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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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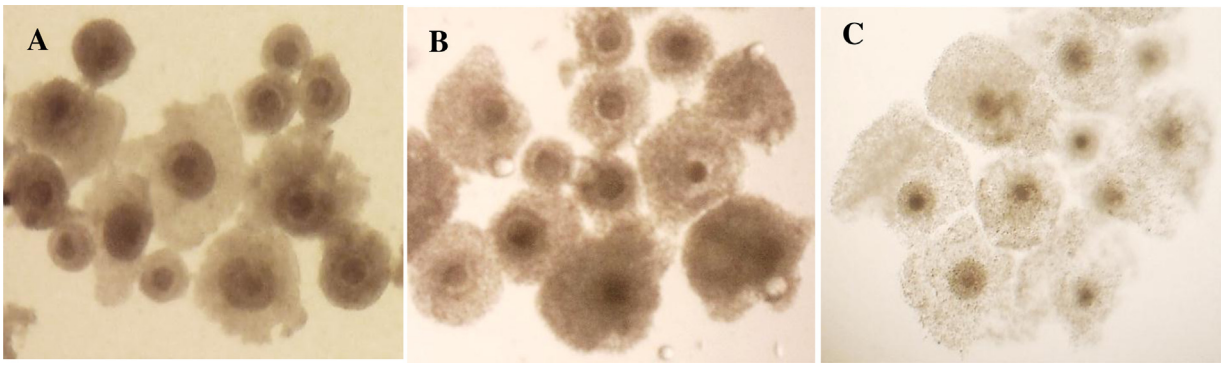

Fig. 1. Degree of *cumulus* cells expansion in sheep COCs evaluated under a stereomicroscope. (A) Absence of expansion. (B) Partial expansion. (C) Total expansion (×100 magnification).

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

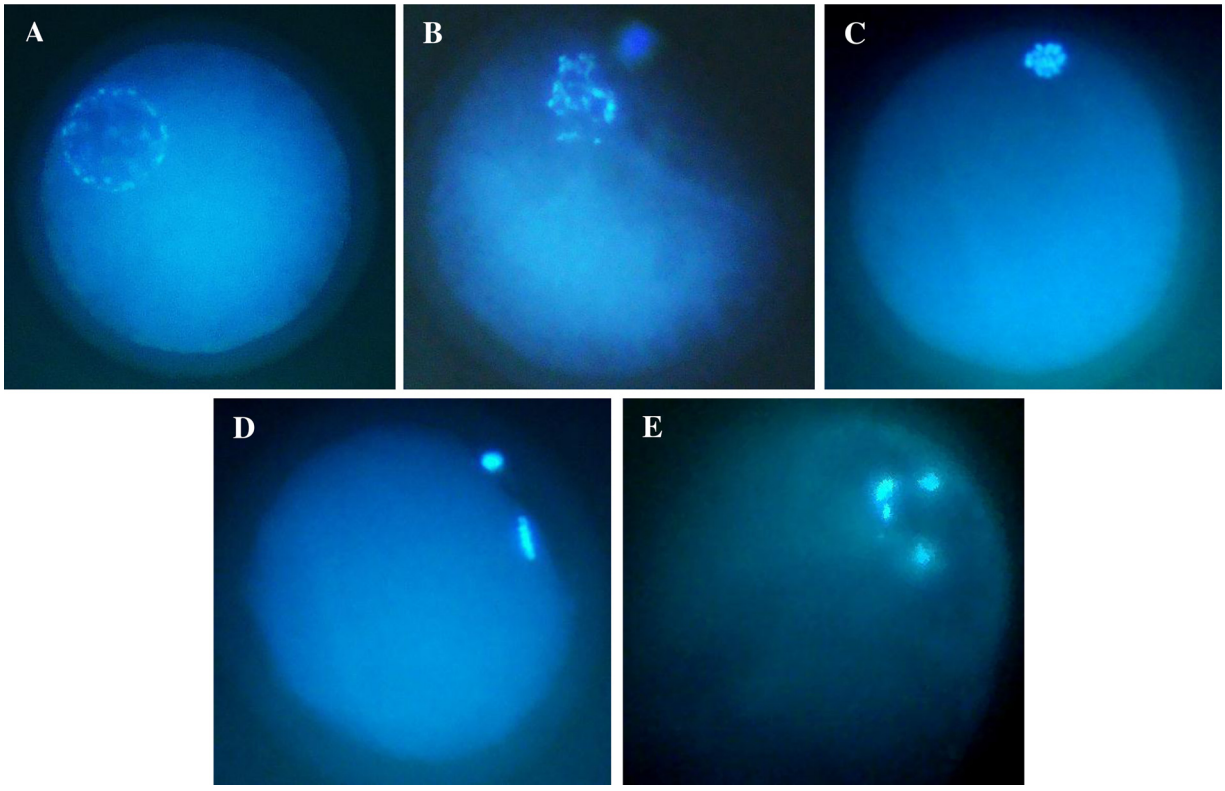

Fig. 2. Nuclear configuration in sheep oocytes stained with Hoechst 33342 and evaluated under a fluorescence inverted microscope: (A) GV: germinal vesicle; (B) GVBD: germinal vesicle breakdown; (C) MI: metaphase I; (D) MII: metaphase II; (E) Deg: degenerate (×200 magnification).

lysis buffer in their respective 1.5 mL sterile tubes. Total RNA was extracted from four pools of 25 oocytes and their corresponding *cumulus* cells using the RNeasy Mini Kit (Qiagen, Mississauga, ON, CA) according to manufacturer's instructions and eluted in 30 μL RNAse-free water. After incubation with DNase I (Invitrogen, São Paulo, Brazil), total RNA was reverse transcribed with Sensiscript and Omniscript RT Kit (Qiagen, Mississauga, ON, CA) for oocyte and *cumulus* samples, respectively, using Oligo-dT (Invitrogen) primers for both.

2.5. Relative quantification by real-time PCR

The expression of SOD1, NLRP5, ZAR1, BMP15 and GDF9 genes was evaluated in oocytes whereas the expression of GREML1, PTX3, GLUT1, PTGS2, ALK5 and ALK6 genes was analyzed in *cumulus* cells. The BAX and BCL2 genes were evaluated in both cell types. Real time RT-PCR analysis was performed using the ABI Prism 7500 Sequence Detection System with Power Sybr Green PCR Master Mix (Applied Biosystems). Reactions were performed in 25 μL volumes and PCR cycling conditions were 95 °C for 10 min followed by 40 cycles of denaturing at 95 °C for 10 s and annealing for 1 min. The primers sequences, fragment size and annealing temperatures for each gene are shown in Table 1.

Each sample was analyzed in duplicate, and the specificity of PCR products was assessed by melting curve analysis and amplicon size determination by electrophoresis in agarose 2%. Among three genes tested (ACTB, GAPDH, CYC-A) with geNorm program, Cyclophilin-A (CYC-A) was selected as the most stable housekeeping gene. The relative expression of each gene was calculated using the ΔΔCt method with efficiency correction (Pfaffl, 2001). Mean efficiency values for each gene were calculated from the amplification profile of individual samples with LinRegPCR software (Ramakers et al., 2003).

2.6. Experimental design

The experimental design was completely randomized with two experiments and five treatments (Immature, Control 6 h, Rosco 6 h, Control 6 + 18 h and Rosco 6 + 18 h) each one. The experiment 1 was developed to evaluate the efficiency of roscovitine to temporarily inhibit the meiosis of sheep oocytes. The experiment 2 aimed to analyze the effect of meiotic inhibition on relative abundance of mRNA in oocytes and *cumulus* cells.

2.6.1. Experiment 1 – meiosis inhibition and reversibility

At the end of 6 h culture, a sample of COCs from Control and Rosco was evaluated for *cumulus* expansion and nuclear maturation under a stereomicroscope and by Hoechst 33342 staining, respectively. Another sample of COCs was *in vitro* matured for a further 18 h in inhibitor-free medium and submitted to the same analysis cited above. Immature oocytes were stained soon after removal from follicle to evaluate the nuclear status before the culture (0 h).

2.6.2. Experiment 2 – gene expression analysis

After 6 h of culture in the absence (Control) and presence of roscovitine (Rosco) as well as after the *in vitro* maturation for a further 18 h in inhibitor-free medium, the oocytes and corresponding *cumulus* cells from each treatment were stored at −80 °C aiming the subsequent analysis of gene expression by qRT-PCR. An additional sample of immature oocytes and their *cumulus* cells was stored soon after removal from follicle (0 h).

2.7. Statistical analysis

The data of *cumulus* expansion and oocyte nuclear status were transformed into square root of $x + 0.5$ and subjected to analysis of variance (ANOVA) according to factorial system with five treatments (Immature, Control 6 h, Rosco 6 h, Control 6 + 18 h and Rosco

Table 1
Detail of primer sequences used for qRT-PCR in sheep *cumulus*-oocytes complexes.

Gene	Primer Sequences (5′–3′)	Cell analyzed	Annealing temperature (°C)	Size (pb)	Genbank Acession no.
CYC-A	F: 5′-GCTGGCCCCAACACAAACGG-3′ R: 5′-AGCGCTCCATGGCTTCCACA-3′	Oocyte/*cumulus*cells	58.5	127	AY251270.1
ZAR1	F: 5′-TATCCGCTGGGAAAGTGCCTATGT-3′ R: 5'-TGATATCCTCCACTCGGTAAGGGT-3′	Oocyte	59	116	HM037367.1
NLRP5	F: 5′-CTGACCTCTGAAGGCTGCAA-3′ R: 5′-GAACACAGCATCGCCATTCC-3′	Oocyte	59.5	116	XM_004015912.1
GDF9	F: 5′-GGCGCTTCCCAACAAATTCTTCCT-3′ R: 5′-TGGTTCAGCAAGGACCAAGTCTCA-3′	Oocyte	59.5	153	NM_001142888
BMP15	F: 5′-TAACCAGTGTTCCCTCCACCCTTT-3′ R: 5′-ATCCACCAGCTCACTGACAAGGTT-3′	Oocyte	60	184	NM_001114767.1
SOD1	F: 5′-CTGGGCAATGTGAAGGCTGACAAA-3′ R: 5′-TTTCCACCTCTGCCCAAGTCATCT-3′	Oocyte	60	143	FJ546075.1
BCL2	F: 5′-TTCGCCGAGATGTCCAGTCA-3′ R: 5′-TCCGAACTCAAAGAAGGCCACGAT-3′	Oocyte/*cumulus*cells	59	129	AY423861.1
BAX	F: 5′-TCTACTTTGCCAGCAAACTGGTGC-3′ R: 5′-AAGGAAGTCCAATGTCCAGCCCAT-3′	Oocyte/*cumulus*cells	60	92	AY609317.1
PTGS2	F: 5′-GCCCAGCACTTCACCCATCAATTT-3′ R: 5′-AAAGGCGACGGTTATGCTGTCTCT-3′	*Cumulus*cells	60	136	NM_001009432.1
PTX3	F: 5′-GTTTCAGTGCCTGCATTTGGGTCA-3′ R: 5′-TCTCTCCACCCACCACAAGCATTA-3'	*Cumulus*cells	60	141	AM492193.1
GLUT1	F: 5′-GCCTTCACTGTCGTTTCGCTGTTT-3′ R: 5′-CACAAAGCCAAAGATGGCCACGAT-3′	*Cumulus*cells	60	174	U89029.1
GREMLIN	F: 5′-CTGAAGCAGACCATCCACGA-3′ R: 5′-GGATGTGCCTGGGGATGTAG-3′	*Cumulus*cells	60	100	XM_004023159.1
ALK5	F: 5′-ACCTAATTCCACGAGACAGGCCAT-3′ R: 5′-GCAATGACAGCTGCCAGTTCAACA-3′	*Cumulus*cells	60	160	AY656799.1
ALK6	F: 5′-TGCTGGTCCAGAGGACAATAGCAA-3′ R: 5′-TCATGCCTCATCAACACCGTCTGA-3′	*Cumulus*cells	60	187	NM_001009431.1

6 + 18 h), three parameters in the case of *cumulus* expansion (total, partial and absence) and five parameters in the case of oocyte nuclear status (GV, GVBD, MI, MII, Deg). Five replicates were performed for each parameter assessed. The means were compared by Tukey test at 5% probability. *P*-values less than 0.05 were considered statistically significant.

Regarding the gene expression, data of each gene were transformed into square root of $x + 0.5$ and individually subjected to analysis of variance (ANOVA) according to completely randomized design with five treatments (Immature, Control 6 h, Rosco 6 h, Control 6 + 18 h and Rosco 6 + 18 h) and four replicates. The means were compared by Tukey test at 5% probability. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Experiment 1 – meiosis inhibition and reversibility

According to Table 2, all immature COCs evaluated immediately after follicular aspiration (0 h) had compact *cumulus* cells, which significantly differed from that observed in the other treatments. After 6 h of culture, the percentage of COCs with absence of *cumulus* expansion in the Control and Rosco significantly prevailed over the rate of partial and total expansion. This rate of COCs with compact *cumulus* cells was also significantly higher than that observed after IVM for 18 h. In contrast, the proportion of COCs from Control and Rosco with total *cumulus* expansion after IVM for 18 h significantly prevailed over that of partial expansion and compact *cumulus* cells. The rate of COCs with partial expansion did not significantly differ among treatments.

As shown in Table 3, almost all oocytes were at GV stage immediately after follicular aspiration (0 h). At 6 h, the rate of oocytes maintained at GV with roscovitine was similar to that of Immature and significantly higher than that recorded in the corresponding Control. In contrast, the rate of oocytes from Control at GVBD and MI after culture for 6 h was significantly higher than that reported in the other treatments. After IVM for 18 h, the percentage of oocytes from Control and Rosco that reached MII stage was significantly higher than that recorded at 0 and 6 h. With respect to degenerate oocytes, no significant difference was observed among treatments.

3.2. Experiment 2 – gene expression analysis

The effect of roscovitine on gene expression in sheep *cumulus* cells is reported in Fig. 3. The expression level of BAX gene after treatment for 6 h with roscovitine was similar to that observed at 0 h but significantly lower than that of corresponding Control. After IVM for 18 h, however, the relative abundance of this transcript in the Rosco treatment significantly increased and reached value greater than that of Control 6 + 18 h. In contrast, the BCL2 mRNA level in the Control significantly increased after culture for a further 18 h but did not differ from Rosco 6 + 18 h. The relative abundance of ALK5 transcript in the roscovitine treatment was down-regulated after IVM for 18 h. However, the value reached was similar to that of Immature and Control treatments. With respect to PTX3 and ALK6 genes, the relative expression was significantly up-regulated in the Rosco and Control treatments after additional culture for 18 h. No significant difference was observed among treatments regarding the expression of GLUT1, GREM1 and PTGS2 genes.

The pattern of gene expression in sheep oocytes treated for 6 h with roscovitine is shown in Fig. 4. The relative abundance of the ZAR1, NLRP5 and SOD1 transcripts was not affected by roscovitine, but was significantly down-regulated after IVM for 18 h in the Control and Rosco treatments. The mRNA levels for BAX and GDF9 in the Control significantly decreased after additional culture for 18 h. The reached values, however, were similar to those recorded in the Rosco and Immature treatments. The BMP15 and BCL2 expression remained stable in all treatments.

4. Discussion

The spontaneous meiosis resumption of oocytes removed from their follicles interrupts the transcription and storage of RNAs required to proper maturation, fertilization and initial embryogenesis (Vigneron et al., 2004). This incomplete cytoplasmic maturity is one the most crucial factors affecting the potential of *in vitro*

Table 2
Cumulus expansion degree of sheep COCs *in vitro* cultured for 6 h with (Rosco) and without (Control) 75 μM roscovitine followed by IVM for 18 h. Immature (0 h): stained soon after aspiration.

Cumulus expansion degree	Treatments, *n* (%)				
	Immature (0 h)	Control (6 h)	Rosco (6 h)	Control (6 + 18 h)	Rosco (6 + 18 h)
Total	0 (0.0)^{bB}	0 (0.0)^{bC}	0 (0.0)^{bC}	67 (57.8)^{aA}	62 (59.6)^{aA}
Partial	0 (0.0)^{bB}	20 (19.6)^{aB}	19 (17.6)^{aB}	28 (24.1)^{aB}	25 (24.0)^{aB}
Absence	122 (100.0)^{aA}	82 (80.4)^{bA}	89 (82.4)^{bA}	21 (18.1)^{cB}	17 (16.3)^{cB}
No. COCs	122	102	108	116	104

*Significant differences are indicated by lowercase letters within lines and capital letters within columns ($P < 0.05$). Standard error (S): 0.045 and coefficient of variation (CV): 5.0%.

Table 3
Nuclear maturation stage of sheep oocytes *in vitro* cultured for 6 h with (Rosco) and without (Control) 75 μM roscovitine followed by IVM for 18 h. Immature (0 h): stained soon after aspiration.

Nuclear Maturation Stage	Treatments, *n* (%)				
	Immature (0 h)	Control (6 h)	Rosco (6 h)	Control (6 + 18 h)	Rosco (6 + 18 h)
GV	107 (87.7)^{aA}	42 (46.7)^{bA}	85 (86.7)^{aA}	15 (16.0)^{cB}	16 (16.0)^{cB}
GVBD	13 (10.7)^{bB}	21 (23.3)^{aB}	13 (13.3)^{bB}	10 (10.6)^{bBC}	11 (11.0)^{bBC}
MI	0 (0.0)^{cC}	27 (30.0)^{aB}	0 (0.0)^{cC}	12 (12.8)^{bB}	10 (10.0)^{bBC}
MII	0 (0.0)^{bC}	0 (0.0)^{bC}	0 (0.0)^{bC}	56 (59.6)^{aA}	59 (59.0)^{aA}
Deg	2 (1.6)^{aBC}	0 (0.0)^{aC}	0 (0.0)^{aC}	1 (1.1)^{aC}	4 (4.0)^{aC}
No. oocyte	122	90	98	94	100

*Significant differences are indicated by lowercase letters within lines and capital letters within columns ($P < 0.05$). Standard error (S): 0.04 and coefficient of variation (CV): 4.8%. GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II, Deg: degenerate.

embryo development which is still low compared to that obtained *in vivo* (Rizos et al., 2002). In this context, attempts to arrest the meiosis *in vitro* with CDK inhibitors have been proposed in several species in order to provide additional time to oocyte capacitation (Mermillod et al., 2000; Han et al., 2006; Romar and Funahashi, 2006; Franz et al., 2003). However, the consequences of this artificial meiotic inhibition on gene expression of COCs have not been extensively investigated (Leal et al., 2012), especially in sheep.

So, the present study aimed to evaluate the effect of temporary meiotic arrest promoted by roscovitine on relative abundance of transcripts associated with developmental competence in sheep oocytes and *cumulus* cells. The absence of *cumulus* expansion in the immature COCs selected indicates that meiosis have not resumed *in vivo* (Dekel et al., 1981). According to Isobe et al. (1998), under gonadotropin stimulation, *cumulus* cells synthesize hyaluronic acid-rich matrix which is deposited into intercellular spaces, resulting in the rupture of Gap junctions between oocyte and *cumulus* cells, and consequent meiotic resumption. This process of *cumulus* expansion, however, occurs in a gradual manner. In appropriate supplemented medium, *cumulus* cells remain compacted until about 12 h of culture, when the expansion becomes evident and continuously increases up to end of culture (Hyttel et al., 1986). This kinetics of *cumulus* expansion was also observed in our study since no expansion occurred at 6 h, but a significant rate of COCs had total *cumulus* expansion after IVM for 18 h.

Our findings also demonstrate that some of investigated genes in *cumulus* cells had an expression profile consistent with the *cumulus* expansion pattern observed in the Control and Rosco treatments. The relative abundance of transcripts PTX3 (pentraxin 3) and ALK6 (actin receptor-like kinase 6) was significantly up-regulated at the end of 24 h culture. Similar expression pattern was observed for ALK6 in ovine oocytes *in vitro* matured (Kyasari et al., 2012). In bovine COCs, Assidi et al. (2008) also found basal expression of PTX3 at 6 h IVM, which, according to Wisniewski and Vilcek (2004), tends to increase until maximal *cumulus* expansion be achieved. With respect to PTGS2 (prostaglandin endoperoxide synthase 2), despite the evident expression after culture for a further 18 h, it did not differ from that observed at 0 and 6 h. The absence of PTGS2 mRNA variation during IVM was also reported by Kyasari et al. (2012) in sheep COCs. However, in the presence of EGF, a marked increase of PTGS2 mRNA was recorded in bovine oocytes (Vigneron et al., 2003).

In contrast, the relative abundance of transcripts ALK5 (actin receptor-like kinase 5) GREM1 (Gremlin), and GLUT1 (glucose transport 1) remained stable during this study. Similar ALK5 expression pattern was reported in sheep (Kyasari et al., 2012). However, the expression kinetics of GREM1 and GLUT1 during IVM has not yet been well established. The up-regulation of BCL2 mRNA and absence of variation of BAX mRNA observed in *cumulus* cells of Control after IVM for 18 h are in accordance with that reported by Filali et al. (2009) in human COCs. According to these authors, the ratio BCL2 (anti-apoptotic)/BAX (pro-apoptotic) determines cell survival, and the prevalence of BCL2 mRNA expression in *cumulus* cells is directly related to oocyte competence. While BCL2/BAX and Glut1 are involved, respectively, in the apoptosis control and glucose metabolism (Filali et al., 2009; Purcell and Moley, 2009), the others investigated genes (PTX3, PTGS2, ALK5 and ALK6) play important functions on *cumulus* expansion (Kyasari et al., 2012).

In *cumulus* cells treated with roscovitine, the up-regulation of BAX expression observed after additional culture for 18 h was followed by increase of BCL2 mRNA, which suggests that balance BCL2/BAX and consequent cellular viability were both maintained. Besides, the similarity between Control and Rosco treatments with respect to *cumulus* expansion and gene expression profile in *cumulus* cells indicates that roscovitine did not affect these events. Likewise, Leal et al. (2012) observed that transcripts pattern in *cumulus* cells of bovine COCs was unaffected by 100 μM butyrolactone. However, Vigneron et al. (2003) reported that 25 μM roscovitine reversibly inhibited the *cumulus* expansion and PTGS2 expression in bovine COCs even in the presence of EGF. These divergences among authors are probably related not only to drug concentration but also to inhibitor exposure time (Han et al., 2006; Sananmuang et al., 2010).

The high proportion of oocytes at GV stage (87.7%) after follicle aspiration suggests that time interval for recovery and selection of COCs was adequate and did not induce the meiotic resumption before culture. Similar results were observed in goat (Han et al., 2006) and pig (Romar and Funahashi, 2006) oocytes. Moreover,

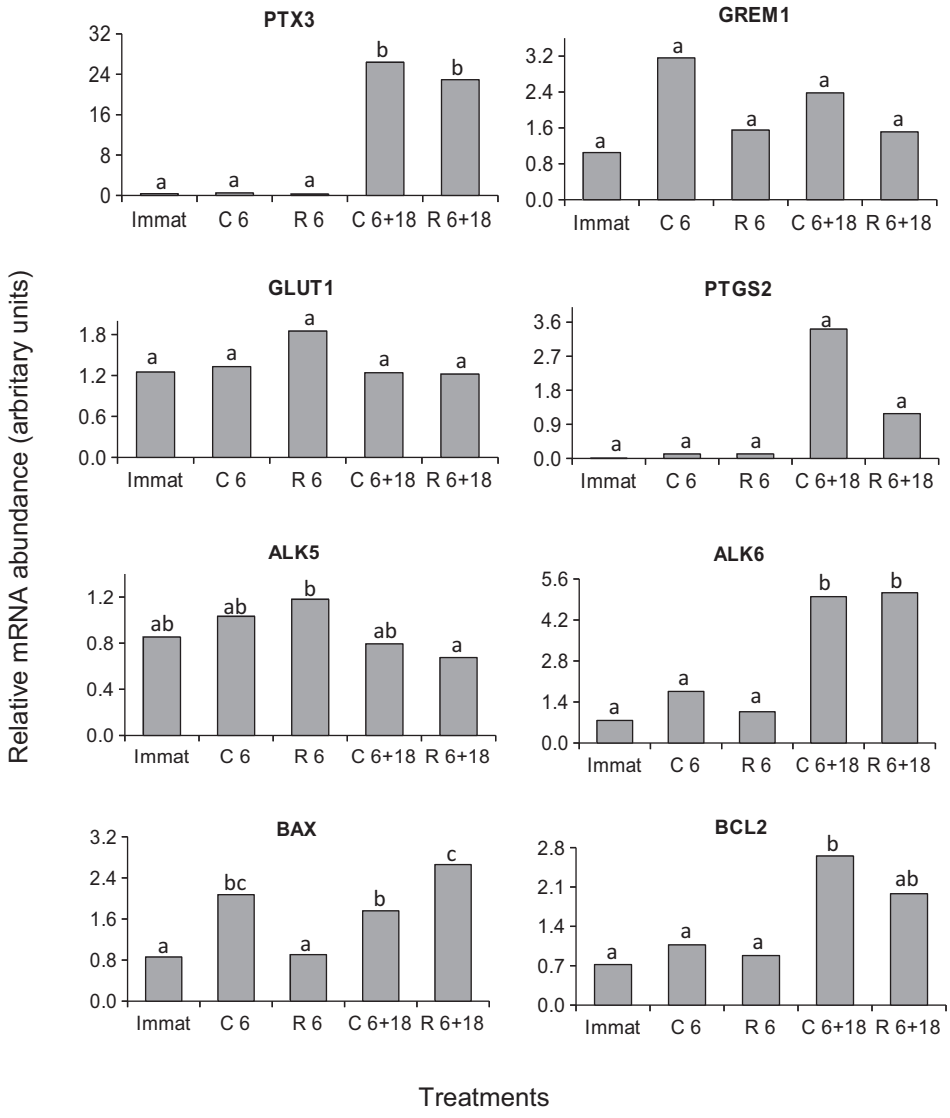

Fig. 3. Relative expression of the PTX3, GREM1, GLUT1, PTGS2, ALK5, ALK6, BAX and BCL2 genes in sheep *cumulus* cells *in vitro* cultured for 6 h with (Rosco; R6) and without (Control; C6) 75 μM roscovitine followed by IVM for 18 h (C 6 + 18 and R 6 + 18). Immature (0 h): stained soon after aspiration.*Values with different superscripts letters differ significantly ($P < 0.05$).

the significantly high rate of GV in the Rosco treatment in contrast to evident meiosis progression observed in the Control at 6 h of culture demonstrates that 75 μM roscovitine was efficient to arrest the meiosis in sheep oocytes. This meiotic inhibition, however, was completely reversed and a significant rate of oocytes reached the MII after 18 h IVM. The reversible meiotic inhibition was also recorded in cat (Sananmuang et al., 2010), goat (Han et al., 2006), bovine (Mermillod et al., 2000) and horse (Franz et al., 2003) oocytes treated with roscovitine at different doses and exposure time. The low rate of degenerate oocytes also indicates that culture conditions were suitable.

With respect to gene expression profile in oocytes, the relative abundance of ZAR1 (zygote arrest 1), NLRP5 (PYD domains – containing protein 5, or MATER) and SOD1 (superoxide dismutase 1) was significantly down-regulated in the Control and Rosco treatments after IVM for 18 h. Similarly, a significant decrease of ZAR1 and NLPR5 mRNA was reported by Bebbere et al. (2008) in sheep oocytes *in vitro* matured. However, the SOD1 mRNA level, which is considered crucial for protection against free radicals, remained stable in canine oocytes (Turathum et al., 2010). Presuming that reduction of mRNA levels indicate translation (Bebbere et al., 2008), the expression decrease of ZAR1, NLRP5 and SOD1 observed in our study suggests participation of these proteins during oocyte maturation. The maternal effect genes (ZAR1 and NLRP5) are still recognized by their importance during oocyte-embryo transition (Bebbere et al., 2008).

The significant decrease of GDF9 (growth and differentiation factor 9) expression during IVM for 18 h in contrast with stable BMP15 (bone morphogenetic protein 15) mRNA level observed in the Control is in accordance with that reported by Bebbere et al. (2008) in sheep oocytes. Probably, the expression profile of GDF9 is directly related to its function in the regulation of *cumulus* expansion and oocyte meiotic maturation (Reyes et al., 2013), while BMP15 seems to be more important in later development stages (Bebbere et al., 2008). Besides, the ratio BCL2/BAX observed in oocytes from Rosco and Control treatments reveals that cellular viability was maintained (Feugang et al., 2011) even in the presence of inhibitor.

The gene expression profile observed in our Control reinforces, therefore, the evidence that oocyte transcriptional activity is inter-

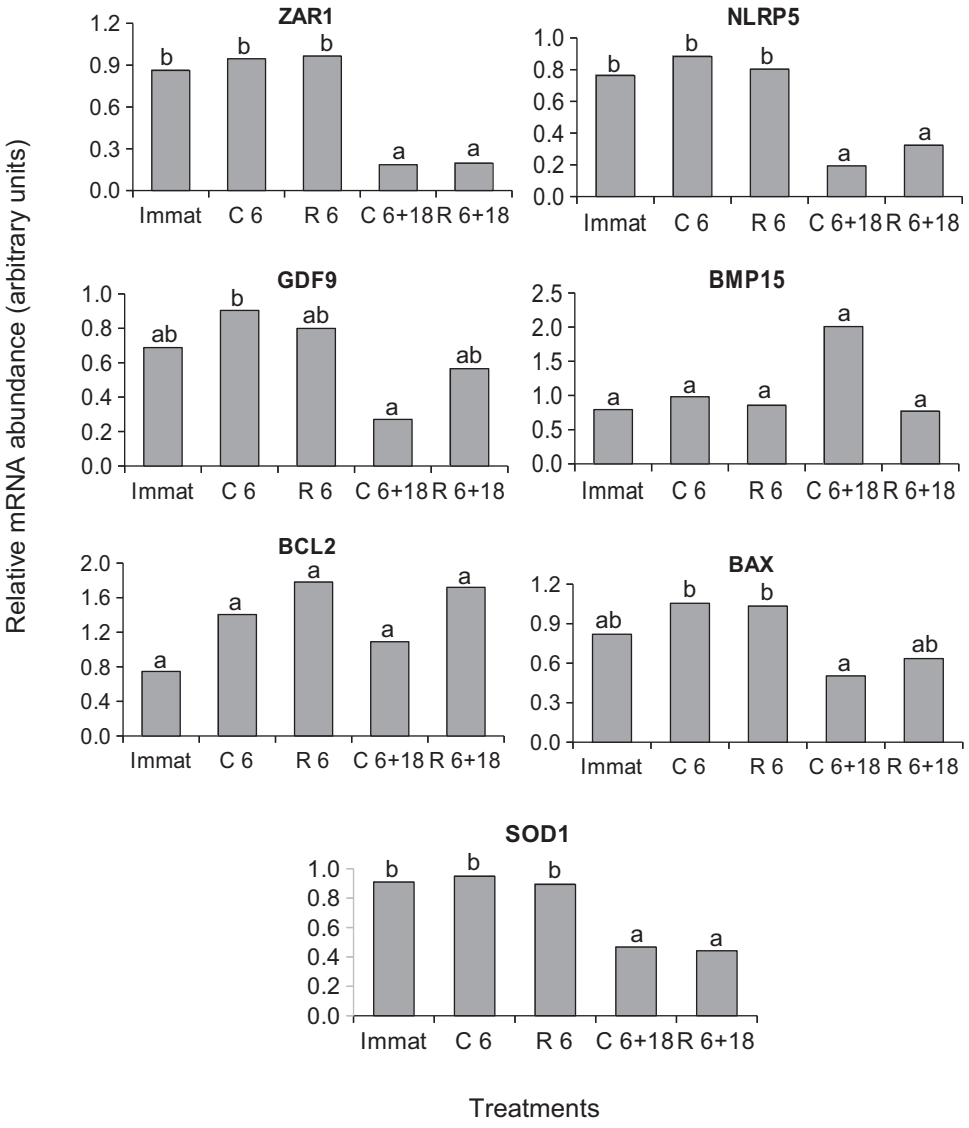

Fig. 4. Relative expression of the ZAR1, NLRP5, BMP15, GDF9, SOD1, BAX and BCL2 genes in sheep oocytes *in vitro* cultured for 6 h with (Rosco; R6) and without (Control; C6) 75 μM roscovitine followed by IVM for 18 h (C 6 + 18 and R 6 + 18). Immature (0 h): stained soon after aspiration. *Values with different superscripts letters differ significantly ($P < 0.05$).

rupted with meiosis resumption (Vigneron et al., 2004). According to these authors, maternal genes tend to remain stable or decline because of translation during IVM. Our findings also demonstrate that meiotic arrest promoted by roscovitine neither improved nor impaired the expression of investigated genes in sheep oocytes. Likewise, Leal et al. (2012) reported that most studied genes in bovine oocytes meiotically inhibited with butyrolactone had the same expression pattern of corresponding control. In contrast, significant increase of poly (A) mRNA content was reported by Lequarre et al. (2004) in bovine oocytes arrested at GV, which suggest neotranscription. However, in other cells types, roscovitine significantly suppressed the mRNA synthesis (Ljungman and Paulsen, 2001). We assumed that this discrepancy of results must be related to inhibition time, studied genes, methods of mRNA detection and species particularities.

The efficiency of temporary meiotic arrest and absence of deleterious effect on *cumulus* expansion, nuclear maturation and gene expression also suggest that roscovitine provides a useful and efficient method for transportation or manipulation of sheep COCs at onset of maturation. Furthermore, the maintenance of oocytes at GV stage may have a great impact on research and commercial application considering that oocytes sometimes are collected in places far from laboratory and their viability is limited (Hashimoto et al., 2003). According to Gharibi et al. (2013), the temporary arrest of meiosis also has implication in the synchronization of oocyte maturation and improvement of *in vitro* oocyte developmental competence.

In conclusion, we can infer that roscovitine, at the concentration and exposure time studied, was efficient to reversibly arrest the meiosis in sheep oocytes without negatively affect or improve the *cumulus* expansion and the relative abundance of investigated transcripts. Further investigations have been performed to evaluate the benefits of roscovitine treatment on *in vitro* development of sheep embryos and its effects on cellular ultrastructure.

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