UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS BOTUCATU-SP

EXPRESSÃO GÊNICA E POTENCIAL DE DESENVOLVIMENTO *IN VITRO* DOS COMPLEXOS *CUMULUS*-OÓCITOS OVINOS TRATADOS COM ROSCOVITINA

LETÍCIA FERRARI CROCOMO

Botucatu, São Paulo
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CROCOMO, L.F. **Expressão gênica e potencial de desenvolvimento *in vitro* dos complexos *cumulus*-oócitos ovinos tratados com roscovitina.** Botucatu, 2015, 129p. Tese (Doutorado) - Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista - UNESP.

Esta pesquisa visou avaliar o perfil de expressão de determinados genes e o potencial de desenvolvimento *in vitro* de complexos *cumulus*-oócitos (COCs) de ovinos temporariamente bloqueados no estadio de vesícula germinativa (GV) com uso da roscovitina. Neste contexto, diferentes composições de meio (com e sem soro fetal bovino e gonadotrofinas), tempos (0, 6, 12 e 20 horas) e métodos de cultivo (com e sem cobertura de óleo mineral) foram testados com intuito de garantir a máxima eficiência de inibição meiótica promovida pela roscovitina a 75µM. A avaliação do grau de expansão das células do *cumulus,* em estereomicroscópio*,* e do estadio da maturação nuclear, por meio da coloração com Hoescht 33342, revelou que a presença ou ausência de LH, FSH e soro fetal bovino não interferiu no potencial de ação da roscovitina. No entanto, máxima eficiência inibitória foi observada em meio suplementado apenas com soro fetal bovino. Foi constatado ainda que, na ausência de óleo mineral, quantidade significativamente maior de oócitos tratados com roscovitina foi mantida em GV o que reforça a hipótese de lipossolubilidade do referido inibidor. Embora o potencial de inibição meiótica da roscovitina tenha se mantido nos diferentes tempos de cultivo, a exposição prolongada dos COCs ao inibidor afetou de maneira irreversível a expansão do *cumulus*. Em todas as situações, a inibição meiótica foi completamente reversível após cultivo por 18 horas em meio de maturação livre de inibidor. Sendo assim, para análise da expressão gênica e desenvolvimento embrionário *in vitro*, foi preconizado o tratamento com roscovitina a 75µM por 6 horas na presença de soro e sem óleo mineral. Nestas condições assim como após a reversibilidade por 18 h, a quantidade relativa da maioria dos genes investigados nas células do *cumulus* e nos oócitos foi similar ao observado nos COCs não inibidos (controle). Do mesmo modo, não foi constatada diferença entre o tratamento com roscovitina e o controle com relação à taxa de blastocistos e à qualidade dos embriões obtidos. Portanto, o bloqueio temporário dos COCs de ovinos no estadio de GV com roscovitina a 75 µM não melhora, mas também não afeta a expressão gênica e o potencial de desenvolvimento embrionário *in vitro.*

Palavras-chave: inibidor da meiose, maturação nuclear, genes, embrião, ovelha.

ABSTRACT

CROCOMO, L.F. **Gene expression and *in vitro* development potential of ovine *cumulus*-oocyte complexes treated with roscovitine.** Botucatu, 2015, 129p. Tese (Doutorado) - Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista - UNESP.

This study aimed to evaluate the expression profile of certain genes and the potential of *in vitro* development of sheep *cumulus*-oocytes complexes (COCs) temporarily arrested at germinal vesicle (GV) stage using roscovitine. In this context, different medium compositions (with and without fetal bovine serum and gonadotropins), times (0, 6, 12 and 20 hours) and methods of culture (with and without mineral oil overlay) were tested in order to ensure maximum efficiency of meiotic inhibition promoted by 75μM roscovitine. The evaluation of *cumulus* expansion degree, under stereomicroscope, and nuclear maturation stage, by staining with Hoechst 33342, revealed that the presence or absence of LH, FSH and fetal bovine serum did not influence the action potential of roscovitine. However, maximum efficiency of meiotic inhibition was observed in medium supplemented with fetal bovine serum. It was also found that, in the absence of mineral oil overlay, significantly higher quantity of oocytes treated with roscovitine was kept at GV, which reinforces the hypothesis of lipo-solubility of this inhibitor. Although the potential of meiotic inhibition of roscovitine had remained at different culture times, the prolonged exposure of COCs to inhibitor irreversibly affect the *cumulus* expansion. In all cases, the meiotic arrest was completely reversible after culture for a further 18 h in inhibitor-free medium. Therefore, for analysis of gene expression and *in vitro* embryo development, it was chosen the treatment with 75μM roscovitine for 6 h under serum-supplemented medium and without mineral oil overlay. Under these conditions as well as after the reversibility for 18 h, the relative amount of most investigated genes in *cumulus* cells and oocytes was similar to that observed in the uninhibited COCs (control). Similarly, no difference was found between roscovitine treatment and control with respect to blastocyst rate and quality of embryos obtained. Therefore, the temporary arrest of sheep COCs at GV stage using 75 μM roscovitine does not improve, but also does not affect the gene expression and the potential of *in vitro* embryo development.

Key-words: meiosis inhibitor, nuclear maturation, gene, embryo, sheep

INTRODUÇÃO

Ainda durante a vida intra-uterina, após intensas divisões mitóticas, as oogônias se diferenciam em oócito e iniciam a meiose, a qual é bloqueada no estadio diplóteno da prófase I, identificado morfologicamente pela vesícula germinativa (GV) (HIRSHFIELD, 1991). Assim permanecem até a puberdade quando, próximo à ovulação, a meiose é retomada *in vivo* pelo estímulo gonadotrófico, sendo caracterizada pela quebra da vesícula germinativa (GVBD) (GOSDEN et al. 1997).

Durante este período de bloqueio meiótico, os oócitos aumentam expressivamente de volume e sofrem importantes transformações citoesqueléticas e moleculares que conferem potencial para suportar os demais estadios de desenvolvimento. Tais transformações envolvem não somente a diferenciação e deslocamento de organelas citoplasmáticas, mas principalmente, a transcrição e estoque de mRNAs que serão mobilizados em momentos específicos para síntese proteica (BREVINI-GANDOLFI & GANDOLFI, 2001; SIRARD et al., 2001). Concomitantemente, as células da granulosa proliferam, se diferenciam e estabelecem íntima comunicação com os oócitos através das junções intercelulares comunicantes do tipo GAP, com transferência bidirecional de substâncias de baixo peso molecular, constituindo o complexo *cumulus*-oócito (COC) (TANGHE et al., 2002).

Com a retomada e progressão das divisões meióticas, no entanto, embora o oócito mantenha a capacidade de tradução gênica e síntese proteica, a atividade transcricional é interrompida sendo restabelecida somente no início do desenvolvimento embrionário, com a ativação do genoma, quando o embrião passa a produzir seus próprios mRNAs e proteínas até se tornar auto-suficiente (MINAMI et al., 2007). Deste modo, os processos de maturação oocitária, fertilização e embriogênese inicial são controlados pelos transcritos maternos, os quais são armazenados no ooplasma em sua forma traducionalmente inativa, caracterizada pela presença da cauda poli-A curta, sendo traduzidos quando requeridos (BREVINI-GANDOLFI & GANDOLFI, 2001).

Em contrapartida, quando os complexos *cumulus*-oócitos são removidos do ambiente folicular, ocorre reinício meiótico espontâneo independente de estadio de maturação citoplasmática (GILCHRIST, 2008). Deste modo, os COCs destinados à maturação *in vitro* ainda não completaram *in vivo* todas as transformações citoesqueléticas e moleculares necessárias para aquisição de competência (GILCHRIST & THOMPSON, 2007). De acordo com Marteil et al. (2009) a baixa qualidade dos

oócitos está diretamente relacionada à menor eficiência da produção *in vitro* de embriões

Sendo assim, a manutenção temporária dos COCs sob condições capazes de impedir a progressão meiótica têm sido proposta como estratégia para restabelecer a sincronia entre os processos de maturação nuclear e citoplasmática e consequentemente melhorar o potencial de desenvolvimento embrionário *in vitro* (LE BEUX *et al*., 2003). Neste contexto, a roscovitina se destaca como inibidor da meiose devido à ação específica e reversível sobre o fator promotor da maturação (MPF), cuja ativação é necessária para o reinício meiótico (MEIJER e tal., 1997) .

Dessa forma, dada a relevância do tema e a necessidade de ampliação do conhecimento referente à produção *in vitro* de embriões ovinos, esta pesquisa foi proposta para analisar a eficiência e a reversibilidade da inibição meiótica promovida pela roscovitina em oócitos de ovino, assim como avaliar o efeito deste inibidor sobre a expansão das células do *cumulus,* expressão gênica e desenvolvimento embrionário *in vitro*.

REVISÃO DE LITERATURA

1. Maturação oocitária

De acordo com Eppig (1996), a maturação oocitária envolve todos os eventos nucleares e citoplasmáticos ocorridos desde o reinício da meiose até o estadio de metáfase II (MII), os quais garantem ao oócito competência para completar seu desenvolvimento, ser fertilizado e sustentar a embriogênese inicial (HYTTEL et al., 1997). Devido a isto, consiste numa das etapas mais importantes da produção de embriões tanto *in vitro* como *in vivo* (MERMILLOD et al. 1999).

Este processo envolve basicamente dois eventos que, apesar de distintos, são interligados e, *in vivo*, ocorrem simultaneamente em determinados momentos (FERREIRA et al., 2009): a maturação nuclear, que se inicia com a quebra de vesícula germinativa e termina apenas quando a meiose é finalizada, sendo marcada pela segregação dos cromossomos e extrusão de 2 corpúsculos polares, e a maturação citoplasmática, caracterizada por alterações oocitárias morfológicas e funcionas (TOSTI, 2006). A regulação da maturação envolve uma sequência de vias bioquímicas que ainda não estão completamente esclarecidas e são caracterizados pela fosforilação, desfosforilação e atividade de diversas moléculas (EPPIG, 1996).

1.1. Maturação nuclear

De acordo com Silva (2008), a maturação nuclear ou meiótica corresponde à divisão reducional dos cromossomos, que tem como objetivo a produção dos gametas haplóides aptos para fecundação. Este processo se inicia ainda na vida uterina, sendo que a maioria dos mamíferos já nasce com uma reserva de oócitos bloqueados no estadio de vesícula germinativa caracterizado pela presença de núcleo vesicular com nucléolo distinto circundado por filamentos de cromatina descondensados (HEWITT & ENGLAND, 1997).

1.1.a. Reinício meiótico *in vivo*

O reinício da maturação meiótica é desencadeado por diferentes mecanismos que envolvem um estímulo positivo, caracterizado pela onda pré-ovulatória do hormônio luteinizante (LH) e consequente síntese de substâncias indutoras da meiose pelas células da granulosa, e a remoção do sinal inibitório, caracterizado pelos fatores inibidores da meiose secretados pelas células foliculares, que são solúveis no meio folicular e agem nos oócitos via células do *cumulus* (EPPIG, 1991; RICHARD & SIRARD, 1996).

In vivo, a onda pré-ovulatória de LH é considerada responsável primária pela indução da maturação oocitária. No entanto, esta gonadotrofina não atua diretamente no oócito, uma vez que este não apresenta receptores para LH (PENG et al., 1991). Ao invés, sua ação é mediada por fatores parácrinos secretados pelas células da granulosa LH-responsivas, e pelo transporte de mensageiros celulares destas células para os oócitos através das junções "Gap" comunicantes (GJCs) (GILULA *et al*., 1978).

Deste modo, a regulação gonadotrófica da GVBD é atribuída à perda da comunicação intercelular entre o COCs e as células foliculares, com consequente redução da transferência de substâncias inibidoras da maturação, à produção de fatores indutores da meiose pelas células do *cumulus*, à oscilação da concentração de segundos mensageiros no oócito e nas células do *cumulus*, e à ativação de proteínas reguladoras do ciclo celular no oócito (DEKEL et al., 1981).

1.1.b. Reinício meiótico *in vitro*

Quando o COC é removido do ambiente folicular, ocorre reinício "espontâneo" da meiose independente do estadio de maturidade citoplasmática e do estímulo gonadotrófico (PINCUS & ENZMANN, 1935). Para Byskov et al. (1997), isto ocorre

devido à interrupção da transferência de fatores inibidores da GVBD para o oócito decorrente da perda das GJCs entre os COCs e as células foliculares.

Além disso, COCs coletados de pequenos folículos antrais ainda não sofreram todas as alterações moleculares e celulares necessárias para suportar a maturação e a fase inicial da embriogênese (GILCHRIST & THOMPSON, 2007), resultando na assincronia entre maturação nuclear e citoplasmática. Sendo assim, o cultivo dos COCs na presença de substâncias capazes de inibir reversivelmente a meiose consiste numa estratégia para melhorar a qualidade dos oócitos maturados *in vitro* (HASHIMOTO et al., 2002).

O reinício da meiose, tanto *in vivo* como *in vitro,* é caracterizado pela GVBD, estadio no qual ocorre dissolução da membrana nuclear e condensação da cromatina (CURCIO et al., 2006). Posteriormente, o oócito passa pelos estadios de metáfase I (MI), anáfase I e telófase I, completando a primeira divisão meiótica, com formação do primeiro corpúsculo polar (DEKEL, 2005). Em seguida, ocorre progressão ao estadio de MII, caracterizado pela presença dos cromossomos arranjados no centro do fuso e corpúsculo polar no espaço perivitelínico. Esta habilidade em reiniciar e completar a meiose é adquirida gradualmente durante a foliculogênese (SILVA, 2008).

Os mecanismos envolvidos no bloqueio e o reinício das divisões meióticas ainda não estão totalmente esclarecidos, mas se reconhece a importância de determinadas proteínas como a MAPK e MPF, além de segundos mensageiros como cAMP, entre outros fatores (GULER et al., 2000). É importante salientar ainda que os eventos celulares e moleculares que regem a maturação oocitária *in vivo* e *in vitro* diferem entre si em determinados aspectos (GILCHRIST & THOMPSON, 2007).

1.1.c. Adenosina 3´- 5´ monofosfato cíclico (cAMP)

O bloqueio dos oócitos no estadio de prófase I e o posterior reinício meiótico está correlacionado com mudanças na concentração intraoocitária do segundo mensageiro cAMP, o qual é derivado da adenosina trifosfato (ATP) através da ação da enzima adenilato ciclase (KAWAMURA et al., 2004). A adenilato ciclase, por sua vez, é ativada ou inibida através das proteínas G que correspondem a um conjunto de proteínas acopladas aos receptores da membrana celular, as quais respondem ao estímulo hormonal (MARZZOCO & TORRES, 2007). De acordo com DEKEL et al. (1981) a cAMP pode ser produzida nos oócitos ou transferida a eles pelas células do *cumulus.*

A ação da cAMP é exercida através da regulação de proteínas como a quinase A (PKA). Estas PKAs são classificadas ainda em tipo I e tipo II de acordo com o predomínio das subunidades reguladoras (RI ou RII) (BEEBE & CORBIN, 1986). Segundo Downs e Dunn (1995), as PKAs tipo I localizam-se no interior dos oócitos e estão relacionadas com o bloqueio meiótico, enquanto que nas células do *cumulus* foi detectada PKA tipo II, relacionada ao reinício da meiose e expansão do *cumulus*.

A cAMP tem uma meia-vida muito curta, sendo degradada, no interior das células onde se formou, pela ação da enzima fosfodiesterase (PDE). Segundo Tsafriri et al. (1996), existem dois subtipos de PDE (3 e 4), presentes no oócito e nas células foliculares, respectivamente, sendo que a regulação seletiva destas fosfodiesterases pelo estímulo gonadotrófico determina o reinício meiótico (THIBAULT et al.,1987). A atividade das PDEs pode ainda ser inibida pelas hipoxantinas presentes no fluido folicular, resultando em acúmulo de cAMP intraoocitário e consequente manutenção do oócito no estadio de GV (AKTAS et al., 2003).

Estudos sugerem que as gonadotrofinas promovem elevação da concentração de cAMP com consequente ativação da PKAII nas células do *cumulus*, resultando na expressão de substâncias indutoras da GVBD e na expansão do *cumulus* (SU et al., 2003). Ainda segundo Dekel et al. (1981), a perda da comunicação entre os COCs e as células foliculares interrompe a transferência de cAMP para os oócitos, resultando na redução da concentração intraoocitária de cAMP e decréscimo da atividade da PKAI, com consequente retomada da divisão meiótica (KAWAMURA et al., 2004).

1.1.d. Proteína Quinase ativada por Mitógenos (MAPK)

A MAPK, também conhecida como quinase regulada por sinal extracelular (ERK), pertence à família das proteínas kinases serina-treonina e está envolvida no reinício e progressão da meiose, reorganização dos microtúbulos, formação do fuso meiótico, manutenção do oócito em MII e expansão do *cumulus* (SU et al., 2003). Esta proteína está presente tanto no oócito quanto nas células do *cumulus* e, em ambos os tipos celulares, é ativada através da fosforilação dos resíduos de tirosina e treonina pela MEK, também designada MAPKK (proteína quinase ativadora da MAPK), a qual é ativada por meio da fosforilação mediada pelas proteínas MOS no oócito, e RAS/RAF nas células do *cumulus* (FISSORE et al., 1996; SAGATA, 1997).

Segundo Sagata (1997), a MAPK promove a ativação e estabilização do MPF nos oócitos através da inibição de reguladores negativos e da ativação de enzimas como

a cdc25 fosfatase. Em oócitos de bovinos, a ativação da MAPK e do MPF ocorre praticamente ao mesmo tempo, pouco antes da GVBD, sendo que a atividade da MAPK aumenta gradualmente ao longo da maturação oocitária e se mantém alta até o estadio de MII (WEHREND & MEINECKE, 2001). Níveis elevados de MAPK e MPF são necessários para manutenção dos oócitos no estadio de MII, sendo que a fertilização ou ativação partenogenética promove queda abrupta na concentração intraoocitária dessas duas quinases e conduz a conclusão da meiose (OH et al. 1998).

De acordo com Fan e Sun (2004), a ativação e inativação da MAPK também está relacionada à variação na concentração de cAMP e PKA tanto no oócito quanto nas células do *cumulus*. Vale ressaltar ainda que, enquanto *in vivo,* a ativação da MAPK é necessária para o reinício meiótico, *in vitro*, este processo ocorre espontaneamente com a remoção do oócito do ambiente folicular (SILVA, 2008).

1.1.e. Fator Promotor da Maturação (MPF)

O MPF é um composto dimérico, que pertence à família das proteínas quinases, sendo constituído por uma subunidade catalítica, a quinase dependente de ciclina denominada cdk1 ou $p34^{cdc2}$, que controla a divisão celular, e uma subunidade reguladora, a ciclina B1. Em sua forma inativa, pré-MPF, a subunidade catalítica se apresenta fosforilada nos resíduos Thr14 (treonina-14) e Tyr 15 (tirosina-15). A ativação do MPF requer a desfosforilação destes resíduos por ação da enzima cdc 25 fosfatase e associação das subunidades $p34^{cdc2}$ e ciclina B1 (GAUTIER et al., 1988).

Estudos indicam que a elevada concentração de cAMP intraoocitária e ativação da PKAI inibe a desfosforilação dos resíduos Thr14 e Tyr 15 e reprime a síntese de ciclina B1(JOSEFSBERG et al., 2003). Segundo Matfen et al. (1994), quando ativa, a PKAI bloqueia a cascata MEK-MAPK resultando na inativação da enzima cdc25, com consequente inatividade do MPF. Em contrapartida, sob baixa concentração intraoocitária de cAMP e consequentemente de PKAI, ocorre a ativação do MPF, o qual promove a fosforilação das proteínas do envoltório nuclear, dissolução do nucléolo; condensação cromossômica e reorganização do citoesqueleto (DEKEL, 1996).

De acordo com Josefsberg et al. (2003) a atividade do MPF apresenta um padrão oscilatório ao longo da maturação oocitária. Sua ativação é necessária para o reinício da meiose, ocorrendo um pouco antes da GVBD, e sua atividade elevada é requerida para progressão até o estadio de MI, no qual alcança nível máximo, sofrendo declínio antes

da extrusão do primeiro corpúsculo polar. Posteriormente, a concentração intraoocitária de MPF se mantém elevada até a fertilização (MONDADORI et al 1999).

1.2. Maturação citoplasmática

Os complexos eventos que ocorrem durante a maturação oocitária, não envolvem somente a correta dinâmica de separação cromossômica durante a maturação nuclear, mas também a redistribuição das organelas citoplasmáticas, o estoque de mRNA e a transcrição de fatores necessários para o adequado desenvolvimento (FERREIRA et al., 2009).

A capacidade do oócito em bloquear a poliespermia no caso de fertilização, promover a descondensação do espermatozóide já no interior do ooplasma, formar o pronúcleo após a fertilização, e sustentar as fases iniciais do desenvolvimento embrionário é determinado pela maturação citoplasmática (PICTON et al., 1998).

1.2.a. Modificações estruturais no oócito

Em síntese, ao longo da folículo-oogênese até próximo ao reinício meiótico ocorre formação das GJCs entre o oócito e as células somáticas circundantes, desenvolvimento e deslocamento do complexo de Golgi para a periferia do oócito, produção dos grânulos corticais e das proteínas da zona pelúcida, desenvolvimento do retículo endoplasmático liso e formação das gotas lipídicas, diferenciação e aumento da quantidade de mitocôndrias, formação e estoque de ribossomos, além da transcrição e armazenamento de mRNAs materno para a síntese proteica (HYTTEL et al., 1997).

Com a retomada da maturação nuclear, organelas como as mitocôndrias, que no oócito imaturo se localizavam perifericamente, migram para o centro da célula. O complexo de Golgi diminui o seu desenvolvimento e praticamente desaparece, enquanto o retículo endoplasmático, que durante o estadio de GV se encontrava distribuído uniformemente no ooplasma, se desloca para região cortical conforme há progressão para o estadio MII (HYTTEL et al., 1997, FERREIRA et al., 200). Além disso, os grânulos corticais, que nos oócitos imaturos, estão distribuídos em grumos por todo ooplasma, migram para o córtex oocitário formando uma monocamada estratégica contra a poliespermia no oócito em MII (THIBAULT, 1987).

Este direcionamento de moléculas e organelas no interior das células é controlado pelos filamentos do citoesqueleto, que também são responsáveis pela segregação dos cromossomos na meiose e mitose, e pela divisão celular (ALBERTS et

al., 2004). Além disso, com a maturação oocitária também ocorre expansão das células do *cumulus* e mudanças morfológicas nas GJCs (BEVERS et al., 1997).

1.2.b. Síntese e estoque de mRNAs

Segundo Sirard (2001), a adequada síntese e estoque de mRNAs e proteínas conferem ao oócito competência para finalizar a maturação e suportar os eventos celulares subsequentes, tais como fertilização, formação dos pronúcleos e embriogênese inicial. A transcrição de mRNAs ocorre durante o desenvolvimento folicular, enquanto o núcleo celular se encontra em quiescência meiótica, e cessa com a retomada da meiose, uma vez que os cromossomos se tornam condensados e "inativos" (BREVINI-GANDOLFI & GANDOLFI, 2001). Com o reinício da meiose, embora a habilidade de síntese proteica não seja afetada, o oócito perde a capacidade de transcrição gênica, a qual é restabelecida apenas com a ativação do genoma embrionário (BLONDIN & SIRARD, 1995).

Os mRNAs persistem no ooplasma em sua forma estável, porém inativa, sendo mobilizados sob sinais específicos conforme requeridos. A eficiência do estoque destes transcritos assim como a sua reativação oportuna são regulados pela poliadenilação (BREVINI-GANDOLFI & GANDOLFI, 2001; SIRARD, 2001). Neste processo, adeninas são adicionadas à porção terminal 3´ do mRNA, resultando na liberação de moléculas repressoras acopladas à porção 5´, com consequente início da tradução (TOMEK et al., 2002). Assim sendo, mRNAs com cauda poli-A curta são traducionalmente inativos devido a sua baixa estabilidade, enquanto que o aumento desta cauda, por ação da enzima poli-a polimerase, determina a tradução do mRNA e síntese proteica (FERREIRA et al., 2009).

2. Principais transcritos implicados na aquisição de competência oocitária

Os principais mRNAs expressos nos oócitos durante o estadio de GV estão envolvidos em processos considerados fundamentais para o adequado desenvolvimento como: regulação do ciclo celular, sinalização celular, metabolismo, foliculogênese, controle da apoptose, proteção contra o estresse oxidativo, entre outros (WRENZYCKI et al., 2007). Dentre os genes que condificam estes transcritos destacam-se o MATER 1 (*maternal antigen that embryos require*), ZAR1 (*Zygote Arrest 1*), GDF 9 (*growth and differentiation factor 9*), BMP15 (*bone morphogenetic protein 15*), Bax e Bcl-2 (Hanrahan et al. 2004; Bebbere et al., 2008).

Além destes, o HAS2 (*hyaluronic acid syntethase-2*), COX2 (*cyclooxygenase*), PTX3 (*pentraxin 3*) e GREM1 (Gremlin) expressos nas células do *cumulus* são reconhecidos por desempenharem importante função no processo de expansão do *cumulus*, o qual está diretamente relacionado ao reinício da meiose oocitária (Shimada et al., 2006).

2.1. MATER e ZAR1

A fase inicial do desenvolvimento embrionário é suportada por transcritos maternos sendo que, com a ativação do genoma (EGA), os embriões se tornam autossuficientes em termos transcricional e passam a sintetizar os mRNA e proteínas de que necessitam (MINAMI et al., 2007). O momento específico da EGA é variável entre espécies ocorrendo por volta do estadio de 8 a 16 células em ovinos (CROSBY et al., 1988). Neste contexto se destacam os transcritos MATER também chamado de NLRP5 (*NLR family, Pyrin domain containing 5*) e ZAR1, ambos codificados pelos genes de efeito materno (MEG) (BEBBERE et al., 2008).

Expressos no oócito a partir do estadio de folículo primário, tanto o Zar1 quanto o MATER desempenham importante função durante a embriogênese inicial (BEBBERE et al., 2008). Tal constatação é reforçada pelos estudos realizados por Tong et al. (2000) e Wu et al. (2003) em camundongos segundo os quais, na ausência do MATER e ZAR 1, embora ocorra adequado desenvolvimento folicular, ovulação e fertilização com formação de zigotos normais, os embriões obtidos não se desenvolvem além do estadio de 2 células e tendem à degeneração. Sendo assim, o bloqueio no desenvolvimento observado em embriões cultivados *in vitro*, o qual coincide com a EGA, está possivelmente relacionado com a menor quantidade de MATER e ZAR1 relatada em oócitos de bovino maturados *in vitro* (PEREIRA et al., 2010).

Com relação ao perfil de expressão, Bebbere et al. (2008), em seus estudos com ovinos, constataram decréscimo do nível de MATER e ZAR1 ao longo da maturação oocitária assim como durante a MET. Considerando a provável relação entre os processos de tradução e degradação do mRNA, estes mesmos autores sugerem possível envolvimento das proteínas codificadas pelos transcritos MATER e ZAR1 tanto na EGA quanto na progressão da meiose oocitária. Resultados similares têm sido constatados em camundongos (WU et al., 2003) e suínos (UZBEKOVA et al., 2006). No entanto, a exata função dos genes de efeito materno ainda permanece desconhecida.

2.2. GDF9 e BMP15

No que diz respeito à aquisição de competência oocitária e foliculogênese, estudos recentes demonstraram que o oócito não é uma estrutura passiva. Pelo contrário, através da síntese de fatores de crescimento, o oócito regula inúmeras funções das células da granulosa como proliferação, diferenciação, expressão de receptores, esteroidogênese e expansão do *cumulus* (SHIMADA et al., 2006). Dentre os fatores parácrinos secretados pelo oócito estão o GDF9 e a BMP15, os quais pertencem à família dos fatores de crescimento transformante beta (TGFβ) e são considerados importantes peptídeos reguladores intraovarianos (GILCHRIST et al., 2008).

Além disso, evidências indicam possível implicação destes fatores na maturação oocitária, luteinização, ovulação e determinação da fertilidade em mamíferos (HANRAHAN et al., 2004; GILCHRIST et al., 2008). Em ovinos foi constatado que a ocorrência natural de mutações nos genes que codificam BMP15 e GDF9 aumenta a taxa de ovulação em portadores heterozigotos, e induz a esterilidade em portadores homozigotos devido ao bloqueio da foliculogênese. Tal constatação justifica a variação genética da taxa de ovulação e fertilidade observada entre raças de ovinos e até mesmo entre ovinos de uma mesma raça (HANRAHAN et al., 2004).

Nas células da granulosa, a ação de ambos os fatores é mediada pela ativação da via de sinalização intracelular constituída pelas proteínas SMAD. Ao se ligar aos receptores proteína morfogenética óssea (BMPRII) e ativina semelhante à quinase 5 (ALK5), o GDF9 ativa a via de sinalização constituída pelas proteínas SMAD 2/3. Já a BMP15 promove a ativação das proteínas SMAD 1/5 e 8 ao interagir com os receptores BMPRII e ALK6. Estas SMADs, por sua vez, quando fosforiladas e ativadas, se translocam para o núcleo aonde interagem com reguladores transcricionais e induzem a expressão de genes específicos (GILCHRIST et al., 2008). Nas células do *cumulus*, esta via bioquímica desempenha importante função no controle da expressão de genes implicados na expansão do *cumulus* (DRAGOVIC et al., 2007).

Com relação ao perfil de expressão, ambos os genes que codificam o GDF9 e a BMP15, são expressos durante o bloqueio meiótico oocitário em GV, sendo detectados já no folículo primordial em ovinos (JUENGEL et al., 2002). Ainda nesta espécie, Bebbere et al. (2008) constataram redução gradual do mRNA GDF9 durante a maturação oocitaria, embriogênese inicial e EGA, com persistência até o estadio de mórula. Já o mRNA BMP15 se manteve constante até o estadio embrionário de 8 células após o qual houve significativa redução. Em ambos os casos, não houve

compensação embrionária após a EGA. Por outro lado, os genes que codificam os receptores BMPRII e ALK6 nas células do *cumulus* de COCs ovinos tiveram sua expressão incrementada ao longo da maturação oocitária devido, provavelmente, ao recrutamento promovido pelos seus ligantes específicos. No entanto, não foi constatada variação na expressão do receptor ALK5(KYASSARI et al., 2012).

2.3. PTGS2, PTX3, HSA2, GREMLIN

O *cumulus* consiste num grupo de células da granulosa em íntima associação com o oócito por meio das junções comunicantes GAP. Através da transferência de substâncias de baixo peso molecular, estas células controlam importantes eventos oocitários como a progressão da maturação. Do mesmo modo, importantes funções da granulosa são reguladas pelos fatores sintetizados pelo oócito (TANGHE et al., 2002).

Sob estímulo da onda pré-ovulatória de LH, *in vivo*, e com a remoção dos COCs do ambiente folicular, *in vitro,* ocorre a expansão das células do *cumulus*, a qual envolve a síntese de uma matriz extracelular rica em ácido hialurônico resultando em aumento de volume do complexo *cumulus*-oócito. Este processo está diretamente implicado no reinício meiótico oocitário devido à perda das junções GAP e interrupção da transferência de moléculas reguladoras para o oócito (ISOBE et al., 1998).

A mucificação do *cumulus* é imprescindível ainda para ovulação uma vez que facilita a extrusão e captura do oócito pelas fimbrias e auxilia seu deslocamento até o sítio de fertilização no infundíbulo. Além disso, auxilia a fertilização por criar um microambiente favorável à capacitação, reação acrossomal e penetração do espermatozoide no oócito (TANGHE et al., 2002). De acordo com Schoenfelder & Einspanier (2003) a matriz extracelular rica em ácido hialurônico também confere proteção ao oócito e serve como reservatório de fatores de crescimento.

In vivo, ao interagir com seus receptores específicos nas células foliculares, o LH induz a síntese de peptídeos ampiregulina (AREG), epiregulina (EREG) e betacelulina (BTC), os quais pertencem à família dos fatores de crescimento epidermal (EGF) (HSIEH et al., 2005). Estes fatores EGF-like, por sua vez, interagem com seus receptores nas células do *cumulus* e, através da ativação das MAPKs, sinalizam a expressão de genes implicados na expansão do *cumulus,* entre os quais se destacam: HAS2, COX2, PTX3 e GREM1 (PANGAS et al., 2004; SHIMADA et al., 2006).

A expressão destes genes, no entanto, depende não somente da ação isolada dos peptídeos AREG, EREG, e BTC, mas também do estímulo gerado pelos fatores

parácrinos secretados pelo oócitos e da interação entre estas moléculas. Evidências sugerem que o GDF9 e BMP15 estimulam a expressão de receptores para os fatores EGF-like no *cumulus* por meio da ativação das proteínas SMAD (DRAGOVIC et al., 2007).

Dentre os genes alvo dos fatores EGF-like, o HAS2 é considerado crucial uma vez que codifica a enzima hialurona sintetase 2 responsável pela síntese do ácido hialurônico que consiste no arcabouço viscoelástico da matriz extracelular implicada na mucificação do *cumulus* (SCHOENFELDER & EINSPANIER, 2003). Com relação a COX2 também designada prostaglandina endoperoxidase sintetase 2 (PTGS2), embora sua ação específica não esteja totalmente determinada, se reconhece sua implicação na síntese de prostaglandina E2 (PGE2) e importância na expansão do *cumulus* (NUTTINCK et al., 2002). Além disso, Lim et al. (1997) observaram que a deleção do gene COX2 em camundongos resulta em falhas na ovulação, maturação oocitária e fertilização. Quanto ao perfil de expressão, Nuttinck et al. (2002) detectaram incremento progressivo do mRNA COX2 ao longo da maturação com máxima expressão ao final de 24 horas de cultivo, em concomitância com a constatação da expansão do *cumulus*. Este padrão também foi acompanhado por aumento da concentração de PGE2 no meio de cultivo.

A estrutura e estabilidade da matriz extracelular são mantidas ainda pela proteína PTX3, cuja expressão nas células do *cumulus* também é regulada pelos fatores EGF-like. Evidências demonstram que na ausência desta proteína não ocorre formação da matriz extracelular, resultando em falhas na expansão do *cumulus* e infertilidade (SALUSTRI et al., 2004). Em COCs de bovinos maturados *in vitro* foi constatado pico de expressão com 12 horas de cultivo o qual se manteve até o final de 22 horas (CAIXETA, 2012). Além disso, a PTX3 exerce atividade antiproteolítica protegendo o oócito e a matriz extracelular da ação de proteases sintetizadas pelo próprio oócito e células do *cumulus* algumas horas após a ovulação (SALUSTRI et al., 2004).

Com relação ao GREM1, sua expressão também é rapidamente induzida pelo estímulo gonadotrófico e se mantém ao longo de toda maturação oocitária, sendo o GDF9 a principal molécula regulatória deste processo. No entanto, apesar do envolvimento na expansão do *cumulus*, na prevenção da luteinização prematura, no desenvolvimento folicular, e na ovulação, a função e modo de ação exatos do GREM 1 ainda não estão definidos (PANGAS et al., 1004).

2.4. BAX e BCL2

O desenvolvimento é controlado não apenas pela proliferação e diferenciação celular, mas também pela eliminação de células indesejáveis ou prejudiciais. Este processo de morte celular programada denominado apoptose é determinado geneticamente e envolve alterações morfológicas evidenciadas pela desestabilização da membrana plasmática, agregação da cromatina, condensação e fragmentação nuclear e citoplasmática, acometendo qualquer tipo celular (LIU et al., 2000).

Nos ovários, a apoptose está implicada tanto na degeneração e perda de quantidade expressiva de oogônias e oócitos ao longo da oogênese como no desenvolvimento folicular normal (DE FELICI et al., 1999). Dentre os transcritos envolvidos neste processo se destacam o Bcl-2, com função anti-apoptótica, e o Bax, com função pró-apoptótica, sendo que o equilíbrio entre estas moléculas determina se a célula permanecerá ou não viável (YANG & RAJAMAHENDRAN, 2002).

A ação anti-apoptótica do Bcl-2 é demonstrada pela quantidade significantemente menor de folículos primordiais em camundongos com deleção deste gene (RATTS et al.,1995) e pela menor porcentagem de atresia folicular quando este gene está superexpresso (MORITA et al., 1999). No sistema de maturação *in vitro*, estes mesmos autores constaram incidência marcadamente menor de apoptose em oócitos com elevada expressão de Bcl-2. Além disso, a superexpressão deste gene conferiu ao oócito resistência e proteção contra quimioterápicos adicionados ao meio de cultivo (MORITA et al., 1999). Já a ação pró-apoptótica do Bax é evidenciada pela relação direta entre a expressão deste gene e a elevada perda de oócitos por apoptose. Evidências demonstram ainda que oócitos com menor expressão do gene Bax apresentam maior habilidade em se manter viável com menor probabilidade de degeneração (DE FELICI et al.,1999).

Esta mesma relação foi observada em embriões por Liu et al. (2000) de modo que aqueles considerados não viáveis e fragmentados apresentaram significativa prevalência de Bax em contraste aos embriões viáveis nos quais apenas o Bcl2 foi constatado. Sendo assim, o adequado estoque materno de mRNA Bcl-2 assim como o restabelecimento da transcrição deste gene a partir da EGA confere ao embrião competência para suprimir a ação do Bax e prosseguir seu desenvolvimento de maneira adequada. Segundo este mesmos autores, a proporção Bcl2:Bax e consequente viabilidade ou morte celular é influenciada ainda por estímulos externos e/ou alterações intracelulares. Aspectos intrínsecos à produção *in vitro* de embriões como a

manipulação dos gametas e condições de cultivo também favorecem a apoptose (JURISICOVA et al., 1998).

3. Inibidores da maturação nuclear oocitária *in vitro*

Quando os oócitos são removidos do ambiente folicular, ocorre reinício espontâneo da meiose independente da maturidade citoplasmática (PINCUS & ENZMANN, 1935). Na tentativa de restabelecer a sincronia entre a maturação nuclear e citoplasmática e consequentemente melhorar o potencial de desenvolvimento oocitário *in vitro*, a pré-maturação dos COCs sob condições capazes de impedir a progressão meiótica tem sido proposta com intuito de fornecer tempo adicional para que os oócitos sofram as modificações necessárias para suportar as posteriores etapas de desenvolvimento (PONDERATO et al., 2001). Diversos inibidores da meiose veem sendo estudados em diferentes espécies animais (LE BEUX et al., 2003).

Segundo Gilchrist (2008), o tempo de inibição meiótica pode se estender até 24 horas sem comprometer o potencial de desenvolvimento oocitário. A reversibilidade do bloqueio meiótico e consequente retomada da maturação nuclear depende ainda do posterior cultivo *in vitro* dos COCs em meio de maturação livre de inibidores, sendo que os oócitos devem alcançar o estadio de MII com correta organização do fuso meiótico e adequada expansão das células do *cumulus* (MOTLIK et al., 1998).

De acordo com Le Beux et al. (2003), os inibidores da meiose devem apresentar três importantes características: promover eficaz bloqueio da maturação nuclear, apresentar efeito totalmente reversível, e não alterar a capacidade de desenvolvimento dos oócitos. A determinação da adequada concentração do fármaco empregado e do tempo de exposição dos COCs ao inibidor é imprescindível para obtenção de melhores resultados uma vez que tanto o potencial de ação quando a toxicidade são dose e tempo-dependentes (ALBARRACÍN et al., 2005)

3.1. Roscovitina

A Roscovitina é um inibidor específico das quinases dependentes de ciclina, que compete com o ATP pelo seu sítio de ligação na subunidade catalítica do fator promotor da maturação, impedindo a desfosforilação desta subunidade e consequente ativação do MPF (MEIJER et al., 1997). De acordo com Wu et al. (2002), devido a especificidade de sua ação, a roscovitina não suprime a síntese e fosforilação de outras proteínas

implicadas na progressão da maturação oocitária. No entanto, quando em altas concentrações, podem interferir na atividade da MAPK (MEIJER et al., 1997).

Vigneron et al. (2004) assim como Donnay et al. (2004) e Barretto et al. (2007) constataram que a roscovitina na concentração de 25 uM promoveu eficaz e reversível bloqueio meiótico em COCs de bovinos. Já Albarracín et al. (2005), relataram que o bloqueio meiótico promovido por este fármaco é dose-dependente, sendo que melhores resultados foram obtidos na presença 50 e 100 µM de roscovitina. No entanto, devido a constatação de alterações estruturais nos COCs cultivados *in vitro* por 24 horas na presença de 100 µM de roscovitina, estes autores estabeleceram 50 µM como a concentração mínima capaz de promover adequada e reversível inibição meiótica. Alterações ultraestruturais irreversíveis como degeneração das células do *cumulus*, inchaço das mitocôndrias, redução do núemro de grânulos corticais e aumento da quantidade de vesículas com conteúdo eletrodenso também foram relatadas em COCs de bovinos (LONERGAN et al., 2003) e ovinos (CROCOMO et al., 2013) tratados com roscovinita nas concentrações de 125 e 100 µM, respectivamente.

Esta discrepância entre os autores com relação à concentração ideal de roscovitina para o bloqueio meiótico pode estar relacionada à espécie animal em estudo, à origem dos ovários, às condições de cultivo *in vitro* dos COCs, e à metodologia empregada para avaliação dos COCs, entre outros fatores (ALBARRACÍN et al., 2005).

Ponderato et al. (2001) constataram que a pré-maturação dos COCs de bovinos com roscovitina não melhorou e também não reduziu o potencial de desenvolvimento embrionário. Já Hashimoto et al. (2002) relataram melhores taxas de desenvolvimento embrionário e sugeriram que modificações nas condições de cultivo *in vitro* podem melhorar os resultados. Donnay et al. (2004), no entanto, constataram significativo decréscimo na taxa de blastocistos, e atribuíram-no ao provável envelhecimento celular, devido à exposição prolongada dos COCs às condições de cultivo *in vitro.*

Trabalhando com COCs de bovinos, Adona & Leal (2004) verificaram que o cultivo *in vitro* por 18 a 20 horas após 24 horas de inibição meiótica com roscotivina foi suficiente para que mais de 80% dos oócitos atingissem o estadio de MII, de maneira semelhante ao relatado por Donnay et al. (2004), o que demonstra que a maturação nuclear é acelerada após o tratamento com este fármaco.

A razão para esta aceleração ainda não está totalmente esclarecida, mas acredita-se que durante o bloqueio da meiose, os oócitos acumulam fatores relacionados ao controle da progressão do ciclo celular e que nem todos os eventos envolvidos na

maturação são bloqueados (VIGNERON et al., 2004). As conseqüências desta aceleração parecem não ser prejudiciais ao desenvolvimento embrionário, já que alguns estudos relatam taxas de desenvolvimento embrionário semelhantes às obtidas em oócitos que não foram inibidos (PONDERATO et al., 2001).

Apesar das inúmeras pesquisas, o emprego de substâncias farmacológicas e fisiológicas para inibir a meiose oocitária ainda não tem aplicabilidade prática, uma vez que não promovem significativo incremento no potencial de desenvolvimento embrionário, sendo destinados, até o momento, apenas como ferramenta de estudo dos mecanismos envolvidos na aquisição de competência oocitária.

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HIPÓTESE

A manutenção temporária dos complexos *cumulus*-oócitos de ovinos no estadio de vesícula germinativa com o uso da roscovitina confere tempo adicional para a transcrição de genes, no oócito e nas células *cumulus*, essenciais para aquisição de competência oocitária, resultando em maior potencial de desenvolvimento embrionário *in vitro*.

OBJETIVOS

1- Avaliar o efeito das gonadotrofinas e do soro fetal bovino, presentes no meio de maturação, sobre o potencial de inibição meiotica reversível da roscovitina e sua ação sobre a expansão do *cumulus* em COCs de ovinos cultivados *in vitro*;

2- Avaliar a eficiência da roscovitina na inibição reversível da meiose em oócitos de ovinos cultivados *in vitro* na presença e ausência de óleo mineral, assim como avaliar seus efeitos e a interferência do óleo sobre a expansão do *cumulus*;

3- Avaliar o potencial de inibição meiótica reversível da roscovitina em oócitos de ovinos ao longo do cultivo *in vitro*, assim como investigar o efeito do tempo de exposição dos COCs à roscovitina sobre o processo de expansão do *cumulus*;

4- Avaliar o efeito da inibição meiótica promovida pela roscovitina sobre o perfil de expressão dos genes SOD, NLRP5, ZAR1, GDF9, BMP15, Bax e Bcl-2, nos oócitos, e PTX3, GREM1, PTGS2, GLUT1, ALK5, ALK6, Bax e Bcl-2 nas células *do cumulus*;

5 - Avaliar o potencial de desenvolvimento embrionário *in vitro* e a qualidade dos embriões obtidos após o tratamento dos COCs de ovinos com roscovitina.

CAPÍTULO 1

Trabalho submetido à revista "Small Ruminant Research"

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Meiotic arrest of sheep oocytes using roscovitine under different medium compositions

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Abstract

Attempts to maintain the meiotic arrest under suitable culture conditions have been performed in order to improve the quality of oocytes *in vitro* matured. Therefore, our study aimed to evaluate the potential of roscovitine, an inhibitor of cyclin-dependent kinases, to reversibly arrest the meiosis of sheep oocytes in medium supplemented with gonadotropins and/or serum. *Cumulus*-oocyte complexes (COCs) were treated, for 20 h, with 75 µM roscovitine (Rosco) in basic maturation medium (Basic) containing 10% fetal bovine serum (FBS) and 0.1 IU/mL FSH plus 0.1 IU/mL LH (Enriched). After this, COCs were *in vitro* matured (IVM) for a further 18 h in the presence of gonadotropins and serum. At 20 and 38 h of culture, *cumulus* expansion and nuclear maturation were assessed under stereomicroscope and by Hoechst 33342 staining, respectively. Our findings demonstrate that total *cumulus* expansion occurred only in medium containing gonadotropins and serum. In the presence of roscovitine, however, the *cumulus* expansion was inhibited in a not fully reversible manner, independently of medium composition. The GV rate in the Rosco FBS group (64.6%) was significantly higher than that of Rosco Enriched (51.8%); however, both did not differ from that observed in the Rosco Basic (56.2%). In all roscovitine treatments, the meiotic arrest was reversible after the additional culture for 18 h in inhibitor-free medium. Besides, significant proportion of oocytes from Basic, FBS and Enriched groups reached the MII stage after 20 and 38 h of culture. Therefore, we can infer that, regardless of medium composition, the roscovitine reversibly inhibit the meiosis in sheep oocytes. However, its action on *cumulus* expansion is not fully reversible.

Key words: Inhibitor, Nuclear maturation, *Cumulus* expansion, Gonadotropin, Serum, Ovine

1. Introduction

In most mammals, the oocyte becomes arrested at diplotene stage of prophase-I until shortly before the ovulation when, in response to LH surge, the meiosis resumes and progresses until the metaphase II (Mehlmann, 2005). Concomitantly to these nuclear events, structural and molecular changes essential to acquisition of developmental competence, which involve the redistribution of cytoplasmic organelles and storage of mRNA and proteins, also occur (Ferreira et al., 2009). These interlinked processes of nuclear and cytoplasmic maturation are directly controlled by regulatory

molecules transferred from *cumulus* cells to oocyte through gap junction communications (Barrett and Albertini, 2010).

In contrast, the removal of oocytes from their follicles and transfer to a suitable culture medium induces the spontaneous meiotic resumption independently of the stage of cytoplasmic maturation (Pincus and Enzmann, 1935). However, only the correct dynamic of chromosomes separation is not enough to ensure the subsequent embryo development (Ferreira et al., 2009). Besides, oocytes destined to *in vitro* maturation display a heterogeneous developmental potential since they are retrieved from follicles at diverse stages of folliculogenesis (Gharibi et al., 2013).

In this context, temporary arrest of meiosis with pharmacological inhibitors has been proposed as a promising alternative to restore the synchrony between nuclear and cytoplasmic maturation, increase the homogeneity of oocyte population and improve the quality of oocytes *in vitro* matured (Mermillod et al., 2000; Gharibi et al., 2013). Due to its specific action on M-phase promoting factor, the roscovitine, an inhibitor of cyclin-dependent kinases (CDK), has been used to reversibly block the spontaneous meiosis resumption in several animal species as bovine (Mermillod et al., 2000; Lagutina et al., 2002), goat (Han et al., 2006), cat (Sananmuang et al., 2010), pig (Marchal et al., 2001) and horse (Franz et al., 2003).

In most studies, the inhibitory action of roscovitine is analyzed in *cumulus*-oocytes complexes cultured in the absence of LH, FSH and serum since these substances facilitate and accelerate the meiosis progression *in vitro* (Mattioli et al., 1991; Accardo et al., 2004). The negative interference of hormones and serum on meiotic arrest promoted by CDK inhibitors was already reported by Barretto et al. (2010) and Marques et al. (2011) in bovine oocytes. In sheep oocytes, however, the effect of roscovitine on nuclear maturation and its possible interaction with certain components of medium has not been investigated.

So, considering that, the present study aimed to evaluate the potential of roscovitine to reversibly inhibit the meiosis of sheep oocytes *in vitro* cultured in the presence and absence of gonadotropins and bovine fetal serum. The effect of this inhibitor on *cumulus* cells expansion under different medium compositions was also examined.

2. Materials and Methods

This study was performed at the School of Veterinary Medicine and Animal Science of UNESP, Botucatu, Brazil. All chemicals 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 the laboratory within 1-2 h in sterile saline solution (0.9 % NaCl) at 32 ºC. All visible follicles with a diameter of 2-6 mm were aspirated with a 20 gauge needle attached to a 10 mL syringe containing 0.5 mL pre-incubated Hepes-buffered TCM199 supplemented with 50 IU/mL heparin. Only COCs with several intact *cumulus* cell layers and homogeneous ooplasm were selected under stereomicroscopy (Shirazi et al., 2010).

2.2. Oocyte culture and roscovitine treatment

After washes in Hepes-buffered TCM199, the selected COCs were transferred to maturation medium and *in vitro* cultured for 20 h at 5% CO_2 and 38.5 ºC. According the medium composition, COCs were randomly allocated into different experimental groups:

Basic: COCs were cultured in basic maturation medium comprised of TCM 199 with Earle's salts, 0.3 mM sodium pyruvate, 75 µg/mL penicillin and 100 µM cysteamine;

FBS: basic maturation medium plus 10 % fetal bovine serum (Gibco 10437);

Enriched: basic maturation medium supplemented with 10 % fetal bovine serum, 0.1 IU/mL FSH (Folltropin®, Bioniche Co.) and 0.1 IU/mL LH (Lutropin-V®, Bioniche Co., Belleville, ON, Canada);

Rosco Basic: same medium of Basic group added of 75 µM roscovitine;

Rosco FBS: same medium of FBS group added of 75 µM roscovitine;

Rosco Enriched: same medium of Enriched group added of 75 µM roscovitine

In each replicate, about 20-25 COCs from each experimental group were culture in 100 µL droplets of maturation medium. Based in our preliminary tests, the *in vitro* culture was performed in 96 well plates without mineral oil overlay. The stock solution of roscovitine (1 mg/mL) was prepared in dimethylsulphoxide, aliquoted and stored at -20 ºC until use.

2.3. Reversion of meiotic inhibition

After the *in vitro* culture for 20 h, COCs from each experimental group were washed in Hepes-buffered TCM 199 and *in vitro* matured, for a further 18 h, in basic maturation medium supplemented with 10% fetal bovine serum, 0.1 IU/ml FSH and 0.1 IU/mL LH. The IVM was performed in 96 well plates without oil overlay at 5 % CO_2 and 38.5 ºC.

2.4. Assessment of cumulus expansion

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

2.5. Assessment of oocyte chromatin organization

Oocytes were stripped from their *cumulus* cells by repeated pipetting, fixed for 30 min in 4 % paraformaldehyde and 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 as germinal vesicle (GV) germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) (Shirazi et al., 2010). The anaphase I and telophase I stages were considered as MII, because they are intermediate stages of a dynamic process. The oocytes with the extrusion of one or two polar body and the presence of one or two pronucleus were classified as parthenogenetic (Marshall et al., 1998). Those with altered nuclear structure were classified as degenerate.

2.6. Experimental design

The experimental design was completely randomized with factorial arrangement of treatments (3x2) conducted in two experiments, each one with six experimental groups and five replicates for each parameter assessed.

Experiment 1- Effect of medium composition on inhibitory action of roscovitine

After 20 h of *in vitro* culture in the presence and absence of hormone, fetal bovine serum and roscovitine, COCs from each experimental group (Basic, FBS, Enriched, Rosco Basic, Rosco FBS, Rosco Enriched) were evaluated for *cumulus* expansion

degree and nuclear maturation stage under stereomicroscope and by Hoechst 33342 staining, respectively. An additional sample of immature oocytes was stained immediately after removal from follicle to evaluate the meiosis stage before *in vitro* culture (0 h).

Experiment 2- Effect of medium composition on reversion of meiotic inhibition

To evaluate the reversibility of inhibitory action of roscovitine after 20 h of culture under different medium compositions, COCs from each experimental group were *in vitro* matured for a further 18 h in inhibitor-free medium supplemented with gonadotropins. The *cumulus* expansion degree and nuclear status were evaluated at the end of culture for 38 h.

2.7. Statistical analysis

The data were transformed into square root of x + 0.5 and subjected to analysis of variance according to completely randomized design. The means were compared by the Tukey test at 5% probability.

3. Results

Experiment 1- Effect of medium composition on inhibitory action of roscovitine

According to table 1, all immature COCs evaluated immediately after the follicular aspiration (0 h) had compact *cumulus* cells without signs of expansion. After 20 h of culture, the absence of *cumulus* expansion prevailed in the Basic (92.7%), Rosco Basic (86.0%), FBS (84.9%), Rosco FBS (94.7%) and Rosco Enriched (79.3%) groups while the total *cumulus* expansion practically was not observed in any of these groups. In contrast, in the Enriched group there was predominance of total *cumulus* expansion (69.4%) while rate significantly lower of COCs had compact *cumulus* cells (8.2%). The rate of COCs with partial *cumulus* expansion did not significantly differ among experimental groups.

As shown in table 2, the analysis of nuclear status by Hoescht staining revealed that almost all immature oocytes (87.7%) were at GV before the *in vitro* culture (0h). Despite the evident decrease of the GV rate during the *in vitro* culture for 20 h, the proportion of oocytes from roscovitine treatment (Rosco Basic, Rosco FBS and Rosco Enriched) kept at this meiotic stage was significantly higher than that observed in the groups cultured without inhibitor (Basic, FBS, Enriched). In the Rosco FBS, the GV

rate was significantly higher than that recorded in Rosco Enriched; however, both did not differ from that observed in Rosco Basic.

In comparison, significantly higher rate of oocytes from Basic, FBS and Enriched groups reached the MII stage, while only 3.8%, 1.0%, and 8.2% of oocytes from Rosco Basic, Rosco FBS and Rosco Enriched were at this same stage after *in vitro* culture for 20 h. The absence of significant difference among Basic, FBS and Enriched groups with respect to the MII rate indicates the medium composition did not interfere in the meiosis progression of sheep oocytes. Likewise, the rate of oocytes at GVBD, MI and degenerate did not significantly differ among experimental groups (table 2).

Experiment 2- Effect of medium composition on reversion of meiotic inhibition

According to table 3, culture for a further 18h in inhibitor-free medium supplemented with gonadotropins and serum allowed the total *cumulus* expansion in high percentage of COCs from Basic, FBS and Enriched groups which significantly differed from that observed in the others experimental groups. In contrast, significantly higher rate of COCs from roscovitine treatment (Rosco Basic, Rosco FBS and Rosco Enriched) showed partial *cumulus* expansion after 18 h of IVM. Besides, in Rosco Basic and Rosco Enriched, the rate of COCs with compact *cumulus* cells was similar to that with partial expansion.

As shown in table 4, the rate of oocytes at MII after the additional culture for 18 h inhibitor-free medium was similar among experimental groups and significantly higher than that observed in the others meiotic stages. The proportion of oocytes at GV, GVBD and MI was similar among them and did not significantly differ among experimental groups. Likewise, no significant difference was observed among experimental groups with respect to the rate of degenerate and parthenogenetic oocytes.

4. Discussion

Despite the spontaneous meiotic resumption, most oocytes destined to *in vitro* maturation have not undergone all structural and molecular changes necessary to competence acquisition (Gilchrist and Thompson, 2007). In this context, CDK inhibitors, as roscovitine, have been used in several animal species to maintain the meiotic arrest *in vitro* and, consequently, provide additional time to oocyte complete its capacitation (Mermillod et al., 2000; Marchal et al., 2001; Han et al., 2006; Franz et al., 2003; Sananmuang et al., 2010). The efficiency of meiotic arrest, however, depends not only on the inhibitor concentration and exposure time but also of the culture conditions

(Han et al., 2006). Despite the importance of gonadotropins and serum to proper oocyte maturation (Kito and Bavister, 1997), these substances may negatively affect the inhibition potential of roscovitine, as already demonstrated in bovine by Barretto et al. (2011) and Marques et al. (2011). However, in sheep, there is no similar information.

Based on that, our study was developed in order to establish the best culture condition, in terms of medium composition, able to allow the maximum efficiency of meiotic inhibition using roscovitine without detrimental effect on sheep COCs. This is the first report about the effect of this inhibitor on nuclear maturation and *cumulus* expansion in sheep. Due to possible lipo-solubility of roscovitine (Phillips et al., 2002), COCs were cultured without oil overlay to avoid alterations of the inhibitor concentration. Besides, we choose to work with 96 well plates aiming to mimic the volume of medium and ratio of COCs (25 COCs/ 100 µL) used in the conventional maturation system. A humid microenvironment was created, as suggested by Gasperin et al. (2010), in order to maintain the medium osmolality around of 294 mOsm.

All COCs selected to this study had compact *cumulus* cells which indicate that, probably, they have not resumed the meiosis *in vivo* (Dekel et al., 1981). The high proportion of oocytes at GV stage (87.7%) soon after follicle aspiration also suggest that the time interval between transport of ovaries, recovery and selection of COCs was adequate and did not induce the meiotic resumption before the *in vitro* culture. Similar results were observed in goat (Han et al., 2006), pig (Le Beux et al., 2003) and bovine (Barretto et al., 2010) oocytes.

The predominance of COCs with compact *cumulus* cells (92.7%) after culture for 20 h without gonadotropins and serum is in accordance with the reported by Cotterill et al. (2012) and Vigneron et al (2003) in sheep and bovine COCs, respectively, cultured in TCM199 alone. This result reinforces the importance of the appropriate medium supplementation to support the *cumulus* expansion (Chen et al., 1994; Kito and Bavister, 1997). Our findings also demonstrate that fetal bovine serum alone was not enough to promote the *cumulus* expansion, as observed by Mattioli et al. (1991) in pig COCs. However, when the culture was performed in the presence of LH, FSH and serum, significantly high proportion of COCs showed total *cumulus* expansion. Similar results were reported by Cotterill et al. (2012) and Mattioli et al. (1991) in sheep and pig COCs culture under this same condition.

According to Kito and Bavister (1997), the effects of gonadotropins not only on *cumulus* expansion but also on nuclear maturation are modulated by serum. *In vitro*,

gonadotropins stimulate the synthesis of hyaluronic acid by COCs. In the presence of serum, some of its components bind to hyaluronic acid and retains it within the complexes *cumulus*-oocytes, allowing the *cumulus* expansion (Daen et al., 1995). Chen et al. (1994) also demonstrated that the synergistic action of LH and FSH is required in this process. Therefore, the results obtained in the present study reinforce the importance of the interaction between gonadotropins and serum to proper *cumulus* expansion.

Our findings also demonstrate that roscovitine affected the *cumulus* expansion since significantly high rate of COCs treated with this inhibitor had compact *cumulus* cells after 20 h of culture even in the enriched medium. The prevalence of partial *cumulus* expansion after culture for a further 18 h with LH, FSH and serum indicates that roscovitine action on *cumulus* cells was not fully reversible. Likewise, irreversible inhibition of *cumulus* expansion was observed in pig COCs treated with 50 µM roscovitine (Romar et al., 2006). However, in bovine COCs, Vigneron et al. (2003) reported that inhibitory action of 25µM roscovitine on *cumulus* cells was fully reversible. The reversibility of roscovitine inhibition on *cumulus* cells is probably related to inhibitor concentration and time exposure used (Sananmuang et al., 2010; Han et al., 2006; Crocomo et al., 2013). According to Schoevers et al. (2005), it remains unclear if the effect of this inhibitor occurs via oocyte or directly via *cumulus* cells.

Although higher percentage of oocytes cultured with fetal bovine serum alone (FBS) or in association with gonadotropins (enriched) had reached the MII at the end of 20 h, it was not significantly different from that observed in the basic group. The meiosis progression even in the absence of serum and gonadotropins was also reported by Cotterill et al. (2012) in sheep oocytes; however, the MII rate (69.5%) reached was significantly lower than that observed in supplemented medium (85%). Besides, these same authors demonstrated that, with or without serum supplementation, the LH and FSH in association supported high MII rates. In bovine, however, Sanbuissho and Threlfall (1990) reported that FBS alone favored the meiotic oocyte maturation, while the addition of FSH and LH did not improve it.

These results together demonstrate that serum and gonadotropins are not essential to resumption and progression of nuclear maturation *in vitro*. However, the presence of these stimulatory substances facilitates and accelerates this process, allowing better MII rates. Besides, the appropriate medium supplementation favors the *cumulus* expansion and improves the cytoplasmic maturation which is essential to

support the subsequent embryo development (Mattioli et al. 1990; Accardo et al., 2004, Cotterill et al., 2012). We also demonstrate in this study that nuclear maturation was not depend upon *cumulus* expansion in sheep COCs, as already observed in bovine (Sirard et al. 1988) and goat (Han et al., 2006).

In contrast to that reported by Barretto et al. (2010) and Marques et al. (2011) in bovine, gonadotropins and serum seem not interfere in the potential of roscovitine to arrest the meiosis of sheep oocytes since the GV rate in the presence of these substances was similar to that observed in medium without supplementation. Similar GV rate (63%) was observed by Lu et al. (2013) in sheep COCs treated with 150 µM butyrolactone in medium containing gonadotropin and serum. Likewise, Marchal et al. (2001) reported that 92% of pig oocytes were arrested at GV with 25 µM roscovitine in the presence of EGF and serum. In bovine, different concentrations of roscovitine also inhibited about 80% of oocytes in serum-supplemented medium (Kaedei et al., 2010). According to Saeki et al. (1997), the presence of stimulatory substances, as hormones and serum, during the meiotic arrest creates a more suitable environment to promote the oocyte developmental competence.

The significant MII rate observed after the additional culture for 18h in inhibitor-free medium containing gonadotropins indicates that, independently of medium composition, the meiotic inhibition promoted by roscovitine was reversible. Similar MII rate was reported in cat and sheep oocytes pre-matured with 25 µM roscovitine and 150 µM butyrolactone, respectively (Sananmuang et al., 2010; Lu et al., 2013). However, in bovine, goat and pig, higher proportion of oocytes treated with roscovitine reached MII after the *in vitro* maturation (Mermillod et al., 2000; Han et al., 2006; Romar et al., 2006). According to Han et al. (2006), the discrepancies among authors regarding the efficiency of meiotic inhibition and reversibility of this arrest is related not only to the concentration and exposure time to inhibitor but also to the particularities of each animal species studied.

It has been also reported in several species that meiosis progression is accelerated after removal of roscovitine (Marchal et al., 2001; Han et al., 2006; Sananmuang et al., 2010; Kaedei et al., 2010). According to Lagutina et al. (2002), bovine oocytes pre-matured with this inhibitor reached the MII stage about 4 h before than untreated oocytes. It occurs probably due to accumulation of developmentally relevant factors during the meiosis block and suggests that meiotic step may be shorter (Han et al., 2006; Sananmuang et al., 2010). Based on that, it was established that 18h

of culture with gonadotropins could be sufficient to oocyte complete the meiosis, as already reported by Máximo et al. (2012) in sheep. However, the significant incidence of parthenogenesis observed in our study suggests that, probably, the total culture time (38 h) was excessive (Schoevers et al., 2005). In fact, Shirazi et al. (2009) reported that aged ovine oocytes are more susceptible to spontaneous parthenogenetic activation. Despite this, the culture conditions were suitable since the rate of degenerate oocytes was low.

5. Conclusion

In conclusion, we can infer that gonadotropins and serum does not affect the potential of roscovitine to reversibly arrest the meiosis of sheep oocytes. Besides, independently of medium composition, the action of roscovitine on *cumulus* cells is not fully reversible. Further investigations have been performed to evaluate the effect of this inhibitor and benefits of these stimulatory substances on oocyte competence and *in vitro* embryo development.

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Table 1. Effect of roscovitine at 75 µM (Rosco) on c*umulus* expansion of sheep COCs *in vitro* cultured for 20 h in basic maturation medium (Basic) supplemented with fetal bovine serum (FBS) and gonadotropins (Enriched). Immature: 0 h of culture.

Groups	No. COC	*Cumulus* expansion degree, *n* (%)		
		Total	Partial	Absence
Immature	122	0 (0.0) ^{bB}	0 (0.0) ^{bB}	122 (100.0) ^{aA}
Basic	110	2 (1.8) ^{bB}	6 (5.5) ^{abB}	102 (92.7) ^{aA}
Rosco Basic	136	0 (0.0) ^{bC}	19 (14.0) ^{abB}	117 (86.0) ^{aA}
FBS	106	0 (0.0) ^{bC}	16 (15.1) ^{aB}	90 (84.9) ^{aA}
Rosco FBS	114	0 (0.0) ^{bB}	6 (5.3) ^{abB}	108 (94.7) ^{aA}
Enriched	98	68 (69.4) ^{aA}	22 (22.4) ^{aB}	8 (8.2) ^{bB}
Rosco Enriched	121	0 (0.0) ^{bC}	25 (20.7) ^{aB}	96 (79.3) ^{aA}

* Differences indicated by lowercase letters in column and capital letters in the line (P<0.05).

Table 2. Effect of roscovitine at 75 µM (Rosco) on nuclear maturation of sheep oocytes *in vitro* cultured for 20 h in basic maturation medium (Basic) supplemented with fetal bovine serum (FBS) and gonadotropins (Enriched). Immature: 0 h of culture.

Groups	No. COC	Nuclear Maturation Stage, *n* (%)				
		GV	GVBD	MI	MII	Deg
Immature	122	107 (87.7) ^{aA}	13 (10.7) ^{aB}	0 (0.0) ^{bB}	0 (0.0) ^{bB}	2(1.6) ^{aB}
Basic	94	21 (22.3) ^{dB}	7 (7.4) ^{aC}	14 (14.9) ^{aBC}	47 (50.0) ^{aA}	5(5.3) ^{aC}
Rosco Basic	130	73 (56.2) ^{bcA}	14 (10.8) ^{aB}	21 (16.2) ^{aB}	5 (3.8) ^{bB}	17 (13.1) ^{aB}
FBS	88	11 (12.5) ^{dB}	7 (8.0) ^{aB}	9 (10.2) ^{abB}	56 (63.6) ^{aA}	5 (5.7) ^{aB}
Rosco FBS	96	62 (64.6) ^{bA}	11 (11.5) ^{aBC}	11 (11.5) ^{abBC}	1 (1.0) ^{bC}	11 (11.5) ^{aB}
Enriched	89	13 (14.6) ^{dB}	6 (6.7) ^{aB}	11 (12.4) ^{aB}	51 (57.3) ^{aA}	8 (8.3) ^{aB}
Rosco Enriched	110	57 (51.8) ^{cA}	17 (15.5) ^{aB}	19 (17.3) ^{aB}	9 (8.2) ^{bB}	8 (7.3) ^{aB}

* Differences indicated by lowercase letters in column and capital letters in the line (P<0.05). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II, Deg: degenerate.

Table 3. *Cumulus* expansion of sheep COCs treated for 20 h with 75 µM roscovitine (Rosco) in basic maturation medium (Basic) containing fetal bovine serum (FBS) and gonadotropins (Enriched) followed by 18 h of *in vitro* maturation in enriched medium.

Groups	No. COC	*Cumulus* expansion degree (%)		
		Total	Partial	Absence
Basic	111	81 (73.0) ^{aA}	17 (15.3) ^{cdB}	13 (11.7) ^{abB}
Rosco Basic	118	0 (0.0) ^{bB}	70 (59.3) ^{abA}	48 (40.7) ^{aA}
FBS	99	70 (70.7) ^{aA}	26 (26.3) ^{bcdB}	3 (3.0) ^{bB}
Rosco FBS	95	6 (6.3) ^{bB}	67 (70.5) ^{aA}	22 (23.2) ^{abB}
Enriched	104	94 (90.4) ^{aA}	9 (8.7) ^{dB}	1 (1.0) ^{bB}
Rosco Enriched	107	0 (0.0) ^{bB}	53(49.5) ^{abcA}	54 (50.5) ^{aA}

* Differences indicated by lowercase letters in column and capital letters in the line (P<0.05).

Table 4. Meiotic progression of sheep COCs treated for 20 h with 75 µM roscovitine (Rosco) in basic maturation medium (Basic) containing fetal bovine serum (FBS) and gonadotropins (Enriched) followed by 18 h of *in vitro* maturation in enriched medium.

Groups	No. COC	Nuclear Maturation Stage, *n* (%)					
		GV	GVBD	MI	MII	Deg	PG
Basic	103	12(11.7)^{aB}	4(3.9)^{aB}	7(6.8)^{aB}	61(59.2)^{aA}	6(5.8)^{aB}	13(12.6)^{aB}
Rosco Basic	112	19 (17.0)^{aB}	9 (8.0)^{aB}	11(9.8)^{aB}	55(49.1)^{aA}	10 (8.9)^{aB}	8 (7.1)^{aB}
FBS	88	11(12.5)^{aB}	4 (4.5)^{aB}	1(1.1)^{aB}	52(59.1)^{aA}	6(6.8)^{aB}	14(15.9)^{aB}
Rosco FBS	91	13(14.3)^{aB}	10(11.0)^{aB}	11(12.1)^{aB}	46(50.5)^{aA}	6(6.6)^{aB}	5(5.5)^{aB}
Enriched	87	14(16.1)^{aB}	4(4.6)^{aB}	10(11.5)^{aB}	44(50.6)^{aA}	5(5.7)^{aB}	10(11.5)^{aB}
Rosco Enriched	95	17(17.9)^{aB}	10 (10.5)^{aB}	10 (10.5)^{aB}	41(43.2)^{aA}	9(9.5)^{aB}	8(8.4)^{aB}

* Differences indicated by lowercase letters in column and capital letters in the line (P<0.05). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II; Deg: degenerate; PG: parthenogenetic.

CAPÍTULO 2

Trabalho submetido à revista "Reproduction in Domestic Animals"

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FMVZ-UNESP, Botucatu, Brazil**, Effect of oil overlay on inhibition potential of roscovitine in sheep *cumulus*-oocyte complexes.** Letícia Ferrari Crocomo[1], Wolff Camargo Marques Filho[1], Carla Maria Vela Ulian[2], Naiana da Silva Branchini[2], Denise Theodoro da Silva[3], Camila Louise Ackermann[1], Fernanda da Cruz Landim Alvarenga[1], Sony Dimas Bicudo[1]

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Running head: Effect of oil cover and roscovitine on sheep COCs

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Contents

Inhibitors of cyclin dependent kinases, as roscovitine, have been used to arrest the meiosis *in vitro* in order to improve the oocyte developmental competence. In this study, the possible interference of oil overlay on potential of roscovitine to reversibly arrest the meiosis in sheep oocytes as well as its effects on *cumulus* expansion were evaluated. For this, *cumulus*-oocyte complexes (COCs) were cultured, for 20 h, in TCM 199 with 10% fetal bovine serum (control) containing 75 μM roscovitine (rosco). Subsequently, they were *in vitro* matured (IVM) for a further 18 h in inhibitor free-medium with LH and FSH. The culture was performed in petri dishes under mineral oil (+) or in 96 well plates without oil overlay (-) at 38.5 ºC and 5% CO_2. At 20 and 38 h, the *cumulus* expansion and nuclear maturation were evaluated under stereomicroscope and by Hoechst 33342 staining, respectively. There was no *cumulus* expansion in any group at 20 h. After the additional culture with gonadotropins, significant rate of COCs from both control groups (+/-) had total expansion while in both rosco groups (+/-) the partial expansion prevailed. Among the oocytes treated with roscovitine, 65.2% were kept at GV in the absence of oil overlay while 40.6% of them reached MII under oil cover. This meiotic arrest was reversible and proper meiosis progression also occurred in the control groups (+/-). So, our culture system without oil overlay improve the meiotic inhibition promoted by roscovitine without affect the *cumulus* expansion and the subsequent meiosis progression.

Keywords: Nuclear maturation, *Cumulus* expansion, Meiosis inhibitor, Oil, Ovine

Introduction

Mammalian oocytes begin the meiosis during the fetal life and become arrested at diplotene stage of prophase-I until near ovulation, when meiosis is resumed in response to LH surge (Mehlmann 2005). During the meiotic arrest, oocytes also undergo structural and molecular changes which involve the redistribution of organelles and storage of mRNAs and proteins. *In vivo*, these nuclear and cytoplasmic events occur simultaneously at determined times and ensure oocyte competence to successfully undergo the fertilization and sustain the embryonic development (Watson 2007; Ferreira et al. 2009). The support of *cumulus* cells through transfer of regulatory molecules for oocyte via gap junction communications is essential to control of oogenesis and maturation (Eppig et al. 2002).

In contrast, when oocytes are removed from their follicles and transferred to a suitable culture medium, the meiosis spontaneously resumes independently of the LH stimulation and the stage of cytoplasmic maturation (Pincus and Enzmann 1935). However, although the nuclear maturation may be successfully completed *in vitro*, there are a variety of other processes occurring in the cytoplasm that are required for oocyte capacitation (Krisher 2004). Based on that, attempts to maintain the meiotic arrest *in vitro* have been performed in order to offer additional time to competence acquisition and, consequently, improve the quality of *in vitro* matured oocytes (Le Beux et al. 2003).

Among the pharmacological inhibitors already studied, roscovitine, an inhibitor of cyclin dependent kinases (CDK), has been considered the candidate of choice due its specific action on M-phase promoting factor (MPF) (Donnay et al. 2004). By competing with ATP for the binding site $p34^{cdc2}$, roscovitine prevents the MPF activation (Meijer et al. 1997). The reversible meiotic arrest using this inhibitor has been reported in several species as bovine (Mermillod et al. 2000), goat (Han et al. 2006), pig (Romar et al. 2006), cat (Sananmuang et al. 2010), and horse (Consiglio et al. 2010). Despite this, there is still discrepancy among authors with respect to the developmental competence of oocytes treated with roscovitine. Besides, no similar information was found in sheep.

Commonly, the studies with meiosis inhibitors on oocytes are performed in culture medium covered by mineral or paraffin oil. In this condition, however, diverse compounds of medium considered important to oocyte maturation, as steroids and soluble factors, can migrate to oil (Gasperin et al. 2010). Likewise, toxic compounds of oil may be release into the medium affecting the oocyte development (Shimada et al. 2002). Recently, it has been suggested that roscovitine also can be lost into the oil due to its lipid-solubility (Phillips et al. 2002). Therefore, it is thought that roscovitine dose used to inhibit the meiosis under oil layer may be higher than the really necessary and, consequently, harmful to oocytes. Based on that, this study was performed to investigate the ability of roscovitine to reversibly arrest the meiosis in sheep COCs cultured with and without oil overlay. The effect of roscovitine on *cumulus* cell expansion under these conditions was also evaluated.

Materials and Methods

This study was performed at the School of Veterinary Medicine and Animal Science of UNESP, Botucatu, Brazil. All chemicals were purchased from Sigma

Chemical. CO. (Sigma- Aldrich Corp., St. Louis, MO, USA), unless otherwise indicated.

Recovery of *cumulus*-oocyte complexes

Ovaries of adult sheep were collected at slaughterhouse and transported to the laboratory within 1-2 h in sterile saline solution (0.9% NaCl) at 32ºC. All visible follicles with a diameter of 2-6 mm were aspirated with a 20 gauge needle attached to a 10 mL syringe containing 0.5 mL pre-incubated Hepes-buffered TCM199 supplemented with 50 IU/mL heparin. Only COCs with several intact *cumulus* cell layers and homogeneous ooplasm were selected under stereomicroscopy (Shirazi et al. 2010).

Oocyte culture and meiosis inhibition

After washes in Hepes-buffered TCM199, the selected COCs were transferred to basic medium consisting of TCM 199 supplemented with 0.3 mM sodium pyruvate, 75 μg/mL penicillin, 10% fetal bovine serum (Gibco 10437) and 100 μM cysteamine (control). To meiotic inhibition, 75 μM roscovitine (rosco) was added to this medium. About 20 COCs were cultured in 100 μL droplet of medium placed in petri dishes under mineral oil or in 96 well plates without mineral oil. Under these conditions, the culture was performed for 20 h at 38.5ºC and 5% CO_2 in air. The stock solution of roscovitine (1mg/mL) was prepared in dimethylsulphoxide, aliquoted and stored at -20 ºC until use.

Reversion of meiotic inhibition

After culture for 20 h, COCs from each experimental (control and rosco) were washed in Hepes-buffered TCM 199 and *in vitro* matured, for a further 18h, in maturation medium composed of TCM 199, 0.3 mM sodium pyruvate, 75 μg/mL penicillin, 10% fetal bovine serum, 100 μM cysteamine, 0.1 IU/ml FSH (Folltropin®, Bioniche Co.) and 0.1 IU/mL LH (Lutropin-V®, Bioniche Co., Belleville, ON, Canada). The IVM was performed in petri dishes under mineral oil or in 96 well plates without oil at 5% CO_2 and 38.5ºC.

Assessment of *cumulus* expansion

Under a stereomicroscope, COCs were classified according to the degree of *cumulus* expansion as: total *cumulus* expansion (expansion of all layers of cells); partial *cumulus* expansion (expansion of outer layers of cells); absence of *cumulus* expansion

(cells strongly adhered to each other and to the pellucid zone) (Heidari Amale et al. 2011).

Assessment of oocyte chromatin configuration

Oocytes were stripped from their *cumulus* cells by repeated pipetting in HEPES buffered TCM-199 and transferred to droplets of Hoechst 33342 in glycerol (10 µg/mL) on a glass slide. The oocytes were examined under a Leica® DMIRB inverted fluorescence microscope (with excitation/emission filters of 350/461 nm) at 200 X magnification, and classified according to the stage of nuclear maturation as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) (Shirazi et al., 2010). The oocytes with the extrusion of one or two polar body and the presence of one or two pronucleus were classified as parthenogenetic (Marshall et al. 1998). Those with altered nuclear structure were classified as degenerate.

Experimental Design

The experimental design was completely randomized with factorial arrangement of treatments (2x2) with two experiments, each one with two experimental groups (control and rosco) cultured under two different conditions (with or without oil overlay) and five replicates for each parameter assessed. The droplet size and ratio of COCs per droplet of medium (20 COCs / 100 µL) was the same in both experiments and culture conditions.

Experiment 1- *Effect of oil overlay on inhibitory action of roscovitine*

After the *in vitro* culture for 20 h in petri dishes under mineral oil (+) or in 96 well plates without oil overlay (-), COCs from each experimental group (rosco and control) were evaluated for *cumulus* expansion degree and nuclear maturation stage. An additional sample of immature oocytes was stained immediately after removal from follicle to evaluate the nuclear status before culture (0 h).

Experiment 2- *Effect of oil overlay on reversion of roscovitine action*

To evaluate the reversibility of meiotic inhibition, COCs cultured for 20 h under oil overlay (+) were *in vitro* matured for a further 18 h in inhibitor-free medium supplemented with serum and gonadotropins (maturation medium) in the presence of mineral oil. The COCs cultured for 20 h in the absence of mineral oil (-) were IVM for a

further 18 h in maturation medium without oil overlay. The *cumulus* expansion degree and nuclear maturation were evaluated after the culture for 38 h.

Statistical analysis

The data were transformed into square root of x + 0.5 and subjected to analysis of variance according to completely randomized design. The means were compared by the Tukey test at 5% probability.

RESULTS

Experiment 1- Effect of oil overlay on inhibitory action of roscovitine

According to table 1, all immature COCs evaluated immediately after removal from follicle (0 h) had compact *cumulus* cells without signs of expansion. Likewise, in both experimental groups (control and rosco) cultured with or without oil overlay there was predominance of the absence of *cumulus* expansion while significantly lower rate of COCs showed partial *cumulus* expansion. Besides, the total *cumulus* expansion was not observed in any group and culture conditions.

As shown in table 2, the rate of oocytes at GV stage before the *in vitro* culture (0 h) was significantly higher than that observed in the others experimental groups after culture for 20 h. In the absence of oil overlay (-), however, the rate of oocytes kept at GV with roscovitine was significantly higher than that recorded in the rosco group cultured under mineral oil and in both control groups. In contrast, in the presence of oil overlay (+), the GV rate was similar between rosco and control groups. No significant difference was observed between control groups (+/-) with respect to the GV rate.

Independently of the presence or absence of oil overlay, the proportion of oocytes from control groups that reached MII at the end of culture for 20 h was significantly higher than that observed in both rosco groups. However, in the absence of oil overlay, the rate of oocytes from rosco group at MII was significantly lower than that recorded in the rosco group cultured under oil. The proportion of oocytes at GVBD, MI and degenerate was low and did not significantly differ among experimental groups (table 2).

Experiment 2- Effect of oil overlay on reversion of roscovitine action

The *cumulus* expansion pattern observed in the control groups cultured for a further18 h in inhibitor-free medium with and without oil overlay significantly differed from that recorded in both rosco groups. The total *cumulus* expansion predominated in

both controls groups while significantly lower proportion of COCs showed compact *cumulus* cells and partial expansion. In contrast, in the rosco groups cultured with and without oil overlay, the percentage of COCs with partial *cumulus* expansion significantly prevailed over the rate of COCs with compact *cumulus* cell and total expansion (table 3).

In all experimental groups, the rate of oocytes at MII after IVM for 18 h in inhibitor-free medium was significantly higher than that observed in the others meiotic stages. In the presence and absence of oil, the proportion of oocytes at MII was similar between rosco groups and its corresponding control groups. Rate of GV, GVBD, MI, degeneration and parthenogenesis did not significantly differ among groups (table 4).

Discussion

The CDK inhibitor roscovitine has been used in several animal species to prevent the spontaneous meiotic resumption *in vitro* and provide additional time to oocyte complete its cytoplasmic maturation (Mermillod et al. 2000; Han et al. 2006; Romar et al. 2006; Consiglio et al. 2010; Sananmuang et al. 2010). Commonly, the effects of meiosis inhibitor on COCs are evaluated in medium covered with oil. However, it has been suggested that roscovitine, as well as lipophilic compounds of medium, may be lost to oil overlay which could alter its effective concentration and, consequently, affect the oocyte developmental competence (Phillips et al. 2002).

In this context, our study aimed to evaluate, for the first time, the ability of roscovitine to reversibly arrest the meiosis in sheep COCs cultured with and without oil overlay. The main function of oil coverage is control the water evaporation and prevents the increase of salt concentration in the medium (Tae et al. 2006). So, as established by Gasperin et al. (2010), to avoid variation of medium osmolality in the absence of oil overlay, the unused empty holes of the 96 well plates were filled with purified water in order to create a humid microenvironment. Our preliminary tests demonstrated that medium osmolality under this culture system remained around of 294 mOsm, similar to the related by Gasperin et al. (2010). According to Staigmiller (1988), the osmolality of a suitable medium to oocyte maturation must range of 285 to 320 mOsm.

In the present study, only good quality COCs, characterized by compact *cumulus* cells and homogeneous cytoplasm (Rodríguez et al. 2006), were selected. The absence of *cumulus* expansion in the immature COCs indicates that, probably, they have not undergone the endogenous LH stimulation (Dekel et al. 1981). Likewise, the high

proportion of oocytes at GV stage (87.7%) soon after follicle aspiration indicates that the time interval between recovery and selection of COCs was adequate and did not induce the meiotic resumption before the *in vitro* culture. Similar results were observed in pig (Le Beux et al. 2003), goat (Han et al. 2006) and bovine (Barretto et al. 2010) oocytes.

The similarity of the *cumulus* expansion pattern between control groups cultured with (+) and without (-) oil overlay indicate that absence of oil did not interfere in the process of *cumulus* mucification. Likewise, the roscovitine action on *cumulus* cells was not affected by the culture system in 96 well plates without oil overlay since no significant difference was observed between rosco groups (+/-). So, it is thought that *cumulus* expansion pattern observed in this study is probably related to medium composition.

Evidences have demonstrated that LH and FSH facilitate the meiosis resumption, accelerate its progression, and even stimulate the *cumulus* expansion *in vitro* (Mattioli et al. 1991; Chen et al. 1994). The negative influence of these stimulatory substances on meiotic arrest promoted by CDK inhibitors was already reported by Barretto et al. (2010) in bovine oocytes. Considering that, in order to avoid any interference on roscovitine action, gonadotropins were not inserted in the basic medium of our study, as commonly performed in most similar researches (Mermillod et al. 2000; Han et al. 2006; Romar et al. 2006; Consiglio et al. 2010). However, the serum supplementation was maintained due to its important functions in the chelation of heavy metals, pH buffering, scavenging of free radical and growth stimulation (Natsuyama et al. 1993).

Therefore, the prevalence of COCs with compact *cumulus* cells observed in our experimental groups after culture for 20 h demonstrates that only serum supplementation was not enough to support the *cumulus* expansion. According to Eppig (1980) the synergistic action of serum and FSH is essential to promote the *in vitro cumulus* expansion. Besides, the high proportion of COCs from both control groups (+/) with total *cumulus* expansion after culture for a further 18 h in the presence of LH and FSH reinforces the importance of gonadotropins to *cumulus* expansion reported by Chen et al. (1994).

However, the partial *cumulus* expansion observed in both rosco groups (+/-) after IVM for 18 h in inhibitor-free medium demonstrates that roscovitine acted on *cumulus* cells but this action was not fully reversible. In pig COCs, Romar et al. (2006)

also reported that 50 µM roscovitine irreversibly inhibited the *cumulus* expansion. In contrast, in bovine COCs, the inhibition of *cumulus* cells with 25µM roscovitine was fully reversible (Vigneron et al. 2003). According to Mermillod et al. (2000), probably, the *cumulus* expansion is dependent on MPF or another roscovitine-sensitive kinase such as MAPK. Besides, it has been demonstrated that inhibition of *cumulus* cells with roscovitine and its subsequent reversibility may be dose and time exposure-dependent (Lonergan et al. 2003; Han et al. 2006; Sananmuang et al. 2010).

Our results also indicate that meiotic inhibition promoted by 75 µM roscovitine was more efficient in the absence of oil overlay since significantly higher proportion of sheep oocytes cultured under this condition were arrested at GV (65.2%) while 40.6% of oocytes treated with this inhibitor under oil coverage reached the MII. It confirms the possible oil dispersion of roscovitine suggested by Phillips et al. (2002). However, similar GV rate was observed in bovine, pig, goat, and horse oocytes treated with roscovitine at 200 µM, 50 µM, 25 µM, 66 µM, respectively, under oil overlay (Mermillod et al. 2000; Le Beux et al. 2003; Han et al. 2006; Consiglio et al. 2010). According to Choi et al. (2006) it suggests that, probably, the inhibitor dose used in these studies was high enough to suppress the meiosis even in the presence of oil.

However, high doses of roscovitine may be toxic and harmful to COCs (Lonergan et al. 2003; Han et al. 2006; Sananmuang et al. 2010). So, it is thought that culture system without oil overlay proposed in our study could allow efficient meiotic arrest with lower concentration of inhibitors, which would be less detrimental to oocyte. It is noteworthy that effective dose of inhibitor also depends on the particularities of each animal species in addition to *in vitro* culture conditions (Han et al. 2006).

The high rate of MII in both control groups (+/-) at the end of culture for 20 h without gonadotropins demonstrates that absence of oil overlay did not affect the meiotic competence of sheep oocytes. Besides, this result also reinforces the evidence that LH and FSH are not essential to resumption and progression of nuclear maturation *in vitro* (Sanbuissho and Threlfall, 1990). However, according to Sha et al. (2010), the presence of gonadotropins not only accelerates the oocyte meiosis and allows better rates of MII but also favors the *cumulus* expansion and improves the cytoplasmic maturation. Our findings also indicate that nuclear maturation was not depend upon *cumulus* expansion in sheep COCs, as already observed in bovine (Sirard et al. 1988) and goat (Han et al. 2006).

Besides, independently of culture condition (with or without oil overlay), the meiotic arrest promoted by roscovitine was reversible since significant rate of oocytes reached the MII stage after IVM for 18 h in inhibitor-free medium. Similar MII rate was reported in cat and sheep oocytes pre-matured for 24 h with 25 µM roscovitine and 150 µM butyrolactone, respectively (Sananmuang et al. 2010; Lu et al. 2013). However, better MII rates were recorded in bovine, goat and pig oocytes *in vitro* matured after roscovitine treatment (Mermillod et al. 2000; Han et al. 2006; Romar et al. 2006). According to Han et al. (2006) the reversibility of roscovitine arrest is dependent upon not only the drug concentration but also the exposure time to inhibitor.

In several animal species, the meiosis progression after roscovitine removal is accelerated due to accumulation of developmentally relevant factors during the meiosis block. Therefore, it suggests that meiotic steps could be shorter in oocytes treated with this inhibitor (Han et al. 2006; Sanamuang et al. 2010). Based on that, we established that 18 h of IVM could be enough to oocyte complete the meiosis, as reported by Maximo et al. (2012) in sheep. However, the significant rate of parthenogenetic oocytes indicates the culture time was excessive (Schoevers et al., 2005). According to Shirazi et al. (2009) aged ovine oocytes are more susceptible to spontaneous parthenogenetic activation. In contrast, the low rate of degenerated oocytes demonstrates that culture conditions, medium composition and inhibitor dose established in the present study were adequate.

In conclusion, we can infer that roscovitine is more efficient to reversibly arrest the meiosis in sheep *cumulus*-oocytes complexes cultured in 96 well plates without oil overlay. Besides, there are no detrimental effects on *cumulus* cells and meiosis progression in the system culture proposed. Further studies have been performed to evaluate the oocyte developmental competence after roscovitine treatment under the culture conditions here established.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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Table 1. *Cumulus* expansion degree in sheep COCs at 0 h (immature) and 20h of *in vitro* culture in basic maturation medium (control) containing 75 μM roscovitine (Rosco) with or without mineral oil overlay.

Groups	Oil overlay	No. COC	*Cumulus* expansion degree, *n* (%)		
			Total	Partial	Absence
Immature	-	122	0 (0.0) ^{aB}	0 (0.0) ^{aB}	122 (100.0) ^{aA}
Control	+	92	0 (0.0) ^{aB}	7 (7.6) ^{aB}	85 (92.4) ^{aA}
Control	-	106	0 (0.0) ^{aB}	16 (15.1) ^{aB}	90 (84.9) ^{aA}
Rosco	+	106	0 (0.0) ^{aB}	10 (9.4) ^{aB}	96 (90.6) ^{aA}
Rosco	-	114	0 (0.0) ^{aB}	6 (5.3) ^{aB}	108 (94.7) ^{aA}

* Significant differences indicated by lowercase letters in the column and capital letters in the line ($P < 0.05$).

Table 2. Chromatin configuration of sheep oocytes at 0 h (immature) and 20 h of *in vitro* culture in basic maturation medium (control) containing 75 μM roscovitine (Rosco) with or without mineral oil overlay.

Groups	Oil	No. COC	Nuclear Maturation Stage, *n* (%)				
			GV	GVBD	MI	MII	Deg
Immature	-	122	107(87.7)^{aA}	13(10.7)^{aB}	0(0.0)^{bB}	0(0.0)^{cB}	2(1.6)^{bB}
Control	+	86	17(19.7)^{cdB}	2(2.3)^{aC}	9(10.5)^{abC}	54(62.8)^{aA}	4(4.6)^{abC}
Control	-	88	11(12.5)^{dB}	7(8.0)^{aB}	9(10.2)^{abB}	56(63.6)^{aA}	5(5.7)^{abB}
Rosco	+	96	27(28.1)^{cAB}	5(5.2)^{aD}	17(17.7)^{aBC}	39(40.6)^{bA}	8(8.3)^{abCD}
Rosco	-	89	58(65.2)^{bA}	10(11.2)^{aBC}	10(11.2)^{abBC}	1(1.1)^{cC}	10(11.2)^{aB}

* Significant differences indicated by lowercase letters in the column and capital letters in the line ($P < 0.05$). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II, Deg: degenerate.

Table 3. *Cumulus* expansion degree in sheep COCs *in vitro* cultured for 20h in basic maturation medium (Control) containing 75 μM roscovitine (Rosco) followed by IVM for18h in inhibitor-free medium, with or without mineral oil overlay.

Groups	Oil overlay	No. COC	*Cumulus* expansion degree (%)		
			Total	Partial	Absence
Control	+	129	74 (57.4) ^{aA}	36 (27.9) ^{bB}	19 (14.7) ^{aB}
Control	-	99	70 (70.7) ^{aA}	26 (26.3) ^{bB}	3 (3.0) ^{aC}
Rosco	+	117	7 (6.0) ^{bC}	83 (70.9) ^{aA}	27 (23.1) ^{aB}
Rosco	-	95	6 (6.3) ^{bB}	67 (70.5) ^{aA}	22 (23.2) ^{aB}

* Significant differences indicated by lowercase letters in the column and capital letters in the line ($P < 0.05$)

Table 4. Meiotic progression of sheep oocytes *in vitro* cultured for 20h in basic maturation medium (control) containing 75 μM roscovitine (Rosco) followed by IVM for 18h in inhibitor-free medium, with or without mineral oil overlay.

Groups	Oil	No. COC	Nuclear Maturation Stage, *n* (%)					
			GV	GVBD	MI	MII	Deg	PG
Control	+	111	20(18.1)^{aB}	10(9.0)^{aB}	9(8.1)^{aB}	55(49.5)^{abA}	5(4.5)^{aB}	12(10.8)^{aB}
Control	-	88	11(12.5)^{aBC}	4(4.5)^{aBC}	1(1.1)^{aC}	52(59.1)^{aA}	6(6.8)^{aBC}	14(15.9)^{aB}
Rosco	+	108	17(15.7)^{aB}	8(7.4)^{aB}	14(13.0)^{aB}	44(40.7)^{bA}	10(9.3)^{aB}	15(13.9)^{aB}
Rosco	-	91	13(14.3)^{aB}	10(11.0)^{aB}	11(12.1)^{aB}	46(50.5)^{abA}	6(6.6)^{aB}	5(5.5)^{aB}

* Significant differences indicated by lowercase letters in the column and capital letters in the line ($P < 0.05$). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II; Deg: degenerate; PG: parthenogenetic.

CAPÍTULO 3

Trabalho submetido à revista "Zygote (Cambridge Journal Online)"

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http://assets.cambridge.org/ZYG/ZYG_ifc.pdf

Time course of the meiotic arrest in sheep *cumulus*-oocyte complexes treated with roscovitine.

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Running headline: Timing of meiosis inhibition in sheep COCs

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Summary

The temporary meiosis arrest with cyclin-dependent kinases inhibitors, as roscovitine, is a promising alternative to improve the quality of oocytes *in vitro* matured. However, it has been little investigated in sheep. In the present study, the effect of different incubation times with roscovitine on nuclear maturation and *cumulus* expansion of sheep *cumulus*-oocyte complexes (COCs) was evaluated. For this, COCs were cultured for 0, 6, 12 and 20 h in TCM 199 with 10% fetal bovine serum (control) containing 75 μM roscovitine (rosco). After, they were *in vitro* matured for a further 18 h in the presence of LH and FSH. At each culture time, *cumulus* expansion and nuclear maturation were assessed under stereomicroscope and by Hoechst 33342 staining, respectively. In both groups, the absence of *cumulus* expansion prevailed at 0, 6, 12 and 20 h. After the additional culture for 18 h, the total *cumulus* expansion in rosco group was dependent on the exposure time to roscovitine. Significantly high rate of oocytes treated with roscovitine were arrested at GV for 6 (87%), 12 and 20 h (65%). In contrast, 23% GVBD, 54% MI and 62% MII were observed in the control at 6, 12 and 20 h of culture, respectively. In all groups, significant rate of oocytes reached the MII stage after culture for a further18 h. Therefore, we can infer that roscovitine is efficient to reversibly arrest the meiosis of sheep oocytes at different culture times, however, the reversibility of its inhibitory action on *cumulus* cells is exposure-time dependent.
Keywords: Nuclear maturation, *Cumulus* expansion, Inhibitor, Meiosis, Ovine

Introduction

Despite the recent advances, the efficiency of *in vitro* production of sheep embryos is still low compared to that observed *in vivo* (Souza-Fabjan *et al*., 2014). One of the most crucial factors affecting the embryo development potential is the oocyte quality, which is gradually acquired during the folliculogenesis and is directly related to nuclear and cytoplasmic maturation (Rizos *et al*., 2002).

In most mammals, oocytes are kept at diplotene stage of prophase-I from fetal life until near ovulation, when meiosis is resumed in response to LH surge (Mehlmann, 2005). During this meiotic arrest, structural and molecular changes essential to acquisition of developmental competence also occur (Ferreira *et al*., 2009). *In vivo*, these nuclear and cytoplasmic events progress synchronously, ensuring that only competent oocytes reach ovulation (Mermillod *et al*., 2008). In this context, the *cumulus* cells also play a crucial role since the oocyte maturation is basically controlled by low

molecular weight peptides transferred to oocytes through GAP junction communications (Tanghe *et al*., 2002).

However, once removed from follicle and cultured in a suitable medium, the oocytes spontaneously resume the meiosis regardless of their development stage (Pincus & Enzmann 1935). Besides, an extremely heterogeneous population of oocytes, retrieved from follicles at diverse stages of folliculogenesis, is destined to *in vitro* maturation (Gilchrist & Thompson, 2007). So, in order to improve the quality of *in vitro* matured oocytes, the temporary arrest of meiosis has been proposed as strategy to offer additional time for the proper oocyte capacitation (Marchal *et al*., 2001; Han *et al*., 2006).

Among the pharmacological inhibitors, roscovitine is recognized by competing for ATP binding sites of the cyclin-dependent kinases, preventing the activation of cell cycle regulator M-phase promoting factor (MPF) (Meijer *et al*., 1997). Studies performed with oocytes of bovine (Mermillod *et al*., 2000; Lagutina et al., 2002), pig (Romar *et al*., 2006), horse (Franz *et al*., 2003) and goat (Han *et al*., 2006) demonstrated that roscovitine at 25µM, 50µM, 66µM and 200µM , respectively, was efficient to reversibly arrest the meiosis for 22-24h. However, the effect of this inhibitor on oocyte competence is still discrepant among authors and species studied.

In this context, Han *et al*. (2006) observed that the reduction of exposure time of goat oocytes to roscovitine from 24 h to 8 h improved the morulae and blastocyst rates. According to these same authors, meiosis inhibitors used at high concentrations and/or for extended period can be harmful to oocytes and compromise its developmental competence. In sheep, however, there is no similar information. Based on that, the present study aimed to evaluate the ability of roscovitine to reversibly arrest the meiosis of sheep oocytes at different times of culture in order to establish the optimal incubation duration. Besides, the effect of roscovitine on *cumulus* cells expansion was also examined.

Materials and methods

This study was performed at the School of Veterinary Medicine and Animal Science of UNESP, Botucatu, Brazil. All chemicals were purchased from Sigma Chemical. CO. (Sigma- Aldrich Corp., St. Louis, MO, USA), unless otherwise indicated.

Cumulus-oocyte complexes collection

Ovaries of adult sheep were collected at slaughterhouse and transported to the laboratory within 1-2 h in sterile saline solution (0.9 % NaCl) at 32 ºC. All visible follicles with a diameter of 2-6 mm were aspirated with a 20 gauge needle attached to a 10 mL syringe containing 0.5 mL pre-incubated Hepes-buffered TCM199 supplemented with 50 IU/mL heparin. Only COCs with several compact *cumulus* cell layers and homogeneous ooplasm were selected under stereomicroscopy (Shirazi *et al.* 2010).

Oocyte culture and meiosis inhibition

After washes in Hepes-buffered TCM199, the selected COCs were cultured for 6, 12 and 20 h in basic maturation medium comprised of TCM 199 with Earle's salts, 0.3 mM sodium pyruvate, 75 µg/mL penicillin, 100 µM cysteamine and 10% fetal calf serum (Gibco 10437) (control) containing 75 µM roscovitine (rosco). Based in our preliminary tests, about 20 COCs / 100 µL droplets of medium were cultured in 96 well plates without oil overlay at 38.5 ºC and 5% CO_2 in air. The stock solution of roscovitine (1 mg/mL) was prepared in dimethylsulphoxide, aliquoted and stored at -20 ºC until use.

Reversion of meiotic inhibition

At the end of *in vitro* culture for 6, 12 and 20 h, COCs from each experimental group were washed in Hepes-buffered TCM 199 and *in vitro* matured, for a further 18 h, in the basic maturation medium supplemented with 0.1 IU/ml FSH (Folltropin®, Bioniche Co.) and 0.1 IU/mL LH (Lutropin-V®, Bioniche Co.). The *in vitro* maturation (IVM) was performed in 96 well plates without oil overlay at 5 % CO_2 and 38.5 ºC.

Assessment of *cumulus* expansion

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

Assessment of oocyte chromatin organization

Oocytes were stripped from their *cumulus* cells by repeated pipetting in HEPES buffered TCM-199, fixed for 30 min in 4 % paraformaldehyde, and transferred to droplets of Hoechst 33342 in glycerol (10 µg/mL) on a glass slide. The oocytes were examined under a fluorescence inverted microscope (Leica® DMIRB) and classified according to the stage of nuclear maturation as germinal vesicle (GV) germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) (Shirazi *et al.,* 2010). The anaphase I and telophase I stages were considered as MII, because they are intermediate stages of a dynamic process. The oocytes with the extrusion of one or two polar body and the presence of one or two pronucleus were classified as parthenogenetic (Marshall *et al.,* 1998). Those with altered nuclear structure were classified as degenerate.

Experimental design

The experimental design was completely randomized with two experiments. Each experiment consisted of two experimental groups (control and rosco) submitted to different times of culture and five replicates for each parameter assessed.

Experiment 1- Inhibitory action of roscovitine at different times of culture

COCs were *in vitro* cultured for 6, 12 and 20 h in the absence (control) and presence of roscovitine (rosco). At the end of each culture time, *cumulus* expansion degree and nuclear maturation stage of COCs from each experimental group were evaluated under stereomicroscope and by Hoechst 33342 staining, respectively. To evaluate the nuclear status of oocytes before the *in vitro* culture, additional sample of oocytes were exposed (rosco 0 h) or not (control 0 h) to 75 µM roscovitine during the follicular aspiration and immediately stained.

Experiment 2 - Reversibility of inhibitory action of roscovitine

After 6, 12, and 20 h of *in vitro* culture, COCs from each experimental group (control and rosco) were *in vitro* matured for a further 18 h in inhibitor-free medium supplemented with gonadotropins. The *cumulus* expansion degree and nuclear maturation stage were evaluated at the end of 24, 30 and 38 h of oocyte incubation.

Statistical analysis

The data were transformed into square root of x + 0.5 and subjected to analysis of variance according to completely randomized design. The means were compared by the Tukey test at 5% probability.

Results

Experiment 1

According to table 1, significantly high proportion of COCs from control and rosco groups showed absence of *cumulus* expansion at 6, 12 and 20 h of culture, while total *cumulus* expansion was not observed at any time of culture. Likewise, all COCs aspirated with (rosco 0h) and without (control 0 h) roscovitine had compact *cumulus* cells without signs of expansion. The rate of COCs with absence of *cumulus* expansion at 20 h of culture was similar to that observed at 0 and 6 h for control and rosco groups but significantly differed from that of control group at 12 h. In contrast, no significant difference was observed among groups control 12 h, rosco 12 h, rosco 6 h and control 6 h with respect to the rate of COCs with compact *cumulus* cells. The proportion of COCs with partial *cumulus* expansion was similar between rosco and control groups at 6 and 12 h of culture, but significantly differed from that observed in the rosco 20h and at 0 h.

The analysis of nuclear status after the removal of COCs from follicle revealed that almost all oocytes from rosco (92%) and control (87.7%) groups were at GV before the *in vitro* culture. At 6 h, a significant decrease of the GV rate (46.7%) was observed in the control group while significantly higher proportion of oocytes treated with roscovitine (86.7%) remained at this stage. In comparison, the rate of oocytes from control group at GVBD (23.4%) and MI (30.0%) significantly increased at 6 h of culture, while only 13.3% and 0% of oocytes from rosco group were at these same stages, respectively. The GV rate significantly decreased in rosco group at 12 h (65.1%) but, after this, remained constant until the 20 h (65.1%) of culture. In the control group, however, a continuous decrease of GV rate was observed at 12 h (25.5%) and 20 h (14.6%) (Table 2).

At 12 h of culture, there was a significant increase of the MI rate (54.3%) in the control group which was followed by an important reduction at 20 h (9.7%). In comparison, only 18.4 % and 9.4% of oocytes treated with roscovitine reached the MI at 12 and 20 h of culture, respectively. At 20 h, significantly higher proportion of oocytes from control group reached MII (61.2%) while only 1% of oocytes from rosco group

were at this stage. With respect to the GVBD rate, no significant difference was observed between experimental groups at 12 and 20 h. The rate of degenerate oocytes was similar between control and rosco groups at different times of culture (Table 2).

Experiment 2

As shown in table 3, the *in vitro* culture for a further 18h in inhibitor-free medium supplemented with gonadotropins allowed the total *cumulus* expansion in a significantly high rate of COCs from control 12 and 20 h, which was similar to the control and rosco 6 h; but significantly differed from that recorded in the rosco 12 and 20 h. The rate of COCs with partial *cumulus* expansion was significantly higher in the rosco 20 h in comparison to rosco 6 h, control 6, 12 and 20 h, but did not significantly differ from that observed in the rosco 12 h. There was no significant difference among experimental groups with respect to the rate of COCs with compact *cumulus* cells.

According to table 4, the rate of oocytes at MII after the *in vitro* culture for a further 18h in inhibitor-free medium supplemented with gonadotropins was similar among all experimental groups and significantly higher than that observed in the others meiotic stages. Besides, the percentage of GV, GVBD, MI, oocyte degeneration and parthenogenesis was similar among them and did not significantly differ among experimental groups.

Discussion

The asynchrony between nuclear and cytoplasmic maturation observed in oocytes *in vitro* matured is the most crucial aspect affecting the effectiveness of culture systems used for embryo production (Rizos *et al*., 2002). In this context, inhibitors of cyclin-dependent kinases, as roscovitine, has been used in several animal species in order to maintain the meiotic arrest *in vitro* and, consequently, allow additional time to oocyte complete its capacitation (Mermillod *et al*., 2000; Franz *et al*., 2003; Romar *et al*., 2006; Han *et al*., 2006; Sananmuang *et al*., 2010). The efficiency of meiosis inhibition, however, depends upon not only the drug concentration but also the exposure time of oocyte to inhibitor (Han *et al*., 2006). Besides, in sheep, this strategy to improve the quality of oocytes *in vitro* matured has been little investigated.

Based on that, the present study aimed to evaluate the meiotic arrest promoted by roscovitine in sheep oocytes at different times of culture in order to establish the optimal incubation duration. The *cumulus* expansion under these conditions was also

examined. To avoid alterations of the inhibitor concentration, COCs were cultured without oil overlay, since the roscovitine may be liposoluble (Phillips *et al*., 2002). Besides, the culture was performed in 96 well plates aiming to mimic the volume of medium and ratio of COCs (20 COCs/ 100 µL) used in the conventional maturation system. As suggested by Gasperin *et al*. (2010), a humid microenvironment was created in order to maintain the medium osmolality around of 294 mOsm.

Only COCs with compact *cumulus* cells were selected to this study, which indicates that, probably, they have not undergone the LH stimulation *in vivo* (Dekel *et al*., 1981). Our findings demonstrate that follicular aspiration with roscovitine was not necessary since the GV rate in both experimental groups (control and rosco) at 0 h was significantly high. It also suggests that time interval between transport of ovaries, recovery and selection of COCs was adequate and did not induce the meiotic resumption before the *in vitro* culture. Similar results were observed in goat (Han *et al*., 2006), pig (Romar *et al*., 2006) and bovine (Barretto *et al*., 2011) oocytes.

The absence of *cumulus* expansion observed in our control group at 6, 12 and 20h of culture is, probably, related not only to culture time but also to medium composition. As commonly performed in most similar researches, gonadotropins were not inserted in the basic maturation medium to avoid any interference on roscovitine action (Mermillod *et al.,* 2000; Han *et al.*, 2006; Romar *et a*l, 2006; Franz *et al*., 2003). However, based on preliminary studies, the serum supplementation was maintained due to its benefits to culture system and cellular viability (Natsuyama *et al.,* 1993). Our results demonstrate, therefore, that only serum was not enough to promote the *cumulus* expansion, as observed by Mattioli *et al*. (1991) and Accardo *et al*. (2004) in pig and sheep COCs, respectively.

According to Daen *et al*. (1995), although gonadotropins stimulate the synthesis of hyaluronic acid by cumulus cells, some components of serum are necessary to retain it within the *cumulus*-oocyte complexes. So, the synergistic effect of gonadotropins and serum is required to *cumulus* expansion (Kito & Bavister, 1997). It was also evidenced in our study, since significant proportion of COCs from control group had total *cumulus* expansion after the additional culture for 18 h in the presence of LH, FSH and serum. Usually, in appropriate supplemented medium, the *cumulus* cells remain compacted until about 12 h, when the expansion becomes evident and continuously increases up to end of culture (Hyttel, *et al*., 1986; Berg, *et al*., 2002). In the present study, this kinetics of *cumulus* expansion was not observed due to the absence of gonadotropin

supplementation during the first 20 h of culture. We believe that the slight difference observed at 6, 12 and 20 h with respect to *cumulus* expansion is probably due to the heterogeneity of ovaries obtained at slaughterhouse (Coy *et al*., 2005).

The *cumulus* expansion pattern observed in the rosco groups (6, 12 and 20 h) after the additional culture for 18 h in inhibitor-free medium demonstrate that roscovitine acted on *cumulus* cells and the reversibility of this action was time exposure dependent. Likewise, Han *et al*. (2006) reported that rate of goat COCs with total *cumulus* expansion after IVM for 24 h was higher in oocytes pre-matured with roscovitine for 8 h than those blocked for 16 and 24 h. However, tt is not well established if the effect of roscovitine on *cumulus* expansion occurs via oocyte or directly via *cumulus* cells (Schoevers *et al.,* 2005). According to Mermillod *et al*. (2000), the inhibition of *cumulus* expansion even under EGF stimulation indicates that mucification process may be dependent on the MPF or another roscovitine-sensitive kinase such as MAPK.

The kinetics of nuclear maturation observed in our control group with significant rate of GVBD (23%) at 6 h, MI (54%) at 12 h and MII (62%) at 20 h of culture is consistent with that reported by Moor & Crosby (1985) in sheep oocytes. Besides, the significant decrease of GV rate during the first 6 h of culture without inhibitor is also in accordance with that recorded in sheep oocytes by Gharibi *et al*. (2013). Despite this, in contrast to these authors, the meiotic progression in our control group occurred in the absence of gonadotropins. It reinforces the evidence that LH and FSH are not essential to resumption and progression of nuclear maturation *in vitro* (Sanbuissho & Threlfall, 1990). However, the medium supplementation with these substances not only facilitates and accelerates the oocyte meiosis but also favors the *cumulus* expansion and improves the cytoplasmic maturation (Mattioli et al., 1991; Cotterill et al., 2012). As already observed in bovine (Sirard *et al.,* 1988) and goat (Han *et al.,* 2006), our findings also demonstrate that nuclear maturation was not depend upon *cumulus* expansion in sheep COCs.

The significantly high rate of oocytes kept at GV stage in the presence of 75µM roscovitine in comparison with the continuous decrease of GV rate observed in the control group suggests that roscovitine was efficient to prevent the meiosis resumption of sheep oocytes at different times of culture. Likewise, Lu *et al*. (2013) reported that about 60% of sheep oocytes were arrested at GV stage during 8, 16 and 24 h of incubation with 150µM butyrolactone (a CDK inhibitor analogue of roscovitine).

Similar GV rate were also reported in bovine, porcine and cat oocytes treated with different doses of roscovitine for 22-24 h (Mermillod *et al*., 2000; Sananmuang *et al*., 2010; Marchal *et al*., 2001). Besides, we presumed that the profile of meiotic inhibition observed in the present study is related to the action mode of roscovitine, which prevents the activation of the MPF by competing with ATP-binding site on catalytic subunit of this M phase kinase (Meijer *et al.,* 1997).

Our findings also demonstrate that, independently of time exposure, the meiotic inhibition promoted by roscovitine was reversible since significant proportion of sheep oocytes reached the MII stage after additional culture for 18 h in inhibitor-free medium. Similar MII rates were reported in porcine and cat oocytes *in vitro* matured for 22-24h after treatment with different concentrations of roscovitine (12.5 µM; 25 µM; 50 µM) (Marchal *et al*., 2001; Sananmuang *et al*., 2010). Lu *et al*. (2013) also obtained similar MII rate after IVM for 16 and 24 h of sheep oocytes previously treated with 150 µM butyrolactone. However, better MII rates were recorded by Mermillod *et al*. (2000), Han *et al.* (2006), and Romar *et al.* (2006) in bovine, goat and pig oocytes, respectively, pre-matured with roscovitine. This discrepancy of results among authors as well as the oocyte developmental competence is directly related to the inhibitor dose, time of incubation, culture conditions, quality of material obtained at slaughterhouse, besides the particularities of animal species studied (Coy *et al*., 2005; Han *et al*., 2006; Crocomo *et al*., 2013).

It has been demonstrate that nuclear maturation progression is accelerated after inhibitor removal, probably, due to accumulation of some MPF activation factors during the meiosis block (Marchal *et al*., 2001; Han *et al.,* 2006). According to Lagutina *et al.* (2002), the GVBD in bovine oocytes pre-matured with roscovitine initiates about 4 h earlier. Based on that, we established that 18 h of IVM could be enough to oocyte complete the meiosis, as already reported by Maximo *et al.* (2012) in sheep. However, the significant parthenogenesis rate suggests that, probably, the incubation time was excessive (Schoevers *et al*., 2005). In fact, it has been demonstrated that aged ovine oocytes are susceptible to spontaneous parthenogenetic activation (Shirazi *et al*., 2009). Despite this, the low rate of degenerate oocytes in our study indicates that culture conditions were suitable.

Therefore, we can infer that roscovitine, at the studied concentration, is efficient to reversibly arrest the meiosis of sheep oocytes at different incubation durations. However, the reversibility of its inhibitory action on *cumulus* cells is exposure-time

dependent. Further investigations have been performed to evaluate the effect of roscovitine pre-maturation during different times on oocyte developmental competence.

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Table 1. *Cumulus* expansion degree of sheep COCs *in vitro* cultured for 6 h, 12 h and 20 h in basic maturation medium (Control) containing 75 µM roscovitine (Rosco). 0h: evaluation immediately after aspiration with (Rosco) and without (Control) roscovitine.

Groups	Culture Time (h)	No. COC	*Cumulus* expansion degree, *n* (%)		
			Total	Partial	Absence
Control	0	122	0 (0.0) ^{aB}	0 (0.0) ^{dB}	122 (100.0) ^{aA}
Rosco	0	117	0 (0.0) ^{aB}	0 (0.0) ^{dB}	117 (100.0) ^{aA}
Control	6	102	0 (0.0) ^{aC}	20 (19.6) ^{abB}	82 (80.4) ^{bcdA}
Rosco	6	108	0 (0.0) ^{aC}	19 (17.6) ^{abB}	89 (82.4) ^{bcdA}
Control	12	104	0 (0.0) ^{aC}	31 (29.8) ^{aB}	73 (70.2) ^{dA}
Rosco	12	114	0 (0.0) ^{aC}	24 (21.1) ^{abB}	90 (78.9) ^{cdA}
Control	20	106	0 (0.0) ^{aC}	16 (15.1) ^{bcB}	90 (84.9) ^{abcA}
Rosco	20	114	0 (0.0) ^{aB}	6 (5.3) ^{cdB}	108 (94.7) ^{abA}

* Differences indicated by lowercase letters in the column and capital letters in the line ($P<0.05$).

Table 2. Nuclear configuration of sheep oocytes *in vitro* cultured for 6 h, 12 h and 20 h in basic maturation medium (Control) containing 75 µM roscovitine (Rosco). 0h: evaluation immediately after aspiration with (Rosco) and without (Control) roscovitine.

Group	Culture Time (h)	No. COC	Nuclear Maturation Stage, *n* (%)				
			GV	GVBD	MI	MII	Deg
Control	0	122	107 (87.7)^{aA}	13 (10.7)^{bB}	0 (0.0)^{dC}	0 (0.0)^{cC}	2 (1.6)^{abBC}
Rosco	0	113	104 (92.0)^{aA}	7 (6.2)^{bB}	0 (0.0)^{dB}	0 (0.0)^{cB}	2 (1.7)^{abB}
Control	6	90	42 (46.7)^{cA}	21 (23.4)^{aB}	27 (30.0)^{bB}	0 (0.0)^{cC}	0 (0.0)^{bC}
Rosco	6	98	85 (86.7)^{aA}	13 (13.3)^{bB}	0 (0.0)^{dC}	0 (0.0)^{cC}	0 (0.0)^{bC}
Control	12	94	24 (25.5)^{dB}	7 (7.4)^{bCD}	51 (54.3)^{aA}	11(11.7)^{bC}	1 (1.0)^{bD}
Rosco	12	109	71 (65.1)^{bA}	11 (10.1)^{bBC}	11 (18.4)^{bcB}	4 (3.7)^{bcC}	3 (2.7)^{abC}
Control	20	103	15 (14.6)^{eB}	9 (8.7)^{bB}	10 (9.7)^{cdB}	63 (61.2)^{aA}	6 (5.8)^{abB}
Rosco	20	106	69 (65.1)^{bA}	15 (14.2)^{bB}	10 (9.4)^{cdBC}	1 (1.0)^{cC}	11 (10.4)^{aB}

* Differences indicated by lowercase letters in column and capital letters in the line ($P<0.05$). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II; Deg: degenerate.

Table 3. *Cumulus* expansion degree of sheep COCs after the *in vitro* culture for 6 h, 12 h and 20 h in basic maturation medium (Control) containing 75 µM roscovitine (Rosco) followed by 18 h of *in vitro* maturation in inhibitor-free medium.

Groups	Culture Time (h)	No. COC	*Cumulus* expansion degree, *n* (%)		
			Total	Partial	Absence
Control	6 + 18	99	57 (57.6) ^{abA}	24 (24.2) ^{cB}	18 (18.2) ^{abB}
Rosco	6 + 18	87	52 (59.8) ^{abA}	21 (24.1) ^{cB}	14 (16.1) ^{abB}
Control	12 + 18	90	65 (72.2) ^{aA}	17 (18.9) ^{cB}	8 (8.9) ^{abB}
Rosco	12 + 18	102	38 (37.3) ^{bA}	45 (44.1) ^{abA}	19 (18.6) ^{abB}
Control	20 + 18	99	70 (70.7) ^{aA}	26 (26.3) ^{bcB}	3 (3.0) ^{bC}
Rosco	20 + 18	95	6 (6.3) ^{cC}	67 (70.5) ^{aA}	22 (23.2) ^{aB}

* Differences indicated by lowercase letters in the column and capital letters in the line ($P<0.05$).

Table 4. Meiotic progression of sheep oocytes *in vitro* cultured for 6 h, 12 h and 20 h in basic maturation medium (Control) containing 75 µM roscovitine (Rosco) followed by 18 h of *in vitro* maturation in inhibitor-free medium.

Groups	Culture time (h)	No. COC	Nuclear Maturation Stage, *n* (%)					
			GV	GVBD	MI	MII	Deg	PG
Control	6 + 18	76	12(15.8)^{aB}	7(9.2)^{aB}	9(11.8)^{aB}	42(55.3)^{aA}	1(1.3)^{aB}	5(6.6)^{aB}
Rosco	6 + 18	86	13(15.1)^{aB}	9(10.5)^{aB}	8(9.3)^{aB}	49(57.0)^{aA}	3(3.5)^{aB}	4(4.7)^{aB}
Control	12 + 18	88	13(14.8)^{aB}	7(8.0)^{aB}	8(9.1)^{aB}	47(53.4)^{aA}	4(4.5)^{aB}	9(10.2)^{aB}
Rosco	12 + 18	100	13(13.0)^{aB}	7(7.0)^{aB}	16(16.0)^{aB}	57(57.0)^{aA}	2(2.0)^{aB}	5(5.0)^{aB}
Control	20 + 18	88	11(12.5)^{aB}	4(4.5)^{aB}	1(1.1)^{aB}	52(59.1)^{aA}	6(6.8)^{aB}	14(15.9)^{aB}
Rosco	20 + 18	91	13(14.3)^{aB}	10(11.0)^{aB}	11(12.1)^{aB}	46(50.5)^{aA}	6(6.6)^{aB}	5(5.5)^{aB}

* Differences indicated by lowercase letters in column and capital letters in the line ($P<0.05$). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II; Deg: degenerate; PG: parthenogenetic.

CAPÍTULO 4

Trabalho submetido à revista "Research in Veterinary Science"

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

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ABSTRACT

This study aimed to evaluate the effect of temporary meiosis arrest using roscovitine on gene expression in sheep oocytes and *cumulus* cells. 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 the *cumulus* cells (PTX3, GREM1, GLUT1, PTGS2, ALK5, ALK6) and oocytes (ZAR1, NLRP5, SOD1, BMP15, GDF9) was similar between control and roscovitine treatment. Besides, the ratio BCL2/BAX was maintained in both cell types even in the presence of roscovitine. In conclusion, the reversible meiotic arrest promoted by roscovitine at concentration and exposure time studied does not affect but also does not improve the expression of the investigated genes in sheep oocytes and *cumulus* cells.

Key words: mRNA, Nuclear maturation, *Cumulus* expansion, Meiosis inhibitor, Ovine

1. Introduction

The mammalian oocytes enter into meiosis during the intrauterine life and remain arrested at diplotene stage of prophase-I until near ovulation. In response to LH surge, meiotic division resumes and proceeds to metaphase II (Mehlmann, 2005). During this diplotene arrest, oocyte displays an enlarged nucleus, called germinal vesicle (GV), which contains lampbrush chromosomes composed of regions with side loops of decondensed chromatin transcriptionally active (Andraszek and Smalec, 2011). Therefore, the oocytes at GV stage are able to produce and store all mRNA and protein required (Krisher, 2004).

With the 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, oocyte maturation, fertilization and initial embryogenesis depend on the maternal mRNAs synthesized and stored during oogenesis (Brevini-Gandolfi and Gandolfi, 2001; Sirard, 2001). Many of these transcripts are involved with cell cycle regulation, metabolism, (oxidative) stress defense, folliculogenesis and cell signaling, among others (Wrenzycki et al. 2007).

In the course of acquiring competence to resume and complete meiosis, beside the molecular events, structural changes considered of fundamental importance to oocyte capacitation also occur (Ferreira et al., 2009). These processes of nuclear and

cytoplasmic maturation are controlled by low molecular weight peptides transferred from *cumulus* cells to oocyte through Gap junctions (Sugiura and Eppig 2005). Likewise, important functions of granulosa cells are regulated by oocyte-secreted factors (Gilchrist et al., 2006).

In contrast, when oocytes are removed from their follicles and transferred to a suitable culture medium, the meiosis spontaneously resumes regardless of the stage of cytoplasmic maturation (Pincus and Enzmann, 1935). So, most oocytes destined to *in vitro* maturation have not 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 to oocyte complete its capacitation *in vitro* (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 meiosis arrest promoted by roscovitine and its effect on embryo development has been demonstrated in diverse animal species as bovine (Mermillod et al., 2000; Lagutina et al., 2002), goat (Han et al., 2006), cat (Sananmuang et al., 2010), pig (Coy et al., 2005) and horse (Franz et al., 2003). However, the effect of this inhibitor on gene expression in oocytes and *cumulus* cells has not been extensively investigated. Likewise, no information regarding this aspect was reported in sheep COCs. So, 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

This study was performed at the School of Veterinary Medicine and Animal Science of UNESP, Botucatu, Brazil. All chemicals 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 ºC. 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 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. Oocyte culture and meiosis inhibition

After washes in Hepes-buffered TCM 199, the selected COCs were transferred to basic maturation medium composed of TCM 199 with Earle's salts, 0.3 mM sodium pyruvate, 75 µg/mL penicillin, 10% fetal bovine serum (Gibco 10437) and 100 µM cysteamine (control) containing 75 µM roscovitine (rosco). About 25 COCs were *in vitro* cultured, for 6 h, in 100 µL droplets of medium at 38.5 ºC and 5% CO_2 in air. Based in our preliminary tests, the culture was performed in 96 well plates without mineral oil. The stock solution of roscovitine (1mg/mL) was prepared in dimethylsulphoxide, aliquoted and stored at -20 ºC until use.

2.3. Reversion of meiotic inhibition

After 6 h of culture, COCs from each experimental group were washed in Hepes-buffered TCM 199 and *in vitro* matured, for a further 18h, in basic maturation medium supplemented with 0.1 IU/ml FSH (Folltropin®, Bioniche Co.) and 0.1 IU/mL LH (Lutropin-V®, Bioniche Co., Belleville, ON, Canada). The *in vitro* maturation (IVM) was performed in 96 well plates without mineral oil at 38.5 ºC and 5% CO_2 in air.

2.4. Assessment of *cumulus* expansion

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

2.5. Assessment of oocyte chromatin organization

Oocytes were stripped from their *cumulus* cells, fixed for 30 min in 4% paraformaldehyde and transferred to droplets of Hoechst 33342 in glycerol (10 µg/mL) on a glass slide. The oocytes were examined under a fluorescence inverted microscope (Leica® DMIRB) and classified according to the stage of nuclear maturation as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II

(MII) (Shirazi et al., 2010). Those with altered nuclear structure were classified as degenerate.

2.6. 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 the *cumulus* cells were centrifuged for 5 min at 700g and the supernatant was removed. Finally, the oocytes and *cumulus* cells were frozen at -80ºC with 350 µL of RNA extraction lysis buffer in 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 the manufacturer's instructions and eluted in 30 µL RNAse-free water. After incubation with DNase I (Invitrogen, São Paulo, Brazil), the total RNA was reverse transcribed with the Sensiscript and Omniscript RT Kit (Quiagen, Mississauga, ON, CA) for oocyte and *cumulus* samples, respectively, using Oligo-dT (Invitrogen) primers for both.

2.7. Relative quantification by real-time PCR

The expression of SOD1, NLRP5, ZAR1, BMP15 and GDF9 genes was evaluated in oocytes, whereas the expression of GREMLIN, PTX3, GLUT1, PTGS2, ALK5 and ALK6 genes was evaluated in *cumulus* cells. The BAX and BCL-2 genes were evaluated in both cell types.

Real time RT-PCR analysis was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystem) with Power Sybr Green PCR Master Mix (Applied Biosystems). Reactions were performed in 25 µL volumes and PCR cycling conditions were 95 ºC for 10 min followed by 40 cycles of denaturing at 95 ºC for 10 s and then annealing for 1 min. The primers sequences, fragment size and annealing temperatures for each target 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 determined by electrophoresis in agarose 2%. The Cyclophilin-A (CYC-A) was selected as the most stable housekeeping gene through the geNorm program (Vandesompele et al., 2002).

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).

2.8. Experimental Design

The experimental design was completely randomized with two experiments, each one with two experimental groups (control and rosco) submitted to two culture times.

2.8.1. *Experiment 1- Meiosis inhibition and reversibility*

After 6 h of culture, a sample of COCs from control and rosco groups was evaluated for *cumulus* expansion and nuclear maturation. Another sample of COCs from each experimental group was *in vitro* matured for a further 18h in inhibitor-free medium and then evaluated for *cumulus* expansion and nuclear maturation. An additional sample of immature oocytes was stained immediately after removal from follicle to evaluate the nuclear status before the culture (0 h). Five replicates were performed for each parameter assessed.

2.8.2. *Experiment 2- Gene expression analysis*

After 6 h of culture in the absence (control) and presence of roscovitine (rosco) as well as after the culture for a further 18 h in inhibitor-free medium, the oocytes and corresponding *cumulus* cells from each experimental group were stored at -80 ºC to subsequent analysis of gene expression by qRT-PCR. An additional sample of immature oocytes and their *cumulus* cells was stored immediately after removal from follicle (0 h). Four pools of 25 oocytes and corresponding *cumulus* cells were stored for each experimental group and culture times.

2.9. Statistical analysis

The data were transformed into square root of x + 0.5 and subjected to analysis of variance according to completely randomized design. The means were compared by the Tukey test at 5% probability.

3. Results

Experiment 1- Meiosis inhibition and reversibility

According to table 2, all immature COCs evaluated immediately after the follicular aspiration (0 h) had compact *cumulus* cells, which significantly differed from that observed in the others experimental groups. After 6 h of culture, the percentage of

COCs with absence of *cumulus* expansion in both experimental groups prevailed over that of partial and total expansion. Besides, it was significantly higher than the rate of COCs with compact *cumulus* cells observed after IVM for 18 h. In contrast, the proportion of COCs from control and rosco groups 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 between experimental groups and culture times.

As shown in table 3, almost all immature oocytes were at GV stage immediately after the follicular aspiration (0 h). At 6 h, the rate of oocytes maintained at GV with roscovitine was similar to that of immature group and significantly higher than that recorded in corresponding control group. In contrast, the rate of oocytes from control group at GVBD and MI after culture for 6 h was significantly higher than that reported in the others experimental groups and culture times. After IVM for 18 h, the percentage of oocytes from both experimental groups that reached the MII stage was significantly higher than that recorded at 0 and 6 h. With respect to degenerate oocytes, no significant difference was observed between experimental groups and culture times.

Experiment 2- Gene expression analysis

The effect of roscovitine on gene expression in sheep *cumulus* cells is reported in the figure 1. 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 group. After IVM for 18 h, however, the relative abundance of this transcript in the rosco group significantly increased and reached value greater than that of control. In contrast, the Bcl-2 mRNA level in the control group significantly increased after the culture for a further 18 h but did not differ from that of rosco group. The relative abundance of the ALK5 transcript in the roscovitine treatment was down-regulated after the IVM for 18 h. However, the value reached was similar to that of immature and control groups. The relative expression of the PTX3 and ALK6 genes was significantly up-regulated in both experimental groups after IVM for 18 h. With respect to the expression of the GLUT1, GREMLIN and PTGS2 genes, no significant difference was observed between experimental groups and culture times (Fig. 1).

The pattern of gene expression in sheep oocytes treated for 6 h with roscovitine is shown in the figure 2. The relative abundance of the ZAR1, NLRP5 and SOD1 transcripts was not affected by roscovitine treatment, but was significantly down-regulated after the IVM for 18 h in both experimental groups. The mRNA levels for

Bax and GDF9 in the control group significantly decreased after the additional culture for 18 h. The reached values, however, were similar to those recorded in the rosco group and at 0 h of culture. The BMP15 and Bcl-2 expression remained stable in both experimental groups and culture times (Fig. 2).

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 the most crucial factor affecting the developmental competence of oocytes *in vitro* matured (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; Coy et al., 2005; Han et al., 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). Besides, there is no similar information in sheep.

So, the present study aimed to evaluate the effect of temporary meiotic arrest promoted by roscovitine on relative abundance of transcripts associated to developmental competence in sheep oocytes and *cumulus* cells. Due to lipo-solubility of roscovitine (Phillips et al., 2002), COCs were cultured in 96 well plates without oil overlay to avoid alterations of the inhibitor concentration. The volume of medium and ratio of COCs (25COCs / 100µL) was the same of conventional maturation system. Besides, as suggested by Gasperin et al. (2010), a humid microenvironment was created to keep the medium osmolality around of 294 mOsm.

Under gonadotropin stimulation, *in vivo* and *in vitro*, *cumulus* cells start synthetizing a hyaluronic acid-rich matrix, which is deposited into the intercellular spaces, resulting in increase of COCs volume (Chen et al., 1994; Salustri, 2000). As consequence of *cumulus* expansion, Gap junction communications between oocyte and *cumulus* cells are lost, inducing the meiotic resumption (Isobe et al., 1998). Therefore, the absence of *cumulus* expansion in the immature COCs selected to this study indicates that they have not undergone the endogenous LH stimulation and have not resumed the meiosis *in vivo* (Dekel et al., 1981).

Evidences demonstrate, however, that *cumulus* expansion occurs in a gradual manner (Sato and Yokoo, 2005). According to Hyttel et al. (1986), in appropriate

supplemented medium, the *cumulus* cells remain compacted until about 12 h of culture, when the expansion becomes evident and continuously increases up to end of culture. In both experimental groups of our study, the absence of *cumulus* expansion observed at 6 h of culture followed by significant rate of COCs with total *cumulus* expansion after the IVM for 18 h is in accordance with the kinetics of *cumulus* expansion described.

Our findings also demonstrate that some of the investigated genes in the *cumulus* cells had an expression profile consistent with the *cumulus* expansion pattern observed in both experimental groups. 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 of culture. Similar expression pattern was observed for ALK6 in ovine oocytes *in vitro* matured (Kyassari et al., 2012). Likewise, basal PTX3 expression was reported in bovine COCs at 6 h of IVM (Assidi et al., 2008) which, according to Wisniewski et al. (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 h. The absence of PTGS2 mRNA variation during IVM was also reported by Kyassari et al. (2012) in sheep oocytes. 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 oocytes (Kyassari 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 the *cumulus* cells of our control group during IVM for 18 h are in accordance with that reported by Fiali et al. (2009) in human COCs. According these same authors, the ratio Bcl2 (anti-apoptotic)/Bax (pro-apoptotic) determines the cell survival and the prevalence of BCL2 mRNA expression in *cumulus* cells is strongly related to oocyte competence. While Bcl2/Bax and Glut1 are involved, respectively, in the apoptosis control and glucose metabolism (Fiali et al., 2009; Purcell and Moley, 2009), the others investigated genes (PTX3, PTGS2, ALK5 and ALK6) develop important functions on *cumulus* expansion.

Although the Bax expression in *cumulus* cells treated with roscovitine had been up-regulated after the additional culture for 18 h, it was followed by increase of Bcl2 mRNA. It suggests, therefore, that balance Bcl2/Bax and consequent cellular viability

was maintained. Besides, the similarity between control and rosco groups 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 (BL). 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. In contrast, the *cumulus* expansion of porcine COCs was irreversibly inhibited by 50 µM roscovitine (Romar et al., 2006). These divergences among authors are probably related not only to drug concentration but also to exposure time to inhibitor (Han et al., 2006; Sananmuang et al., 2010).

The high proportion of oocytes at GV (87.7%) after follicle aspiration suggest that time interval to recovery and select COCs was adequate and did not induce the meiotic resumption before the culture. Similar results were observed in goat (Han et al., 2006), pig (Romar et al., 2006) and bovine (Barretto et al., 2011) oocytes. Besides, the significantly high rate of GV in the rosco group in contrast to evident meiosis progression observed in the control group at 6 h of culture demonstrate that 75µM roscovitine was efficient to arrest the meiosis of sheep oocytes. This meiotic inhibition was still completely reversible since, in both experimental groups, significant rate of oocytes reached the MII stage after additional culture for 18 h. The reversible meiotic inhibition was also recorded in cat (Sananmuang et al., 2010), goat (Han et al., 2006), pig (Coy et al., 2005), 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 still indicates that our 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 both experimental groups during 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, according to Turathum et al. (2010), the SOD1 mRNA level remained stable in canine oocytes. Presuming that reduction of mRNA levels indicate translation (Bebbere et al., 2008), the expression decrease of ZAR1, NLRP5 and SOD1observed in our study suggest participation of these proteins during oocyte maturation. The maternal effect genes (ZAR1 and NLRP5) are still recognized by their importance during oocyte-

embryo transition, while SOD1 is crucial for protection against free radicals (Mouatassim et al., 1999; 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 our control group is in accordance with that reported by Bebbere et al., (2008) in sheep oocytes. Probably, this 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). Although roscovitine treatment appears to prevent the decrease of GDF9 mRNA level in our study, further investigations are necessary regarding this aspect. Besides, the proper ratio Bcl2/Bax observed in oocytes of both experimental groups reveals that cellular viability was maintained during the culture (Feugang et al., 2001) even in the presence of inhibitor.

The gene expression profile observed in our control group reinforces, therefore, the evidence that oocyte transcriptional activity is interrupted with the meiosis resumption (Vigneron et al., 2004). According these same authors the maternal genes tends to remain stable or decline because of translation during IVM. Our findings also demonstrate that meiotic arrest promoted by roscovitine did not affect but also did not improve the expression of the investigated genes in sheep oocytes. Likewise, Leal et al. (2012) reported that most studied genes in bovine oocytes meiotically inhibited with BL maintained the same expression pattern of control. In contrast, Zhang et al. (2011) observed that cyclin B1, cdc2, c-mos, GDF9 and BMP15 mRNA reached high values in pig oocytes treated with roscovitine. Significant increase of poly (A) mRNA content was also reported by Lequarre et al. (2004) in bovine oocytes arrested at GV stage, which suggest possible neotranscription. However, in other cells types, roscovitine significantly suppressed the mRNA synthesis (Ljungman and Paulsen, 2001). We assumed that this discrepancy among authors is probably related to inhibition duration, studied genes, methods of mRNA detection and species particularities.

In conclusion, we can infer that roscovitine at concentration and exposure time studied is efficient to reversibly arrest the meiosis in sheep oocytes without detrimental effects on *cumulus* expansion and gene expression. Despite this, the meiotic inhibition for 6 h does not improve the relative abundance of the investigated transcripts. Further investigations have been performed to evaluate the effects of roscovitine treatment on *in vitro* development of sheep embryos.

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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
Bcl-2	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

Table 2. *Cumulus* expansion degree of sheep COCs after the *in vitro* culture for 6 h in basic maturation medium (Control) containing 75 µM roscovitine (Rosco) followed by IVM for 18 h in inhibitor-free medium. Immature (0 h): stained immediately after aspiration.

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

*Significant differences indicated by lowercase letters in the column and capital letters in the line ($P < 0.05$).

Table 3. Nuclear maturation stage of sheep oocytes after the *in vitro* culture for 6 h in basic maturation medium (Control) containing 75 µM roscovitine (Rosco) followed by IVM for 18 h in inhibitor-free medium. Immature (0 h): stained immediately after aspiration.

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

*Significant differences indicated by lowercase letters in the column and capital letters in the line ($P < 0.05$). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II, Deg: degenerate.

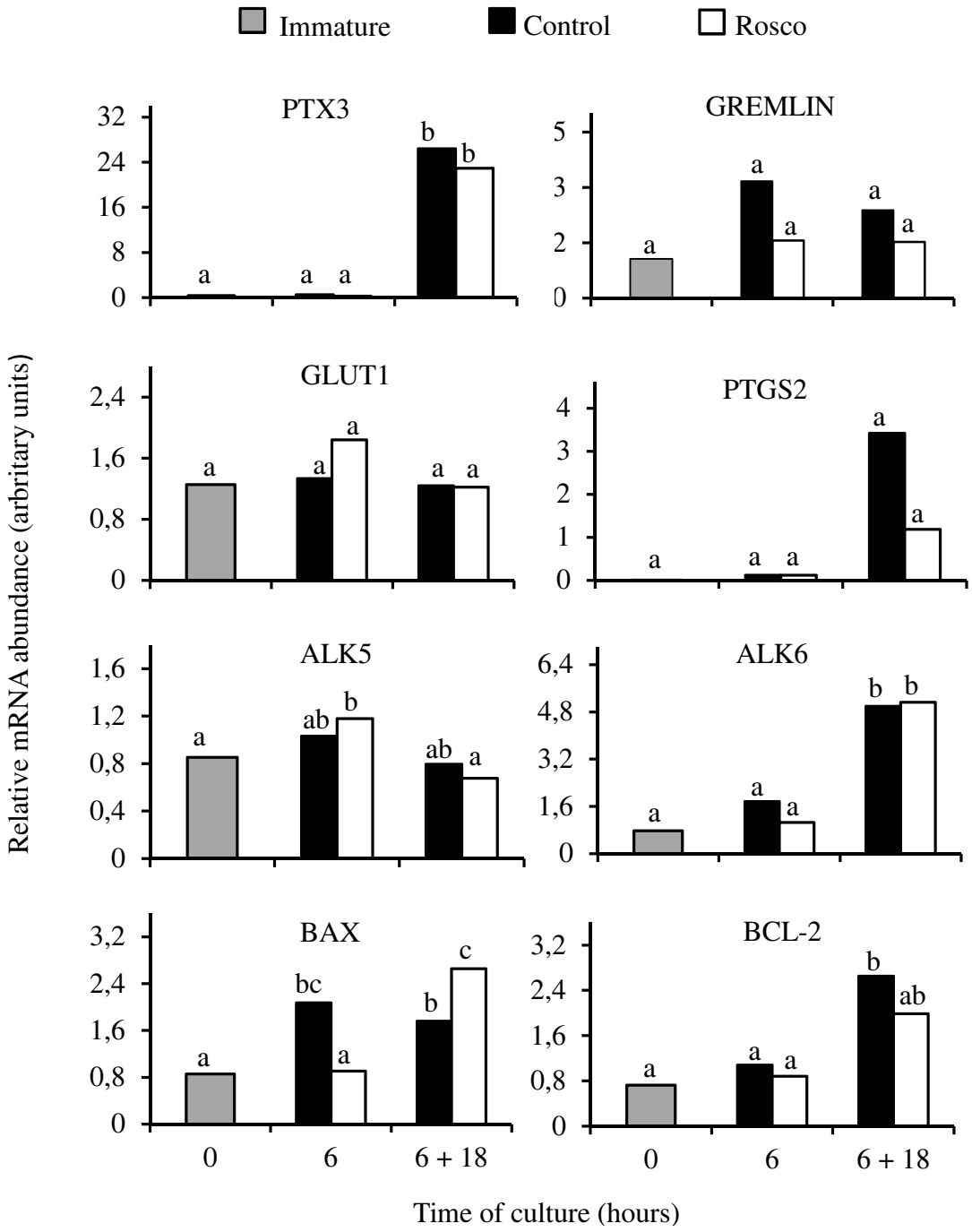

Fig.1. Relative expression of the PTX3, GREMLIN, GLUT1, PTGS2, ALK5, ALK6, BAX and BCL-2 genes in sheep *cumulus* cells after 6 h of treatment with 75 µM roscovitine (Rosco) followed by IVM for 18 h in inhibitor-free medium. Control: oocytes not exposed to meiosis inhibitor. Immature (0 h): stained immediately after aspiration. *Values with different superscripts letters differ significantly ($P < 0.05$).

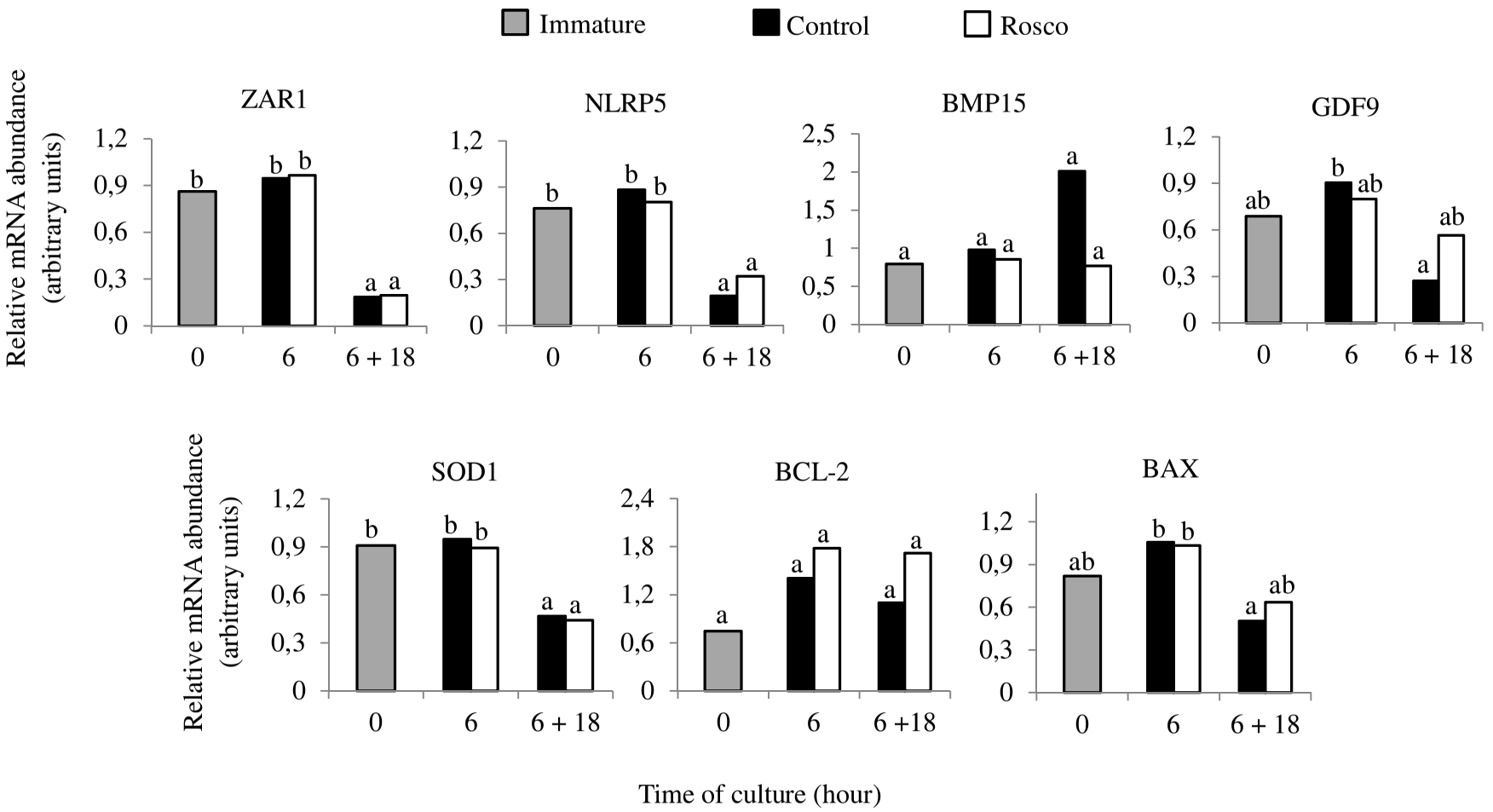

Fig. 2. Relative expression of the ZAR1, NLRP5, BMP15, GDF9, SOD1, BAX and BCL-2 genes in sheep oocytes after 6 h of treatment with 75 µM roscovitine (Rosco) followed by IVM for 18 h in inhibitor-free medium. Control: oocytes not exposed to meiosis inhibitor. Immature (0 h): stained immediately after aspiration. *Values with different superscripts letters differ significantly ($P < 0.05$).

CAPÍTULO 5

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Effects of roscovitine on meiosis progression and developmental competence of sheep oocytes

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Abstract

The temporary meiotic arrest with cyclin-dependent kinase inhibitors has been proposed as strategy to reestablish the synchronization between nuclear and cytoplasmic maturation and, consequently, improve the *in vitro* developmental competence of oocytes. Based on that, the present study aimed to evaluate the potential of roscovitine to reversibly arrest the meiosis in sheep oocytes and its effects on *in vitro* embryo production. *Cumulus*-oocyte complexes (COCs) were cultured, for 6 h, in TCM 199 supplemented with 10% heat-treated oestrus sheep serum, 8 mg/ml of pyruvate and 100 mM cysteamine containing 0 μM (Rosco 0 μM) or 75 μM roscovitine (Rosco 75 μM). Subsequently, they were *in vitro* matured (IVM) for a further 18 h in the presence of LH and FSH. COCs matured *in vitro* for 24 h in medium supplemented with gonadotropins were used as control. At 0 h, 6 h and 24 h, the nuclear status of the oocytes from each experimental group was evaluated by Hoechst staining. The cleavage and blastocyst rate were recorded after the *in vitro* fertilization with frozen/thawed ram semen and embryo culture for 7 days. Blastocysts were evaluated by differential staining. Our results showed that 94.2% of oocytes treated for 6 h with roscovitine were arrested at GV stage. This rate was similar to that observed in immature oocytes (94.9%) and significantly higher compared to that of Rosco 0 μM (40.3%). The rate of oocytes from Rosco 75 μM (91%) and Rosco 0 μM (89%) groups at MII after culture for a further 18 h was similar to that observed in the Control group (92.2%). No significant difference was observed among experimental groups in terms of cleavage rate, development to blastocyst stage and the blastocyst cell number. It is concluded that roscovitine reversibly inhibit the meiosis of adult sheep oocytes without detrimental effect on quality and development of embryos *in vitro* produced.

Key words: Nuclear maturation; Embryo development; Roscovitine; Inhibition; Ovine

1.Introduction

The small ruminants represent an important part of economy of several developing countries. In this context, the reproductive biotechnologies, as *in vitro* embryo production (IVP), could represent a promising alternative to accelerate the genetic improvement and increase the productivity in ruminant livestock (Rodriguez-Martinez, 2012; Souza-Fabjan et al., 2014). However, despite the recent advances, the quality and developmental competence of *in vitro* produced sheep embryos are still low

compared to that obtained *in vivo* (Cognié et al., 2003; Souza-Fabjan et al., 2014). According Krisher (2004), this low development efficiency is directly related to the poor quality of oocytes *in vitro* matured.

In vivo, the oocytes are arrested at vesicle germinal stage during the folliculogenesis until the preovulatory gonadotropin surge, when meiosis is resumed. During this period of meiotic arrest, oocytes undergo structural modifications and store mRNAs and proteins necessary to become competent. These events characterize the cytoplasmic maturation and are considered crucial to support the maturation, fertilization and early embryo development (Sirard et al., 2006; Mermillod et al, 2008).

In contrast, when oocyte is removed from the follicular environment and is cultured *in vitr*o, the spontaneous meiosis resumption occurs independently of the cytoplasmic maturation stage (Pincus and Enzmann, 1935). However, although the nuclear maturation may be successfully complete, it alone is not enough to ensure the subsequent embryo development (Krisher, 2004). Furthermore, oocytes destined to *in vitro* maturation are retrieved from follicles at diverse stages of folliculogenesis (Gharibi et al., 2003) and most of these oocytes have not undergone all required changes to be able to successfully complete the normal development process (Gilchrist and Thompson, 2007).

In this context, the induction of temporary arrest of meiosis during *in vitro* culture has been proposed as a system to reestablish the synchronization of nuclear and cytoplasmic maturation, make the oocyte population more homogeneous and improve the oocyte developmental competence (Gharibi et al., 2003). Recently, the effects of cAMP modulators on *in vitro* matured ovine oocytes resulted in an increase in embryo quality (Rose et al., 2014). However, cAMP is not the only regulatory molecule of the meiotic progression of sheep oocytes. Others important signaling pathways are also involved in this process (Gharibi, et al., 2013).

Among the different pharmacological molecules, the inhibitors of cyclin-dependent kinases, as roscovitine, have been considered a possible candidate due to their specific action on M-phase promoting factor (Donnay et al., 2004), which is considered the main regulator of cell-cycle (Nurse, 1990). The potential effect of roscovitine has been examined in different animal species such as bovine (Donnay et al., 2004; Barreto et al., 2007), goat (Jimenez-Macedo et al., 2006), cat (Sananmuang et al., 2010), pig (Shcoevers et al., 2005) and horse (Consiglio et al., 2010). However, contradictory results have been obtained among authors and species studied. Moreover,

few studies have been conducted in sheep oocytes. In this species, the ultrastructure changes of oocytes treated with roscovitine have already been investigated (Crocomo et al., 2013), but the effect of this inhibitor on potential of *in vitro* development of these oocytes is still unknown.

The present study aimed to evaluate the efficiency of roscovitine to reversibly arrest *in vitro* the nuclear maturation of sheep oocytes and to assess the effect of this inhibitor on *in vitro* embryo production.

2. Materials and methods

This study was performed at the University of Sassari, Italy. All chemicals were purchased from Sigma Chemical. CO. (Sigma- Aldrich Corp., St. Louis, MO, USA), unless otherwise indicated.

2.1. Oocyte recovery and in vitro maturation (IVM)

Ovaries of adult sheep (Sarda sheep 4-6 years old) were collected at local slaughterhouses and transported to the laboratory within 1 h in sterile PBS (Dulbecco's Phosphate Buffered Saline) containing 0.01 % penicillin (100 µg/mL) and streptomycin (100 µg/mL) at 37 °C. COCs were collected through slicing method in sterile Petri dishes containing dissection medium composed of 20 mM Hepes-buffered TCM 199 supplemented with 0.1% (w/v) polyvinyl alcohol (PVA), 50 IU/mL heparin, and antibiotics. Only COCs showing several intact *cumulus* cell layers and uniform ooplasm with homogenously distributed lipid droplets were selected under stereomicroscopy (Olympus IX 70).

In the Control group, COCs were cultured under the conventional method of *in vitro* maturation. For this, COCs were *in vitro* matured in 400 µl of TCM 199 with 10 % (v/v) heat-treated oestrus sheep serum (OSS), 0.1 IU/ml FSH and 0.1 IU/ml LH (Pergonal, Serono Italy), 8 mg/ml of pyruvate and 100 mM cysteamine, overlaid with 300 µl of mineral oil in 4-well dishes (Nunclon, Rosklide, Denemark) at 38.5 °C in a humidified atmosphere of 5 % CO_2 for 24 h.

2.2 Roscovitine treatment and reversibility

To evaluate the meiotic inhibition promoted by roscovitine, the selected COCs were cultured for 6 h in 400 µl of TCM 199 with10 % (v/v) OSS, 8 mg/ml of pyruvate and 100 mM cysteamine (Rosco 0 µM) containing 75 µM roscovitine (Rosco 75 µM).

In this condition, the culture was performed in four-well plate without oil overlay in 5 % CO_2 in air at 38.5ºC. The stock solution of roscovitine (1 mg/mL) was prepared in dimethylsulphoxide, aliquoted and stored at -20 ºC until use. After *in vitro* culture for 6 h, COCs from Rosco 0 µM and Rosco 75 µM groups were washed in Hepes-buffered TCM 199, and cultured at 38.5 °C and 5 % CO_2 for a further 18 h in inhibitor-free medium maturation composed of TCM 199, 10 % (v/v) OSS, 8 mg/ml of pyruvate, 100 mM cysteamine, 0.1 IU/ml FSH and 0.1 IU/ml LH.

2.3. In vitro fertilization (IVF)

After *in vitro* culture for 24 h, COCs from each group (Control, Rosco 0 µM and Rosco 75 µM) were denuded in TCM 199 containing 300 IU/mL of hyaluronidase using a narrow bore pipette. Mature good quality oocytes were fertilized in SOF medium + 2 % OSS for 22 h at 38.5 ºC and 5 % CO_2, 5 % O_2 and 90 % N_2 under mineral oil in four-well Petri dishes. The fertilization was performed with frozen–thawed ram spermatozoa selected by swim-up technique at 1 x 10^6 sperm cells / mL.

2.4. In vitro embryo culture (IVC)

IVF zygotes were cultured in groups for 7 days in four-well Petri dishes in SOF + essential and non-essential amino acids at oviductal concentration (Walker et al. 1996) + 0.4 % BSA, under mineral oil, in maximum humidified atmosphere with 5 % CO_2, 5 % O_2 and 90 % N_2 at 38.5 ºC. The first and second cleavage was registered 24 and 30 h after the start of fertilization, respectively. Development to blastocyst stage was recorded on the 7 th day of culture.

2.5. Assessment of oocyte chromatin organization

Denuded oocytes were fixed for 30 min in 4% paraformaldehyde and transferred to droplets of Hoechst 33342 in glycerol (10µg/mL) on a glass slide. The oocytes were examined under a fluorescence inverted microscope (Olympus IX 70) and the nuclear status was classified according to Shirazi et al. (2010) as germinal vesicle (GV) germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase, telophase and metaphase II (MII). The oocytes with altered nuclear structure were classified as degenerate.

2.6. Differential staining of embryos

The analysis of blastocyst cell number was performed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) of expanded, hatching and hatched blastocysts from each experimental group at day 7 of *in vitro* culture. For this, embryos were exposed, for 30 seconds, to 1 % Triton X-100 in 20 mM Hepes-buffered TCM 199 containing 30 µg/mL propidium iodide (PI). After, they were washed for 3-5 seconds in ethanol 100 % and transferred to a solution of 10 µg/mL Hoechst 33342 in ethanol and glycerol, for 5 min. The embryos were mounted in a glycerol droplet on a glass slide and examined under fluorescence microscope (excitation, 340-380nm; emission, 440-480 nm). The cells number of inner cell mass (ICM) were stained in blue caused by DNA labelling with Hoechst 33342, and the trophectoderm (TE) were stained in red.

2.7. Experimental design

The experimental design used was completely randomized with three experiments, each one with three experimental groups and 5 replicates for each parameter assessed.

2.7.1. Experiment 1 - Potential of meiotic inhibition of roscovitine

The nuclear configuration of oocytes from Rosco 75 µM (n = 112) and Rosco 0 µM (n = 103) groups was evaluated by Hoechst 33342 staining after 6 h of culture with and without roscovitine, respectively. An additional sample of immature oocytes (n = 78) was stained soon after removal from follicle to evaluate the meiosis stage before the *in vitro* culture.

2.72. Experiment 2 - Reversibility of inhibitory action of roscovitine

The nuclear configuration of oocytes from Rosco 0 µM (n = 95) and Rosco 75 µM (n = 115) groups was evaluated by Hoechst 33342 staining after the *in vitro* culture for a further 18 h in inhibitor-free medium supplemented with gonadotropins. In the Control group (n=115), the nuclear configuration was examined after the *in vitro* culture for 24 h without interruption.

2.7.3. Experiment 3- In vitro embryo development

After the *in vitro* maturation for 24h, oocytes from Rosco 0 µM (n = 207), Rosco 75 µM (n = 214) and Control (n = 198) were *in vitro* fertilized. The first and second embryo cleavage were registered after 24 and 30h of the *in vitro* fertilization, respectively. The rate and development stage of embryos (early blastocyst, blastocyst, expanded, hatching and hatched blastocyst.) was recorded on day 7 of *in vitro* culture.

On this same day, a sample of expanded, hatching and hatched blastocysts from each experimental group was submitted to differential staining to evaluate the embryo quality.

2.8. Statistical analysis

The data were transformed into square root of x + 0.5 and subjected to analysis of variance according to completely randomized design. The means were compared by the Tukey test at 5% probability.

3.Results

3.1. Experiment 1 - Potential of meiotic inhibition of roscovitine

According to table 1, the treatment with 75µM roscovitine kept 94.2 % of oocytes at GV stage during the first 6h of *in vitro* culture. This result was similar to that observed in immature group (94.9 %) and significantly higher compared to Rosco 0 µM (40.3 %). Besides, significantly higher rate of oocytes from Rosco 0 µM was at GVBD (37.8 %) and MI (21.9 %) compared to Rosco 75 µM, in which only 5.0 % and 0.8 % of oocytes resumed the meiosis and reached the GVBD and MI stages, respectively. The group of oocytes stained at 0h (immature) had similar proportion of GVBD (5.1 %) and MI (0.0 %) oocytes. In all experimental groups, there was no oocyte at MII, anaphase, telophase and degenerated (Table 1).

3.2. Experiment 2 - Reversibility of inhibitory action of roscovitine

The rate of oocytes at MII in the Rosco 75 µM (91 %) and Rosco 0 µM (89 %) groups were similar to that observed in Control group (92 %). This demonstrates that the *in vitro* culture for a further 18 h in inhibitor-free medium supplemented with gonadotropins was enough to the proper meiosis progression. Moreover, in all experimental groups, the low rate of oocytes that did not complete the meiosis compared to that at MII indicates that the *in vitro* culture conditions were adequate (Table 2).

3.3. Experiment 3- In vitro embryo development

As shown in table 3, the treatment with 75 µM roscovitine (Rosco 75 µM) as well as the *in vitro* culture for 6 h without gonadotropins (Rosco 0 µM) did not affect the percentages of cleavage and blastocyst rate, which was similar to that observed in Control group. Regarding the embryo formation, no significant difference was observed among the experimental groups at day 7 of culture (table 4, Fig. 1). No differences were

observed about the quality of embryos evaluated by the differential staining. The treatment for 6 h with roscovitine did not affect the number of ICM and TE, which was similar to that observed in the Rosco 0 µM and Control groups (Table 5, Fig. 1).

4. Discussion

The oocytes removed from the follicular environment spontaneously resume the meiosis (Pincus and Enzmann, 1935). Most of these oocytes, however, did not undergo all morphological and biochemical changes necessary to acquire developmental competence (Gilchrist and Thompson 2007). In this context, the temporary arrest of meiosis with cyclin-dependent kinases inhibitors has been proposed as strategy to restore the synchrony between the nuclear and cytoplasmic maturation, and improve the quality of oocytes *in vitro* matured (Mermillod et al., 2000, Donnay et al., 2004). Based on that, the present study aimed to evaluate the efficiency of roscovitine to reversibly arrest the nuclear maturation of sheep oocytes and to assess the effect of this inhibitor on *in vitro* embryo production.

In our experimental study, a high rate of germinal vesicle (94.9 %) and absence of oocyte degeneration was found in the oocytes stained immediately after their removal from follicles, which was similar of those reported by Barretto et al. (2010) in bovine and Han et al. (2006) in goat. This result indicates that the time interval between transport of ovaries, recovery and selection of COCs was adequate and did not induce the meiotic resumption before *in vitro* culture.

The significantly higher percentage of oocytes at GV stage in the Rosco 75 µM (94.2 %) in comparison to that observed in the Rosco 0 µM (40.3 %) revealed that the addition of 75 µM roscovitine was enough to inhibit the meiosis of sheep oocytes. Similar efficiency of meiotic inhibition was reported in goat (Han et al., 2006); pig (Le Beux et al., 2003) and bovine (Mermillod et al., 2000) oocytes treated for 24 h with 200 µM, 50 µM and 25µ M roscovitine, respectively. However, the comparison among studies is difficult since different species and different concentrations of inhibitor have been used (Adona and Leal, 2004).

According to Han et al. (2006), the differences encountered between species regarding the concentration of roscovitine effective to inhibit the meiotic resumption during the *in vitro* maturation is probably related to the existence of different regulatory molecules. Besides, Meijer and Kim (1997) demonstrated in bovine oocytes that roscovitine at concentrations higher than 25 µM may also inhibit the MAPK activity.

However, very high doses of this inhibitor can be toxic (Sananmuang et al., 2010). It is important to highlight that the conditions of *in vitro* culture, as composition of medium and incubator temperature, can also affect the efficiency of the inhibitor (Han et al., 2008).

According to Sananmuang et al. (2010), the duration of incubation with meiosis inhibitors can also affect the oocyte developmental competence. Han et al. (2006) demonstrated that reduction of exposure time from 24 h to 8 h of goat oocytes to roscovitine decreased the harmful effects and improved the percentage of blastocysts which reached similar level of the control group. Based on that and our preliminary tests, the present study was performed using inhibition time of 6 h and 75 µM concentration of roscovitine.

After 6 h of *in vitro* culture in absence of roscovitine and gonadotropins, 37.8 % of oocytes and 21.9 % of oocyte were at GVBD and MI, respectively. These results are simila to those obtained by Moor and Crosby (1986) when sheep oocytes were *in vitro* matured in the presence of FSH and LH. According to Sha et al. (2010), although the nuclear maturation may be complete in the absence of gonadotropins, the supplementation of medium with FSH and LH accelerates the meiosis progression and allows better rates of MII.

Our findings also showed that treatment with roscovitine was completely reversible. In fact, the oocytes cultured for 6 h with roscovitine and those from Rosco 0 µM had similar percentage of MII after further culture for 18 h with gonadotropins. This result is consistent with observed in porcine (Romar et al. 2006), goat (Han et al., 2006), and bovine (Adona and Leal, 2004) oocytes *in vitro* matured, after roscovitine exposition, for 44h, 24h and 18h, respectively. Furthermore, the low rate of degenerated oocytes observed in our study is in accordance with the reported by Le Beux et al. (2003) and indicates the conditions of *in vitro* culture were suitable.

Ponderato et al. (2001) demonstrated that bovine oocytes treated with meiosis inhibitors undergone GVBD and reached the MII stage 5 h earlier than those from control. Similar observation has also been reported in goat and cat (Han et al., 2006; Sananmuang et al., 2010). This acceleration of meiosis progression probably occurs due the synthesis and accumulation of some factors developmentally relevant during the meiosis block (Vigneron et al., 2004). Besides, the prolonged culture of COCs leads to structural alterations as ooplasmic fragmentation beyond the parthenogentic activation (Schoevers et al., 2005). Based on that and our preliminary tests, we established that 18

h of culture with gonadotropins, after 6 h of meiotic inhibition, was sufficient to complete the meiosis and to avoid any aging deleterious effect determined by an extension of culture time.

Our findings also indicate that roscovitine did not affect the oocyte developmental competence as evidenced by the similarity of the cleavage rate and development up to blastocyst stage between the Rosco 0 µM and Rosco 75 µM. This result are in accordance with those reported in bovine (Lagutina et al., 2002; Mermillod et al., 2000) and goat (Han et al.2006). Likewise, the quality of sheep embryos obtained in the present study was similar to that reported by Zacchini et al. (2011), as evidenced by the cell number of ICM and TE. In pig, Coy et al. (2005) also observed pregnancy and delivery of 12 healthy piglets after the transfer of embryos derived from oocytes treated with roscovitine. Furthermore, the similarity between Rosco 0 µM and Control groups observed in our study demonstrate that the absence of gonadotropins in the early 6 h of culture did not significantly affect the developmental rates.

Similar result was observed in sheep oocytes treated with 150 µM butyrolactone for 8 h (Lu et al., 2013) and with 1 µM cilostamide for 22 h (Gharibi et al., 2013) followed by IVM for 20 h and 22 h, respectively. According these authors, the inhibition of meiosis was completely reversible without any consequence on the subsequent development and quality of embryos. However, despite the similarity of results, the rate of meiotic inhibition and *in vitro* maturation observed by these authors was lower than that obtained in our study with roscovitine.

In contrast, some authors found in their study with bovine (Adona and Leal, 2004), cat (Sananmuang et al., 2010) and pig (Romar et al., 2006) that treatment with roscovitine prior of IVM compromised the oocyte developmental competence since the rate of blastocyst formation was lower than that of control. This divergence among studies is probably related to the culture and experimental conditions used such as COCs quality, concentration of inhibitor and time of *in vitro* culture established (Coy et al., 2005; Crocomo et al., 2013).

In conclusion our data demonstrated that roscovitine, at conditions established, is able to reversible block the meiosis in oocytes from adult sheep without any remarkable effect on development potential of embryos. Further studies are needed to investigate, at molecular level, the quality of the blastocyst produced *in vitro* after roscovitine treatment and to test their capacity to generate viable and healthy offspring after transfer to recipient animals.

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TABLE 1. Nuclear configuration of sheep oocytes at 0h (immature) and after 6h of *in vitro* culture with (Rosco 75 µM) or without roscovitine (Rosco 0 µM).

Groups	oocytes (n)	Nuclear Maturation Stage (%)						
		GV	GVBD	MI	MII	Anap.	Telop.	Deg.
Immature	78	94.9 ^{aA}	5.1 ^{bB}	0.0 ^{bB}	0.0 ^{aB}	0.0 ^{aB}	0.0 ^{aB}	0.0 ^{aB}
Rosco 0 µM	103	40.3 ^{bA}	37.8 ^{aA}	21.9 ^{aB}	0.0 ^{aC}	0.0 ^{aC}	0.0 ^{aC}	0.0 ^{aC}
Rosco 75 µM	112	94.2 ^{aA}	5.0 ^{bB}	0.8 ^{bB}	0.0 ^{aB}	0.0 ^{aB}	0.0 ^{aB}	0.0 ^{aB}

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; Anap.: anaphase; Telop.: telophase; MII: metaphase II, DEG: degenerated. *Differences indicated by lowercase letters in the columns and capital letters in the lines ($P < 0.05$).

TABLE 2. Meiotic progression of sheep oocytes *in vitro* cultured for 6 h with 75 µM roscovitine followed by IVM for 18 h (Rosco 75 µM). Rosco 0 µM: culture for 6 h without inhibitor followed by IVM for 18 h. Control: IVM for 24 h

Groups	No. COC	Stage of Meiotic Progression, *n* (%)						
		GV	GVBD	MI	MII	Anap.	Telop.	Deg.
Control	115	2 (2)^{aB}	0 (0)^{aB}	6 (5)^{aB}	106 (92)^{aA}	0 (0)^{aB}	0 (0)^{aB}	1 (1)^{aB}
Rosco 0 µM	95	3 (3)^{aB}	0 (0)^{aB}	6 (6)^{aB}	84 (89)^{aA}	0 (0)^{aB}	0 (0)^{aB}	2 (2)^{aB}
Rosco 75 µM	94	0 (0)^{aB}	2 (2)^{aB}	2 (2)^{aB}	85 (91)^{aA}	2 (2)^{aB}	1 (1)^{aB}	2 (2)^{aB}

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; Anap.: anaphase; Telop.: telophase; MII: metaphase II, DEG: degenerated. *Differences indicated by lowercase letters in the columns and capital letters in the lines ($P < 0.05$).

TABLE 3. Effect of 6 h treatment of the sheep oocytes with roscovitine (Rosco 75 µM) followed by 18 h of IVM on cleavage and blastocyst rates. Rosco 0 µM: culture for 6 h without inhibitor followed by IVM for 18 h. Control: IVM for 24 h.

Groups	No. oocytes	Rate of Embryo Development		
		Cleavage post 24 h *n* (%)	Cleavage post 30 h *n*. (%)	Blastocyst *n* (%)**
Control	198	78 (37.2)	142 (70.9)	90 (63.0)
Rosco 0 µM	207	63 (31.7)	142 (68.4)	70 (49.0)
Rosco 75 µM	214	74 (34.7)	153 (71.6)	75 (48.9)

**Cleavage post 24 and 30 h were calculated on the total number of oocytes. Blastocyst rate was calculated on the number of cleaved embryos post 30 h of the IVF. ($P > 0.05$)

TABLE 4. Development stage of blastocyst *in vitro* produced (D7) after 6 h treatment of sheep oocytes with roscovitine (Rosco 75 µM) followed by IVM for 18 h. Rosco 0 µM: culture for 6 h without inhibitor followed by IVM for 18 h. Control: IVM for 24 h.

Groups	Embryo (n)	Development Stages of Blastocyst No. (%)				
		initial	blastocyst	expanded	hatching	hatched
Control	90	4 (4.4) ^{aC}	25 (24.3) ^{aB}	35 (40.8) ^{aA}	24 (28.3) ^{aB}	2 (2.0) ^{aC}
Rosco 0 µM	70	2 (2.7) ^{aC}	8 (13.8) ^{aB}	42 (58.1) ^{aA}	18 (25.3) ^{aB}	0 (0.0) ^{aC}
Rosco 75 µM	75	5 (6.7) ^{aC}	8 (11.9) ^{aB}	40 (50.3) ^{aA}	21 (29.7) ^{aB}	1 (1.4) ^{aC}

*Differences indicated by lowercase letters in the columns and capital letters in the lines ($P < 0.05$).

TABLE 5. Effect of 6 h treatment of sheep oocytes with roscovitine (Rosco 75 µM) followed by 18 h of IVM, on quality of embryos *in vitro* produced at day 7of development. Rosco 0 µM: culture for 6 h without inhibitor followed by IVM for 18 h. Control: IVM for 24 h

Groups	Differential staining of embryos (D7)			
	No. embryo	**Total cells number****	**ICM *n* (%)***	**TE *n* (%)***
Control	18	104,65 ± 7.8	16.6 (16)	88.1 (84)
Rosco 0 µM	32	103,43 ± 8.9	24.5 (23)	79.2 (77)
Rosco 75 µM	30	111,70 ± 1.9	22.9 (21)	89.1 (80)

ICM: inner cell mass, TE: trophectoderm. **Mean ± SEM of the total cells number. *Mean percentage calculated on total cells number. (P > 0.05).

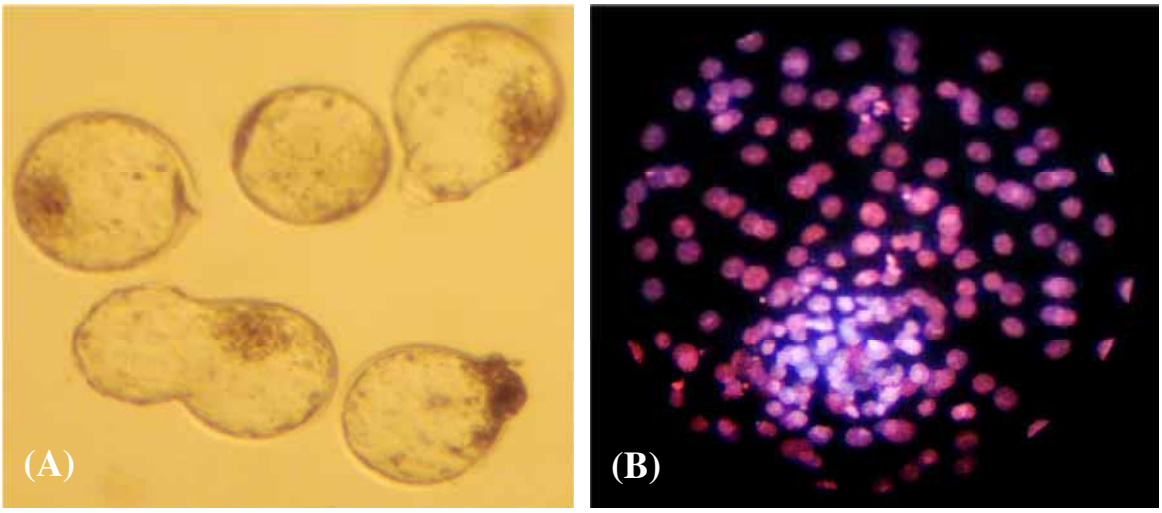

Fig.1. Morphological appearance of sheep blastocysts *in vitro* produced: (A) expanded and hatching blastocysts; (B) expanded blastocyst with differential staining. Nuclei of inner cell mass appear blue (Hoescht 33342) and the trophectoderm cells appear red (PI) (Magnification 200X).

IMPLICAÇÕES

Os resultados obtidos no presente estudo forneceram informações relevantes para melhor compreensão de alguns dos inúmeros eventos fisiológicos implicados nos processos de maturação oocitária e expansão do *cumulus* em COCs de ovinos. A ausência de relação entre a progressão da meiose e a expansão do *cumulus* consiste numa constatação inédita para espécie em questão e incita o desenvolvimento de novas pesquisas com intuito de esclarecer os mecanismos biológicos envolvidos neste contexto. Foi verificado também que, embora a suplementação do meio de maturação com gonodotrofinas e soro fetal bovino seja essencial para a expansão do *cumulus,* estas substâncias não são imprescindível para a progressão da meiose oocitária.

Com relação à metodologia de cultivo *in vitro* dos COCs ovinos foi constatado ainda que o potencial de inibição meiótica da roscovitina é potencializado na ausência da camada protetora de óleo mineral, em virtudade da lipossolubilidade do referido inibidor. No entanto, a exposição dos COCs à roscovitina por períodos superiores a 6 horas promove efeitos deletérios às celuas do *cumulus,* o que prejudica a retomada do processo de expansão. Além disso, a ausência de efeitos benéficos e prejudiciais sobre a competência de desenvolvimento oocitário *in vitro* e sobre a expressão gênica tanto nos oócitos quanto nas células do *cumulus* sugere que o emprego da roscovitina visando o bloqueio meiótico temporário dos oócitos pode ser considerado facultativo conforme as necessidades circunstanciais da pesquisa em questão, como no caso do transporte de COCs por períodos prolongados.

No entanto, apesar da importante contribuição científica obtida com o presente estudo, ainda restam questões referentes ao uso da roscovitina em COCs de ovinos a serem esclarecidas. Aspectos relacionados ao modo de ação da roscovitina sobre as células do *cumulus,* assim como o efeito do tempo de incubação e das diferentes concentrações deste inibidor sobre as características citoesqueléticas e moleculares dos COCs de ovinos ainda precisam ser mais bem avaliados.

Finalmente, a escassez de informação e a discrepância de resultados entre autores e espécies estudadas torna evidente a necessidade de novas pesquisas no sentido de estabelecer condições de cultivo que justifiquem o uso de inibidores da meiose, como a roscovitina, no sistema comercial de produção *in vitro* de embriões visando o aprimoramento biotecnológico e a maior eficiência em termos de taxa e viabilidade dos blastocistos obtidos.

CONCLUSÕES GERAIS

1- As gonadotrofinas e o soro fetal bovino, presentes no meio de maturação, não interferem no potencial da roscovitina em inibir reversivelmente a meiose de COCs de ovinos cultivados *in vitro*. Além disso, independentemente da composição do meio, a ação da roscovitina sobre as células do *cumulus* não é completamente reversível.

2- O sistema de cultivo na ausência de óleo mineral potencializa a inibição meiótica reversível promovida pela roscovitina sem prejudicar o processo de expansão do *cumulus* ou promover efeitos deletérios nos oócitos.

3- A eficiência da inibição meiótica promovida pela roscovitina se mantém ao longo do cultivo *in vitro*. No entanto, a exposição prolongada dos COCs a esse inibidor afeta o processo de expansão do *cumulus* de maneira irreversível.

4- A inibição temporária dos COCs de ovinos no estadio de vesícula germinativa promovida pela roscovitina não melhora, mas também não prejudica, a expressão dos genes investigados tanto nos oócitos quanto nas células do *cumulus*.

5- A roscovitina na concentração de 75 µM é eficiente em inibir reversivelmente a meiose em COCs de ovinos por um período de 6 horas sem afetar potencial de desenvolvimento embrionário *in vitro* nem a qualidade dos embriões obtidos.