A). Clusters of pleomorphic mitochondria were located mainly at the oocyte periphery (Fig. 1C) but some of them were also uniformly distributed by throughout the ooplasm. The mitochondria were rounded or elongated and some of them had an arc-like projection (Fig. 1C). Large amount of smooth endoplasmic reticulum (SER) and lipid droplets were observed either as isolated aggregations throughout the ooplasm or as complex associations with mitochondria, constituting the metabolic or functional units (Fig. 1C). Well developed Golgi complexes (Fig. 1C) with many dilated lamellae associated with small vesicles were detected mainly in the oocyte periphery. Few clusters of spherical and heterogeneous cortical granules (Fig. 1C) were scattered throughout the ooplasm and associated with Golgi complexes. Vesicles with different
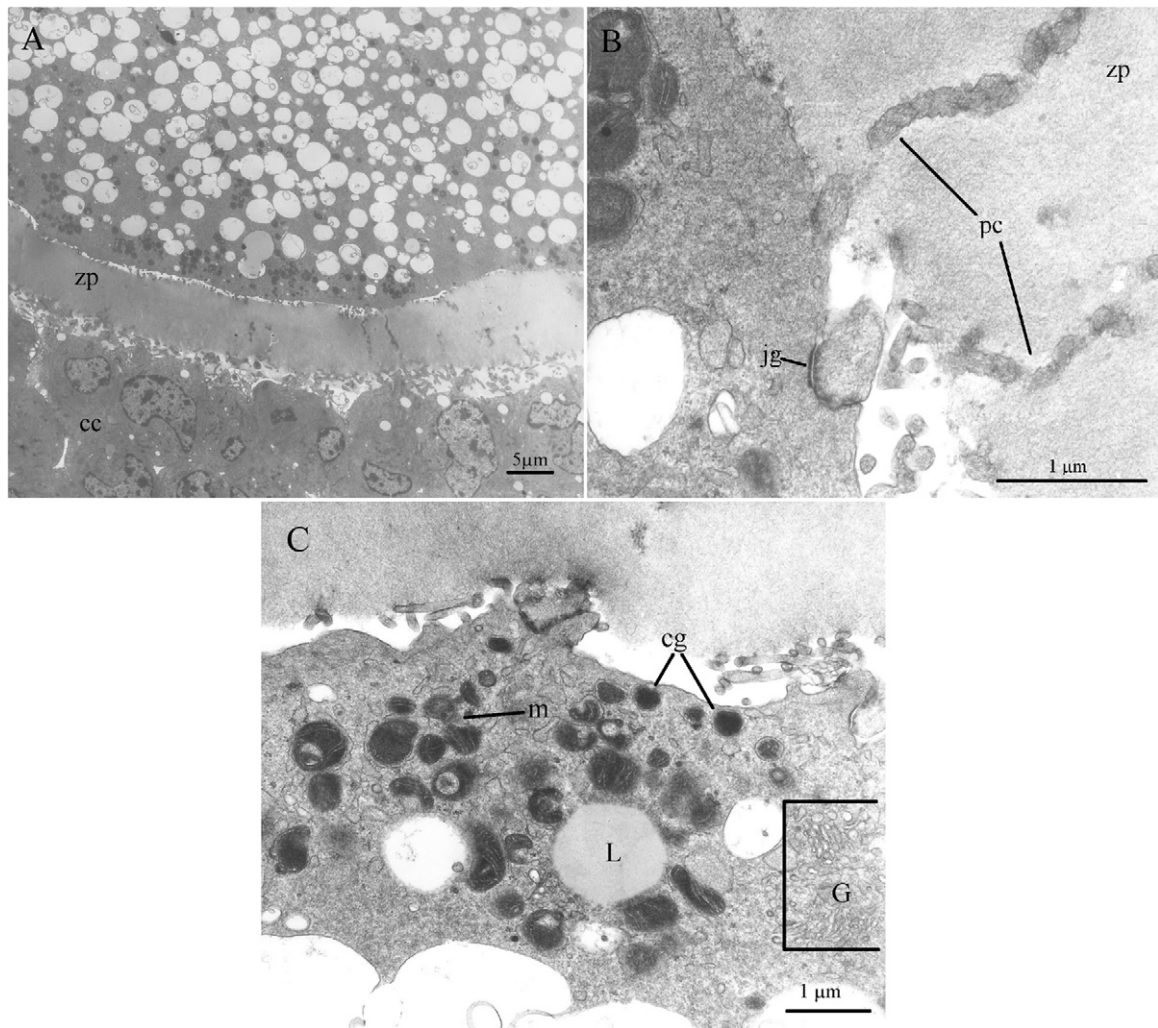
Fig. 1. Immature sheep COCs (group 1). (A) Note the compact *cumulus* cells (cc), narrow perivitelline space, the large amount of lipid droplets and vesicles spread throughout the ooplasm; (B) detail of the *cumulus* cells projections (pc) through the zona pellucid (zp) and gap junctions (jg); (C) clusters of mitochondria (m) rounded, elongated or with an arc-like projection located mainly in the ooplasm periphery. Some of them in association with lipid droplets (L), forming metabolic units. Well developed Golgi complex (G) and few cortical granules (cg) heterogeneous in size and coloration can also be observed.

sizes and electrodensity and some myelin figures were also found.

3.2. Group (2): control 24 h

COCs fixed after 24 h of IVM in inhibitor-free medium showed maturity signs characterized by full expansion of the *cumulus* cells (Fig. 2A); retraction of cytoplasmic projections emitted by *cumulus* cells (Fig. 2A), with some of them remained in the perivitelline space, and disjunction of junctional complexes (Fig. 2B). Perivitelline space was more developed and there were several microvilli on the surface of the cytoplasmic membrane (Fig. 2B). Many pleomorphic mitochondria (Fig. 2C), with the same aspect from those of immature COCs, were spread throughout the ooplasm. There were few lipid droplets (Fig. 2C) but many vesicles and myelin figures (Fig. 2C). As in immature COCs, SER was either as isolated aggregations or as complex association with mitochondria and lipid droplets. There was substantial reduction of quantity and size of Golgi complexes. Large amount of homogeneous cortical granules were isolated and aligned with the cytoplasmic membrane (Fig. 2B).

3.3. Group (3): roscovitine

COCs treated with roscovitine showed some characteristics of maturity and immaturity as: partial *cumulus* expansion (Fig. 3A); retraction of *cumulus* cells projections, some of them visible within the perivitelline space, and substantial reduction of junctional complexes (Fig. 3B). As well as in group 2, the perivitelline space was well developed (Fig. 3B) and numerous pleomorphic mitochondria were spread throughout the ooplasm (Fig. 3C). But, cytoplasmic membrane had few microvilli (Fig. 3B) and the mitochondria seemed to be swollen and whit lesser electrodensity. Some of them had mitochondrial granules
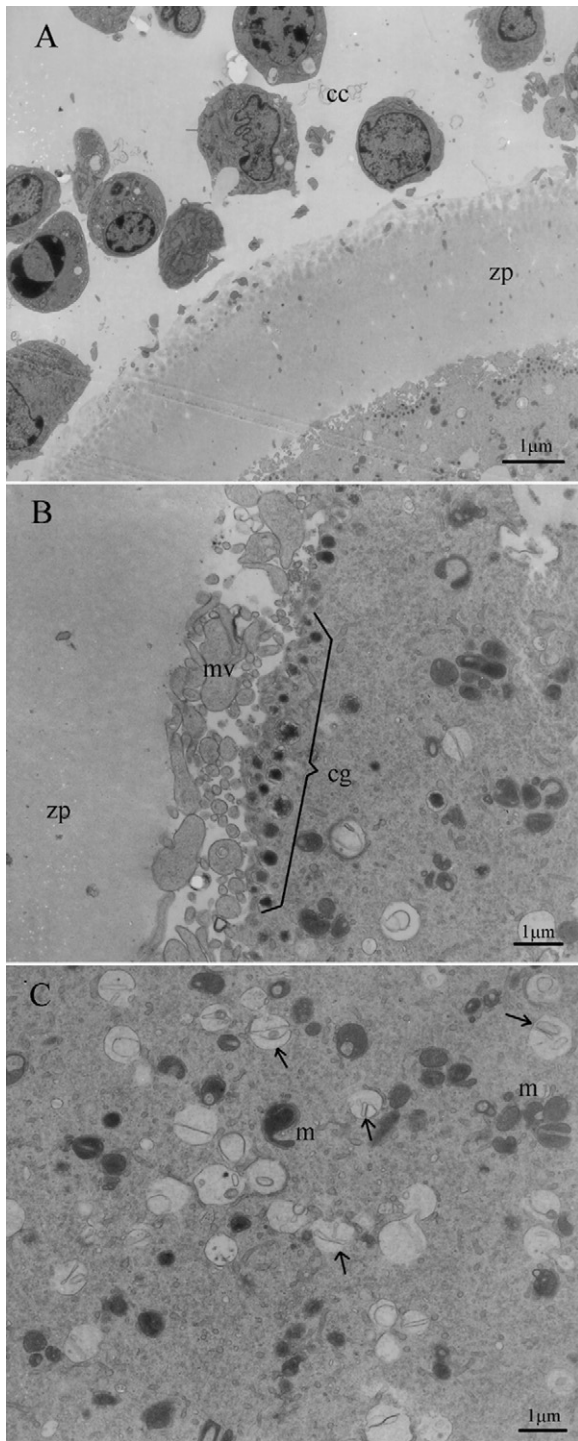
Fig. 2. Sheep COCs *in vitro* cultured for 24 h without inhibitors (group 2). (A) Note the full expansion of the *cumulus* cells (cc) and the absence of projections through the zona pellucid (zp); (B) observe the large amount of cortical granules (cg) in close contact with the cytoplasmic membrane, the absence of junctional complexes, the perivitelline space well developed and filled by microvilli (mv); (C) detail of the ooplasm: pleomorphic mitochondria (m), few lipid droplets, many vesicles and myelinic figures (arrows) can be observed spread throughout the ooplasm. COCs treated for 24 h with cycloheximide (group 4) showed these same characteristics.
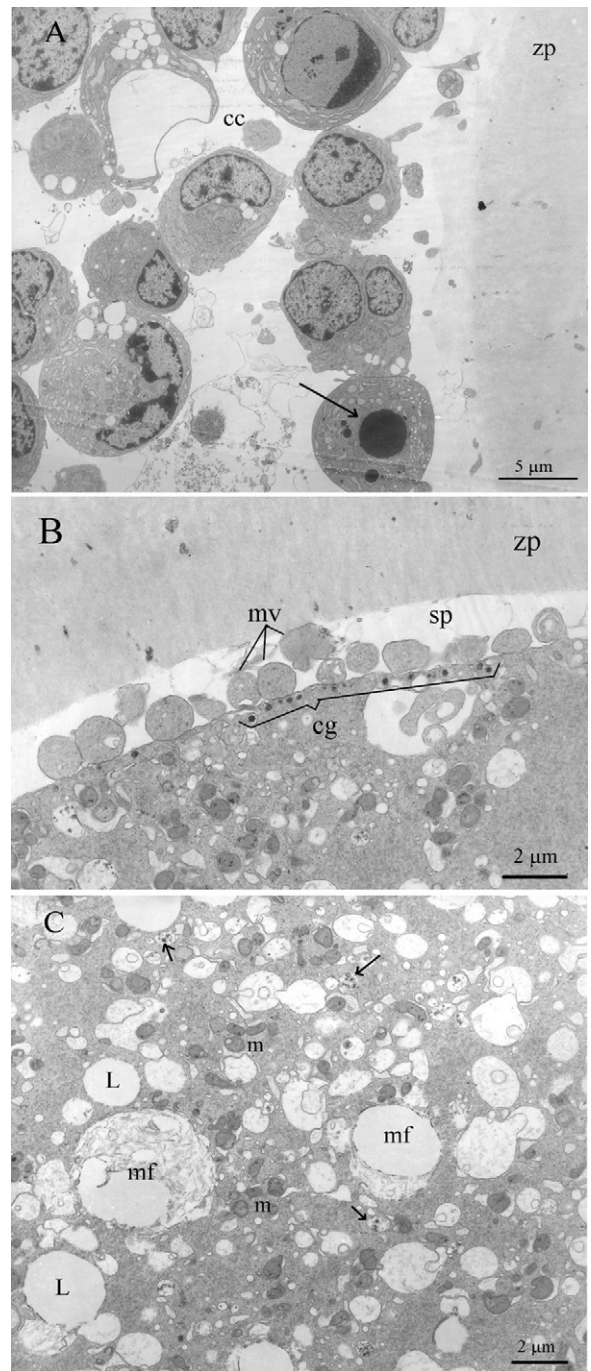
Fig. 3. Sheep COCs treated for 24 h with roscovitine (group 3). (A) Note the partial expansion of the *cumulus* cells and the degeneration signs as nucleus pyknotic (arrow); (B) few cortical granules (cg) in close contact with the cytoplasmic membrane, perivitelline space well developed and few microvilli (mv) can be observed. *Cumulus* cells projections through the zona pellucid (zp) and the junctional complexes are absent; (C) detail of the ooplasm: note the pleomorphic mitochondria (m) and large amount of lipid droplets (L), myelinic figures and vesicles with different size and electrodensity spread throughout the ooplasm. Mitochondria seemed to be swollen, whit lesser electrodensity and granules.
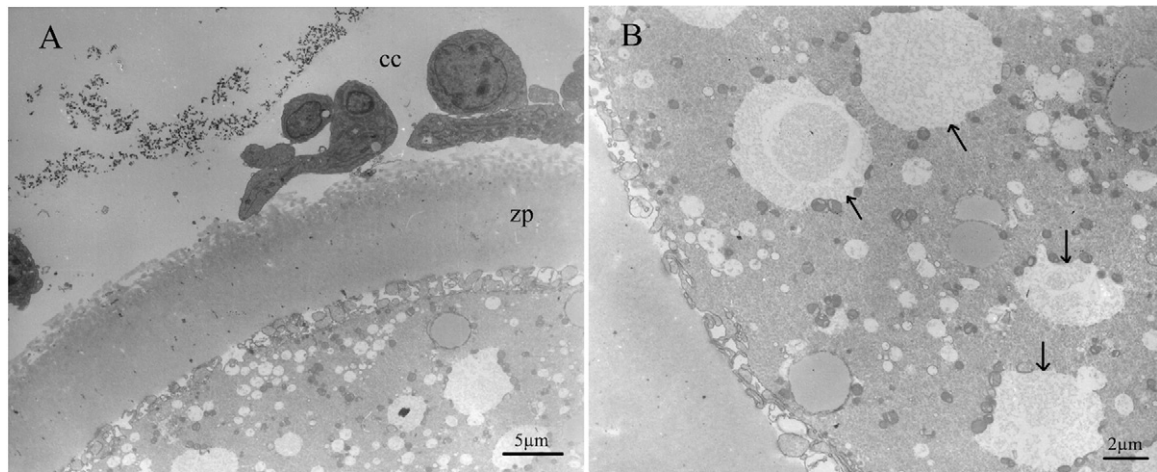
Fig. 4. Sheep COCs *in vitro* cultured for 46 h without inhibitors (group 5). (A) Observe the full expansion, dispersion and loss of *cumulus* cells (cc); (B) detail of the areas of cytoplasmic matrix absence (arrows) that represent degeneration signs in the ooplasm. COCs treated with roscovitine and then *in vitro* culture for additional 22 h in inhibitor-free medium (group 6) showed these same characteristics.

(Fig. 3C). There were many lipid droplets and vesicles with different size and electrodensity, as in immature COCs (Fig. 3C). SER was either associated with mitochondrias or dispersed in the ooplasm. There were more myelin figures than in group 2. Golgi complexes were not found and there were few cortical granules in close contact with the cytoplasmic membrane (Fig. 3B). Signs of degeneration, such as pyknotic nucleus were identified in *cumulus* cells (Fig. 3A), but not in the ooplasm and organelles.

3.4. Group (4): cycloheximide

COCs treated with cycloheximide had the same characteristics of maturity observed in the group (2) (Fig. 2A–C).

3.5. Group (5): control 46 h

COCs fixed after 46 h of *in vitro* culture in inhibitor-free medium revealed maturity signs similar to COCs of

group (2). However, besides the full *cumulus* expansion, there were dispersion and substantial loss of cells (Fig. 4A). Moreover, there were few myelin figures and dilated SER in association with mitochondria and lipid droplets. Evidences of cellular senescence and degeneration were observed in the ooplasm, such as areas of cytoplasmic matrix absence (Fig. 4B), and in *cumulus* cells, characterized by pyknotic nucleus.

3.6. Group (6): roscovitine reversibility

The ultrathin section of COCs revealed characteristics of maturity and immaturity similar to those observed in the group (3) (Fig. 3A–C). However, as in the group (5), there were full expansion, dispersion and substantial loss of *cumulus* cells (Fig. 4A). Degeneration signs were also detected in the *cumulus* cells and ooplasm (Fig. 4B).
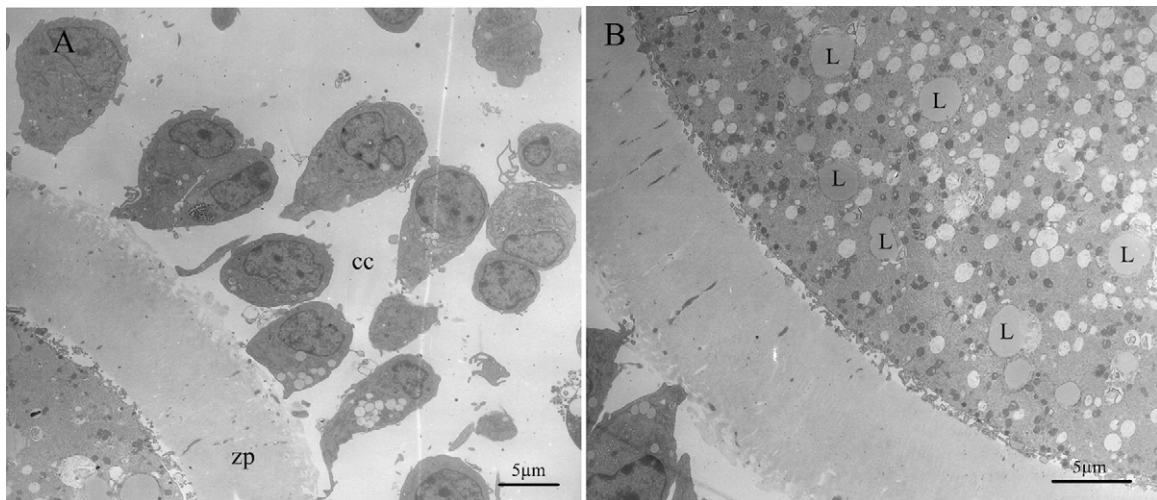

Fig. 5. Sheep COCs treated with cycloheximide and then *in vitro* culture for additional 22 h in inhibitor-free medium (group 7). (A) Detail of the full *cumulus* (cc) expansion without substantial loss of cells; (B) note the large amount of lipid granules (L) and vesicles spread throughout the ooplasm.

3.7. Group (7): cycloheximide reversibility

As in the group (2), signs of maturity were detected (Fig. 2A–C). However, in contrast to group (5), there was not substantial loss of *cumulus* cells (Fig. 5A) and degenerations signs were not observed in the *cumulus* cells and ooplasm. Despite of maturity evidences, large amount of lipid droplets and vesicles were observed throughout the ooplasm (Fig. 5B).

4. Discussion

Meiosis inhibitors have been used to improve the *in vitro* embryo production efficiency in different animal species (bovine: Albarracin et al., 2005; Takayama et al., 2006; pig: Coy et al., 2005; Ye et al., 2005; goat: Han et al., 2006; cat: Sananmuang et al., 2010). However, there is no much information about the effect of these inhibitors on the COCs ultrastructure. Here, we report the morphological changes in sheep COCs *in vitro* cultured with roscovitine and cycloheximide, at acceptable concentrations.

The signs of immaturity and maturity observed in COCs analyzed before (group 1) and after (group 2) the *in vitro* culture for 24 h in inhibitor-free medium, respectively, are consistent with the expected and with the observations of earlier workers. It is assumed that the *in vitro* culture conditions were adequate and allowed the progression of cytoplasmic maturation. These findings are similar with the others mammalian species, like bovine (Nagano et al., 2006); ovine (O'Brien et al., 2005); equine (Alvarenga, 2006) and pig (Cran, 1985).

The studies with meiosis inhibitors in cattle propose a period of 24 h for meiotic inhibition followed by 18–24 h for resumption and progression of meiosis (Adona and Leal, 2004; Albarracin et al., 2005). This experimental model was carried out because there were no studies conducted with meiotic inhibitors in sheep. But, the evidences of cellular senescence, such as degeneration signs in the ooplasm and *cumulus* cells, after *in vitro* culture for 46 h in inhibitor-free medium, indicate that this period was excessive. According to some researchers, the adequate time for IVM of sheep COCs varies from 24 h (Shi et al., 2009) to 26–27 h (Shirazi and Sadeghi, 2007). Máximo et al. (2011) also reported that 18 h was enough for the oocyte to complete maturation.

According to Isobe et al. (1998), the meiotic resumption is directly related to the disruption of gap junctions between the *cumulus* cells and the oocyte. Thus, it is believed that the partial *cumulus* expansion and presence of some junctional complexes in the COCs treated for 24 h with roscovitine (group 3) are directly related to the efficiency of meiotic arrest promoted by this inhibitor, since the *cumulus* cells are involved in the regulation of oocyte maturation. However, roscovitine treatment also resulted in *cumulus* cells degeneration and substantial loss of cells after *in vitro* culture for additional 22 h in inhibitor-free medium. In bovine COCs, Lonergan et al. (2003) also reported absence of *cumulus* expansion, premature loss and degeneration of *cumulus* cells even after *in vitro* culture in absence of roscovitine.

In contrast, treatment with 1 μg/mL cycloheximide did not interfere in *cumulus* cells, which were fully expanded and without degeneration signs at the end of *in vitro* culture for 24 and 46 h. However, there was disjunction of junctional complexes. These findings differ from Saeki et al. (1997) who reported absence of expansion, degeneration and dispersion of *cumulus* cells in bovine COCs after *in vitro* culture for 48 h with 10 μg/mL cycloheximide.

This divergence of results involves many factors like: the action mode and concentration of inhibitors; the time of *in vitro* culture with inhibitors and the animal species under study. It is noteworthy that remains unclear whether the effect of these inhibitors on *cumulus* cell occurs *via* the oocyte or directly *via* the *cumulus* cells (Schoevers et al., 2005).

According to Stojkovic et al. (2001), mitochondria are responsible for the supply of energy that will be consumed during the oocyte maturation and embryo development. So, the number and the movement of these organelles to the areas with high energy consumption are crucial for oocyte and embryo during the critical periods of development. In this study, mitochondria migrated from peripheral position in immature COCs (group 1) to a more disperse distribution throughout the ooplasm in mature COCs (group 2), as described by Hyttel et al. (1986).

The mitochondrial migration was not impaired by both meiosis inhibitors studied, but the roscovitine treatment for 24 h affected the electron density of mitochondria and caused the swelling of them as reported by Lonergan et al. (2003). These changes were not reversible even after *in vitro* culture for additional 22 h in inhibitor-free medium.

In all experimental groups there was mitochondrial pleomorphism which was also described by Senger and Saacke (1970), in bovine, Lucci et al. (2001), in goat, and Máximo et al. (2011) in ovine oocytes. According to Fair et al. (1995), the round shape of mitochondria indicates immaturity. With the progression of oocyte maturation, they become elongated. Moreover, the presence of arc-like projection increases the mitochondrial surface and may be related with high oocyte metabolism during the maturation (Máximo et al., 2011).

Functional or metabolic units were also observed in this study. These structures are involved with the metabolism of lipid and synthesis of lipoproteins, nutrients and growth factors during the critical periods of oocyte development, fertilization and neogenesis of membranes in the first embryonic cleavages (Motta et al., 2000).

As expected, during the oocyte maturation, there was reduction in the number of lipid droplets and increase of the myelin figures, which represent digestive vesicle responsible for metabolism of lipid and degradation of aged and non-functional cellular structures (Kacinskis et al., 2005). The large amount of lipid droplets observed in COCs treated with cycloheximide at the end of *in vitro* culture for 46 h probably corresponds to an individual characteristic of examined oocytes. It is noteworthy that, in this study, ovaries were randomly collected at the slaughterhouse, regardless of physiological female conditions.

The vesicles with different sizes, electrodensity and content observed in all experimental groups are common in sheep oocytes (Cran et al., 1980). However, the nature and functional significance of these vesicles are still unknown.

They appear to be involved with storage and transportation of metabolites (Motta et al., 2000).

As describe by Hyttel et al. (1989), well developed Golgi complexes were found only in immature COCs (group 1) and, after *in vitro* maturation for 24 h (group 2), the cortical granules were in close contact with the cytoplasmic membrane forming a monolayer strategically to prevent polyspermy. In comparison, COCs treated with roscovitine had reduction in the number of cortical granules and increase of electron-dense material in their vesicles, after *in vitro* culture for 24 and 46 h.

5. Conclusion

These findings demonstrate the treatment with 100 μM roscovitine resulted in harmful and irreversible changes in oocytes and *cumulus* cells. While the treatment with 1 μg/mL cycloheximide did not impair cytoplasmic maturation of sheep COCs which showed maturity signs without ultrastructure changes and degeneration signs. However the implications on embryo development remain to be elucidated.

Acknowledgement

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