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Gene expression in sheep *cumulus*-oocyte complexes meiotically inhibited with roscovitine

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ABSTRACT

The majority of mammalian oocytes destined for *in vitro* maturation (IVM) have not undergone all molecular and structural changes necessary for competence acquisition to support the fertilization and early embryogenesis. In this context, different methods able to provide a transient arrest of meiosis resumption have been tested in order to improve the *in vitro* developmental potential of oocytes. Based on that, our study aimed to evaluate the effect of temporary meiosis inhibition using roscovitine on gene expression in sheep oocytes and *cumulus* cells. For this, *cumulus*-oocyte complexes (COCs) were cultured for 6 h in modified TCM199 medium with (Rosco) and without (Control) 75 μM roscovitine. Subsequently, they were *in vitro* matured for a further 18 h in inhibitor-free TCM199 medium supplemented with gonadotropins. At 0, 6 and 24 h of culture, nuclear status of oocytes and expression of selected genes were evaluated by Hoescht staining and qRT-PCR, respectively. The analysis of oocyte chromatin organization revealed that roscovitine efficiently inhibited the meiosis of sheep oocytes for 6 h and its action was completely reversed after 18 h of *in vitro* maturation in inhibitor-free medium. Besides, no detrimental effect on *cumulus* expansion was observed. The expression profile of most investigated genes in *cumulus* cells (PTX3, GREM1, GLUT1, PTGS2, ALK5, ALK6) and oocytes (ZAR1, NLRP5, SOD1, BMP15, GDF9) was similar between Control and Rosco treatments and the ratio BCL2/BAX was maintained in both cell types even in the presence of roscovitine. These results indicate that reversible meiotic arrest promoted by roscovitine, at the concentration and exposure time studied, neither impaired nor improved the expression of investigated genes in sheep oocytes and *cumulus* cells. Moreover, the efficiency of temporary meiotic arrest and the absence of deleterious effect on COCs suggest that roscovitine provides a useful method for transportation or manipulation of sheep oocytes at onset of maturation. However, further investigations are necessary to evaluate the benefits of roscovitine treatment on *in vitro* development of sheep embryos and its effects on cellular ultrastructure.

1. Introduction

Mammalian oocytes enter into meiosis during the intrauterine life and remain arrested at diplotene stage of prophase-I until near ovulation. In response to preovulatory surge of LH, meiotic division resumes and proceeds to metaphase II (Mehlmann, 2005). During this diplotene arrest, oocytes display an enlarged nucleus, called germinal vesicle, which contains lampbrush chromosomes composed of regions with side loops of decondensed and transcriptionally active chromatin (Andraszek and Smalec, 2011). So, oocytes at germinal vesicle stage are able to produce and store all mRNAs and proteins required (Sirard, 2001).

With meiosis resumption, however, this transcriptional activity is interrupted, due to chromatin condensation, and restored only with embryonic genome activation when the embryo begins to transcribe its own mRNAs. Therefore, the processes of oocyte maturation, fertilization and pre-implantation development depend on maternal mRNAs synthesized and stored during oogenesis (Brevini-Gandolfi and Gandolfi, 2001; Sirard, 2001). In the course of

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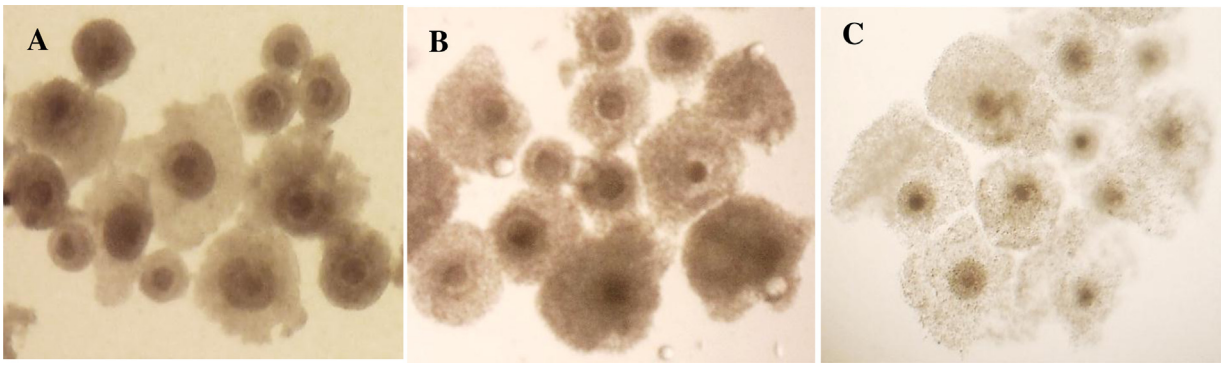

Fig. 1. Degree of *cumulus* cells expansion in sheep COCs evaluated under a stereomicroscope. (A) Absence of expansion. (B) Partial expansion. (C) Total expansion (×100 magnification).

acquiring competence to resume and complete the meiosis, oocytes also undergo important structural changes related to the morphology and distribution of organelles (Ferreira et al., 2009). All these events that characterize the nuclear and cytoplasmic maturation are controlled by low molecular weight peptides transferred from *cumulus* cells to oocyte through Gap junctions. Likewise, many critical functions of granulosa cells are regulated by oocyte-secreted factors (Sugiura and Eppig, 2005).

In contrast, when oocytes are removed from their follicles and transferred to a suitable culture medium, meiosis spontaneously resumes regardless of the cytoplasmic maturation stage (Pincus and Enzmann, 1935). So, most oocytes destined to *in vitro* maturation have not yet undergone all molecular and structural changes necessary to competence acquisition (Gilchrist and Thompson, 2007). In this context, the temporary arrest of meiosis with cyclin-dependent kinase (CDK) inhibitors has been proposed as strategy to provide time enough for the oocyte to complete its capacitation (Mermillod et al., 2000; Han et al., 2006).

The roscovitine is a CDK inhibitor that competes for ATP-binding domain on catalytic subunit of M-phase promoting factor, preventing its activation (Meijer et al., 1997). The reversible meiotic arrest promoted by roscovitine and its effect on embryo development has been demonstrated in several animal species as bovine (Mermillod et al., 2000), goat (Han et al., 2006), cat (Sananmuang et al., 2010), pig (Romar and Funahashi, 2006) and horse (Franz et al., 2003). The action of this inhibitor on gene expression in oocytes and *cumulus* cells, however, has not been extensively investigated. Furthermore, no similar information has been reported in sheep COCs. Based on that, the present study aimed to evaluate the effect of temporary arrest of meiosis, using the CDK inhibitor roscovitine, on relative abundance of transcripts associated with developmental competence in sheep oocytes and *cumulus* cells.

2. Materials and methods

All chemicals used were purchased from Sigma Chemical Co. (Sigma–Aldrich Corp., St. Louis, MO, USA), unless otherwise indicated.

2.1. Collection of cumulus-oocyte complexes

Ovaries of adult sheep were collected at slaughterhouse and transported to laboratory within 1–2 h in sterile saline solution (0.9% NaCl) at 32 °C. All follicles with diameter of 2–6 mm were aspirated with a 20 gauge needle attached to 10 mL syringe containing 0.5 mL Hepes-buffered TCM199 (12340-030; Gibco, Invitrogen Co., USA) supplemented with 50 IU/mL heparin. Only COCs with several intact *cumulus* cell layers and homogeneous ooplasm were selected (Shirazi et al., 2010).

2.2. Meiosis inhibition and reversibility

After washes in Hepes-buffered TCM 199, the selected COCs were transferred to basic maturation medium composed of TCM 199 with Earle's salts (11150059; Gibco, Invitrogen Co., USA), 0.3 mM sodium pyruvate, 75 μg/mL penicillin, 10% fetal bovine serum (10437; Gibco, Invitrogen Co., USA) and 100 μM cysteamine (Control) added of 75 μM roscovitine (Rosco). About 25 COCs were cultured, for 6 h, in 100 μL droplets of medium placed in 96 well plates without oil overlay at 38.5 °C and 5% CO_2 in air. The stock solution of roscovitine (1 mg/mL) was prepared in dimethylsulphoxide, aliquoted and stored at −20 °C until use.

In order to enable the reversibility of roscovitine action, after 6 h of culture, COCs from Rosco treatment were washed in Hepes-buffered TCM 199 and *in vitro* matured, for a further 18 h, in basic maturation medium supplemented with 0.1 IU/mL FSH (Folltropin®, Bioniche Co., Belleville, ON, Canada) and 0.1 IU/mL LH (Lutropin-V®, Bioniche Co.). COCs from Control were submitted to this same procedure. The *in vitro* maturation was performed in 96 well plates without mineral oil overlay at 38.5 °C and 5% CO_2 in air. The inhibitor concentration and culture conditions were based on our preliminary studies (Crocomo et al., 2015a,b,c).

2.3. Assessment of cumulus expansion and oocyte nuclear status

Under a stereomicroscope, COCs were evaluated and classified according to the degree of *cumulus* expansion (Heidari Amale et al., 2011) as: total *cumulus* expansion (significant dispersion of all cells layers); partial *cumulus* expansion (subtle dispersion of outer cells layers); and absence of *cumulus* expansion (cells strongly adhered to each other and to the pellucid zone) (Fig. 1).

For evaluation of oocyte chromatin organization, *cumulus* cells were removed by repeated pipetting and denuded oocytes were transferred to droplets of Hoechst 33342 in glycerol (10 μg/mL) on a glass slide. Under a fluorescence inverted microscope (Leica® DMIRB), oocytes were examined and classified according to the stage of nuclear maturation (Shirazi et al., 2010) as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII). Those with altered nuclear structure were classified as degenerate (Deg) (Fig. 2).

2.4. RNA isolation and reverse transcription (RT)

Oocytes were completely stripped from their *cumulus* cells by repeated pipetting in phosphate-buffered saline (PBS). The PBS droplet containing *cumulus* cells were centrifuged for 5 min at 700 g and supernatant was removed. Finally, oocytes and *cumulus* cells were frozen at −80 °C with 350 μL of RNA extraction