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Programa de Pós-Graduação em Biotecnologia

RENORBIO

EFEITO DO HORMÔNIO FOLÍCULO ESTIMULANTE (FSH) E DA MELATONINA SOBRE O CULTIVO E A MATURAÇÃO *IN VITRO* DE FOLÍCULOS OVARIANOS PRÉ-ANTRAIS E ANTRAIS

VANESSA RAQUEL PINTO DE BARROS

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Universidade Federal Rural de Pernambuco - UFRPE

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Rede Nordeste de Biotecnologia - RENORBIO

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VANESSA RAQUEL PINTO DE BARROS

Efeito do hormônio folículo estimulante (FSH) e da melatonina sobre o cultivo e a maturação *in vitro* de folículos ovarianos pré-antrais e antrais

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Epígrafe

“Jamais considere seus estudos como

uma obrigação, mas como uma oportunidade

invejável para aprender a conhecer

a influência libertadora da beleza

do reino do espírito, para seu próprio

prazer pessoal e para proveito da comunidade

à qual seu futuro trabalho pertencer.”

Albert Einstein

Resumo

O efeito do hormônio folículo estimulante recombinante humano (rhFSH) e da melatonina sobre cultivo e a maturação *in vitro* de folículos ovinos foi avaliado. Este trabalho foi divido em 3 Capítulos. Capítulo I: Folículos secundários foram cultivados em α-MEM^{+} (controle) ou adicionado de diferentes concentrações fixas (500, 750 e 1000 ng/mL) ou concentrações sequenciais de rhFSH (Seq. 1 e Seq 2). Folículos secundários (Capítulo II) e antrais iniciais (Capítulo III) foram cultivados utilizando diferentes concentrações fixas da melatonina (Mel 100, Mel 500 ou Mel 1000 pg/mL) ou uma concentração sequencial (Mel Seq). Cap.I: a sobrevivência folicular foi maior em rhFSH 750 ng/mL do que em α-MEM^{+} e 1000 ng/mL. A adição de rhFSH ao meio promoveu um aumento significativo na percentagem de oócitos totalmente crescidos em todos os tratamentos em comparação ao α-MEM^{+}. A atividade mitocondrial foi maior nos tratamentos com rhFSH do que o controle, exceto o rhFSH Seq. 2. Após a MIV, oócitos de folículos intactos cultivados em rhFSH 750 ng/mL apresentaram um aumento significativo na porcentagem de maturação em comparação ao α-MEM^{+}. Cap.II: Mel 500 pg/mL e Mel 1000 pg/mL apresentaram uma taxa de oócitos totalmente crescidos significativamente maior do que os outros tratamentos. Após a MIV, os níveis de ERO foram menores em Mel 1000 pg/mL em comparação aos demais tratamentos. Cap III: Mel 500 pg/mL apresentou maior taxa de oócitos totalmente crescidos *in vitro* que os outros tratamentos. Após o CIV, os níveis de ERO no oócito foram semelhantes entre Mel 500 pg/mL e MelSeq, sendo ambos menores que os demais tratamentos. A atividade mitocondrial foi similar entre os tratamentos controle, Mel 500 e 1000 pg/mL. Mel 500 pg/mL apresentou uma porcentagem maior de oócitos GVBD do que o grupo controle e percentuais similares aos demais. Folículos cultivados em melatonina seguida de maturação oocitária com adição de 500 pg/mL de melatonina no meio de MIV apresentaram aumento da atividade mitocondrial do que α-MEM^{+}. Conclusões gerais: Após os resultados obtidos, pode-se inferir que utilizando um meio definido, o FSH na concentração de 750 ng/mL mantém a sobrevivência folicular e promove o aumento dos níveis de mitocôndrias ativas, do crescimento e melhora a maturação oocitária. Além disso, foi possível demonstrar que a melatonina no cultivo de folículos pré-antrais e antrais iniciais pode aumentar os níveis energéticos dos oócitos, reduzir a produção de espécies reativas de oxigênio e favorecer a obtenção de oócitos com tamanho apropriado para a maturação *in vitro*.

Palavras – Chave: ERO. Albumina sérica bovina. Mitocôndrias. GSH.

Abstract

The effect of human recombinant follicle stimulating hormone (rhFSH) and melatonin on culture and *in vitro* maturation of ovine follicles was evaluated. This work was divided into 3 Chapters. Chapter I: Secondary follicles were cultured in α-MEM^{+} (control) or added at different fixed concentrations (500, 750 and 1000 ng/mL) or sequential concentrations of rhFSH (Seq.1 and Seq.2). Secondary follicles (Chapter II) and initial anthraxes (Chapter III) were cultured using different fixed concentrations of melatonin (Mel 100, Mel 500 or Mel 1000 pg/mL) or a sequential concentration (Mel Seq). Cap.I: follicular survival was higher in rhFSH 750 ng/mL than in α-MEM^{+} and 1000 ng/mL. The addition of rhFSH in the medium promoted a significant increase in the percentage of fully grown oocytes in all treatments compared to α-MEM^{+}. Mitochondrial activity was higher in rhFSH treatments than control, except rhFSH Seq. 2. Following IVM, intact follicular oocytes cultured in rhFSH 750 ng/mL showed a significant increase in maturation percentage compared to α-MEM^{+}. Cap.II: Mel 500 pg/mL and Mel 1000 pg/mL showed a significantly higher rate of fully grown oocytes than the other treatments. After the IVM, ERO levels were lower in Mel 1000 pg / mL compared to the other treatments. Cap III: Honey 500 pg/mL presented higher rate of fully grown oocytes *in vitro* than the other treatments. After IVC, the levels of ROS in the oocyte were similar between Mel 500 pg/mL and MelSeq, both being lower than the other treatments. Mitochondrial activity was similar between control treatments, Mel 500 and 1000 pg/mL. Mel 500 pg/mL presented a higher percentage of GVBD oocytes than the control group and percentages similar to the others. Follicles cultured in melatonin followed by oocyte maturation with addition of 500 pg/mL melatonin in the IVM medium showed increased mitochondrial activity than α-MEM^{+}. After the results obtained, it can be inferred that using a defined medium, FSH at a concentration of 750 ng/mL maintains follicular survival and promotes an increase in active mitochondria levels, growth and improvement of oocyte maturation. In addition, it was possible to demonstrate that melatonin in the culture of preantral and antral follicles can increase oocyte energetic levels, reduce the production of reactive oxygen species and favor the production of oocytes of appropriate size for *in vitro* maturation.

Keywords: ROS. Bovine serum albumin. Mitochondria. GSH.

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Lista de abreviaturas, siglas e símbolos

a: Antrum (antro)

ANOVA: Analysis of variance (análise de variância)

ATP: Adenosine-5'-triphosphate (trifosfato de adenosina)

BAX: Bcl-2 associated X protein (proteína X associada ao Bcl-2)

Bcl-2: B-cell lymphoma protein 2 (proteína linfoma de célula B2)

bFGF: Basic fibroblast growth factor (fator de crescimento fibroblástico básico)

BMP-15: Bone morphogenetic protein-15 (proteína morfogenética do osso-15)

BMP-2/-4/-6: Bone morphogenetic protein-2/-4/-6 (proteína morfogenética do osso-2/-4/-6)

BMP-7/-8b: Bone morphogenetic protein-7/-8b (proteína morfogenética do osso-7/-8b)

BSA: Bovine serum albumin (albumina sérica bovina)

Ca++: Calcium ion (íon cálcio)

cAMP: Cyclic adenosine-3',5'-monophosphate (adenosina-3',5'-monofosfato cíclico)

cc: Cumulus cells (células do cumulus)

CG: Células da granulosa

CGP: Células germinativas primordiais

c-Kit: Kit ligand receptor (Receptor para kit ligand)

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

CO_2: Dióxido de Carbono

CCOs: Cumulus oocyte complexes (complexos cumulus-oócito)

DAB: Diaminobenzidina

DNA: Deoxyribonucleic acid (ácido desoxirribonucléico)

DNMT1a: gene responsável pela metilação do DNA

EGF: Epidermal growth factor (fator de crescimento epidermal)

ERO: Espécies reativas de oxigênio

FGF: Fibroblast growth fator (fator de crescimento de fibroblasto)

Fig.: Figure (figura)

FIV: Fecundação *in vitro*

Fox12: Forkhead box L2 (proteína forkhead L2)

Foxo3a: Forkhead transcription factor (fator de transcrição Foxo forkhead)

FSH: Follicle stimulating hormone (hormônio folículo estimulante)

FSHr: FSH recombinante

FSHR: FSH receptor (receptor de FSH)

G: Gauge (calibre)

GC: Granulosa cell (célula da granulosa)

GDF-9: Growth differentiation factor-9 (fator de crescimento e diferenciação-9)

GDP: Guanosine diphosphate (guanosina difosfato)

GH: Growth hormone (hormônio do crescimento)

GnRH: Gonadotropin-releasing hormone (hormônio liberador de gonadotrofinas)

GSH: Glutationa peroxidase

GV: Germinal vesicle (vesicula germinativa)

GVBD: Germinal vesicle breakdown (quebra da vesicular germinativa)

H_2 O_2 : Peróxido de hidrogênio

IGF-1/-2: Insulin like growth factor-1/-2 (fator de crescimento semelhante à insulina-1/ 2)

IHQ: Imunohistoquímica

ITS: Insulin, tranferrin and selenium (insulina, transferrina e selênio)

IVM: *In vitro* maturation (maturação *in vitro*)

K+: Íon potássio

KL: Kit ligand

L/l: Litro

LH: Luteinizing hormone (hormônio luteinizante)

LIF: Leukemia inhibitory factor (fator inibidor de leucemia)

M: Molar

MARF1: gene essencial regulador da progressão meiótica durante a oogênese

MEL: Melatonin (Melatonina)

MEM: Minimal essential medium (meio essencial mínimo)

MEM^{+}: Supplemented minimal essential medium (meio essencial mínimo suplementado)

MII: Metaphase II (metáfase II)

MIV: Maturação *in vitro*

mL: Mililitro

mM : Milimolar

MOIFOPA: Manipulação de oócitos inclusos em folículos ovarianos pré-antrais

mOsm/L: Miliosmol/litro

ng: Nanograma

O: Oocyte (Oócito)

$P < 0.05$: Probabilidade de erro menor do que 5%

pg: Picograma

PCR: Polymerase chain reaction (reação em cadeia da polimerase)

RNA: Ribonucleic acid (ácido ribonucléico)

RNAm: Ribonucleic acid messenger (ácido ribonucléico mensageiro)

SEM: Standard error of means (erro padrão da média)

UNIVASF: Universidade Federal do Vale do São Francisco

α-MEM: Alpha minimal essential medium (meio essencial mínimo alfa)

α-MEM^{+}: Supplemented alpha minimal essential medium (meio essencial mínimo alfa suplementado)

µM : Micromolar

% : Percentage (porcentagem)

µg : Micrograma

µL : Microlitro

µm : Micrômetro

± : Mais ou menos

SUMÁRIO

1. INTRODUÇÃO

O cultivo *in vitro* de folículos ovarianos tem despertado interesse porque os milhares de oócitos inclusos nestes folículos têm potencial para produzir um grande número de embriões (FIGUEIREDO e LIMA, 2017). Em mamíferos, como por exemplo, em pequenos ruminantes, apenas alguns dos milhares de oócitos presentes no ovário se desenvolvem até a maturação e, em caso de fecundação, podem gerar descedentes. Porém, o restante destas células gradualmente se torna atrésica. Assim, o desenvolvimento de sistemas de cultivo capazes de proporcionar o crescimento de folículos ovarianos, em que seus oócitos, quando fecundados, adquiram a competência para se desenvolver até o estádio de blastocisto, poderia abrir novas perspectivas na reprodução das fêmeas (PARAMIO e IZQUIERDO, 2016).

O uso de modelos animais, como por exemplo a ovelha, para o estudo reprodutivo *in vitro* é possível devido ao fato de que os folículos desses animais domésticos podem assemelhar-se aos dos seres humanos em termos de taxas de crescimento e tamanho (TELFER e ZELINSKI, 2013). Contudo, para que este objetivo seja alcançado, é necessário saber que o desenvolvimento folicular pode ser regulado por hormônios e fatores de crescimento, que agem de forma endócrina, parácrina e/ou autócrina (HSUEH et al., 2015). Uma substância importante para a foliculogênese é o Hormônio Folículo Estimulante (FSH), o qual já foi utilizado no cultivo *in vitro* de folículos pré-antrais de várias espécies, auxiliando na manutenção da viabilidade (ovinos: COSTA et al., 2010; bovinos: ROSSETTO et al., 2016; caprinos: SILVA et al., 2017) e promovendo o crescimento folicular (bovinos: ITOH et al., 2002; caprinos: BARROS et al., 2013; equinos: AGUIAR et al., 2016). Outros estudos mostraram que folículos secundários ovinos cultivados com FSH hipofisário suíno (pFSH) desenvolveram-se até o estádio antral (CECCONI et al., 1999), além de ter sido observado um aumento da taxa de crescimento diário de folículos secundários bovinos cultivados com FSH recombinante bovino (rbFSH) (RODRIGUES et al., 2010). No entanto, o rbFSH não está facilmente disponível para compra ou comercialização, porém, o FSH recombinante humano (rhFSH) é comercialmente disponível e tem sido utilizado com sucesso na maturação de oócitos ovinos e produção de embriões (ACCARDO et al., 2004). Desta forma, tendo em vista a crescente preocupação em torno da eficácia do FSH recombinante versus FSH pituitário (CALDER et al., 2003; MAGALHÃES et al., 2009), seria importante avaliar os efeitos do rhFSH sobre a cultivo *in vitro* de folículos pré-antrais ovinos isolados.

Outro hormônio que parece exercer influência sobre a reprodução animal é a melatonina, que é produzida principalmente na glândula pineal, sendo sintetizada e secretada

durante a noite (JOCKERS et al., 2016). A presença de receptores para melatonina em ovários de humanos e de ovinos já foi demonstrada (NILES et al., 1999; LEE et al., 2001; SOARES Jr. et al., 2003; KANG et al., 2009; BARROS et al., 2013; BARBERINO et al., 2017), sugerindo que este hormônio participa da foliculogênese. Em células normais, a melatonina e seus derivados são poderosos eliminadores de espécies reativas de oxigênio (ERO) (REITER et al., 2016), pois possuem a capacidade de interagir com radicais como por exemplo, superóxido, hidroxila ou peróxido de hidrogênio, além do óxido nítrico (HARDELAND, 2005). Em camundongos pré-tratados com a melatonina antes do tratamento quimioterápico com a cisplatina, este hormônio atuou na manutenção da morfologia folicular, reduziu a apoptose e os danos mitocondriais (BARBERINO et al., 2017). *In vitro* a adição da melatonina de forma sequencial ao meio de cultivo de folículos pré-antrais secundários manteve a sobrevivência e promoveu o crescimento folicular e oocitário em caprinos (BARROS et al., 2013). Em folículos antrais, a melatonina estimulou a produção de progesterona e androstenediona durante cultivo *in vitro* por 30 horas (TANAVDE e MAITRA, 2003). No entanto, a utilização dessa substância antioxidante no cultivo *in vitro* de folículos secundários e antrais iniciais ovinos ainda não foi realizada.

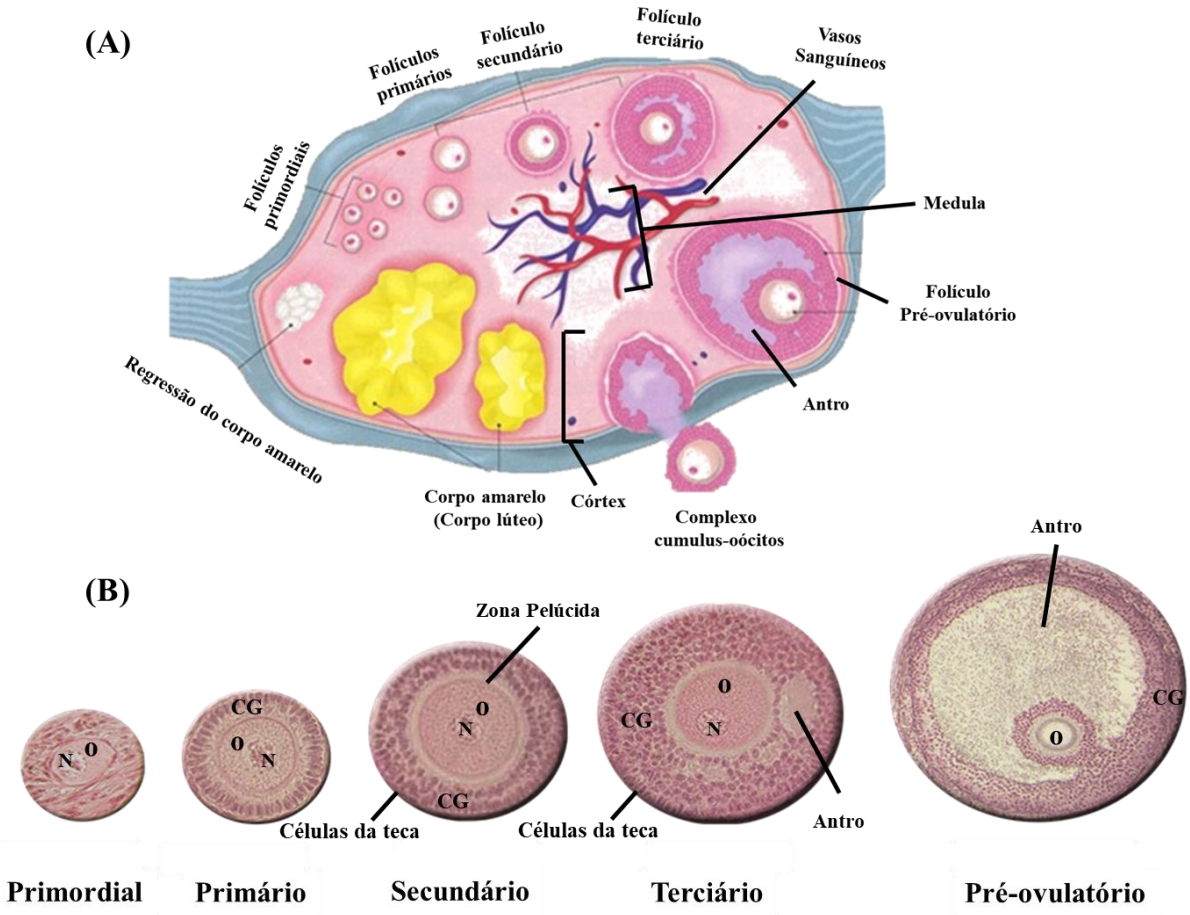
Para uma melhor compreensão da presente tese, será descrita abaixo uma breve revisão de literatura abordando os temas: ovário mamífero, oogênese e foliculogênese, população ovariana e atresia, cultivo *in vitro* de folículos ovarianos, a composição do meio para o desenvolvimento folicular *in vitro* e os hormônios utilizados neste trabalho, FSH e melatonina.

2. REVISÃO DE LITERATURA

2.1. Ovário de mamíferos

O ovário de mamíferos é o órgão reprodutor feminino, cujas principais funções são a formação e a diferenciação de células germinativas e a produção de hormônios esteroides. Este órgão contém os folículos ovarianos, o corpo lúteo, tecido intersticial, a região medular mais interna e a camada mais externa da superfície do epitélio (FENG, TAMADON e HSUEH, 2018), na maioria das espécies. O folículo ovariano é considerado como a unidade fundamental do ovário e é composto pelo oócito e suas células somáticas circundantes (GREEN e SHIKANOV, 2016). De acordo com o nível de desenvolvimento, o folículo pode ser classificado em diferentes estádios, agrupados em duas grandes categorias: folículos ovarianos pré-antrais (folículos primordiais, primários e secundários) e folículos antrais (folículos terciários e pré-ovulatórios) (FIGUEIREDO et al., 2008) (Figura 1).

Figura 1. Imagem ilustrativa da organização do ovário de mamíferos representando a sequência evolutiva desde os folículos primordiais até à formação da cavidade antral e ovulação, corpos lúteos na região cortical e vasos na região medular (A). Ilustração histológica dos folículos ovarianos em diferentes estádios de desenvolvimento no córtex do ovário. O, Oócito; N, Nucleo; CG, Células da Granulosa.



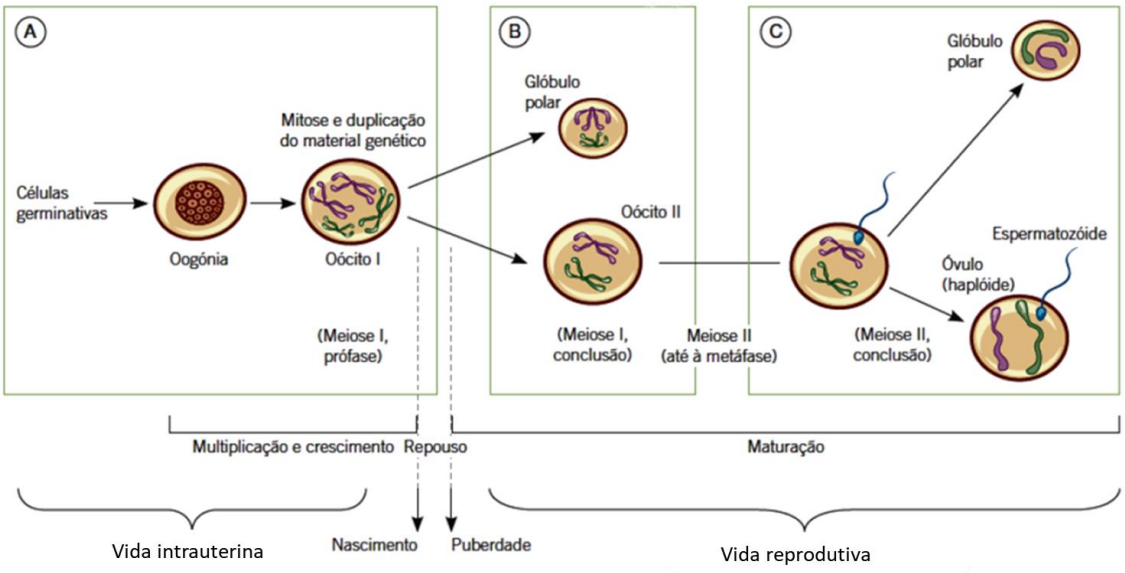
Fonte: http://www.atlasdasaude.pt/publico/content/sistema-reprodutor-feminino

2.2. Oogênese e Foliculogênese

Após o nascimento, no ovário mamífero, nota-se um *pool* de folículos em fase de repouso ou quiescentes, que constitui a reserva ovariana. Essa reserva inclui os folículos classificados como primordiais, além de pequenos folículos primários. Estes folículos representam entre 91 e 98% do total da população folicular ovariana. Em todas as espécies de mamíferos, os folículos deixam o *pool* de folículos quiescentes em um fluxo contínuo, seja por atresia (morte folicular) ou ainda por entrada para a fase de crescimento (GOUGEON, 2010).

A oogênese consiste na etapa em que as células germinativas primordiais (CGP) transformam-se até oócitos maduros, ou aptos à fertilização, tendo seu início ainda na vida intrauterina, e concluindo-se até a formação do ooócido haploide fecundado (MOORE e PERSAUD, 2004) (Figura 2).

Figura 2. Imagem ilustrativa do processo de oogênese. (A) vida intrauterina, (B) puberdade e (C) vida reprodutiva.



Fonte: http://animanmov.blogspot.com/2014/10/oogenese-foliculos-e-trompas-de-falopio.html

As CGP migram do saco vitelínico do endoderma para as gônadas em desenvolvimento cerca de 17 a 21 dias após a fecundação em ovelhas (LEDDA et al., 2010). O fator de crescimento Kit ligand (KL) e seu receptor (cKIT) desempenham um papel crítico na sobrevivência destas células (WILHELM, PALMER e KOOPMAN, 2007). Além disso, as

proteínas da matriz extracelular, como por exemplo, a fibronectina, também desempenham um papel importante na migração das CGP e no início da sua multiplicação (IRVING-RODGERS e RODGERS, 2006). Na gônada em desenvolvimento, essas células iniciam seu processo de multiplicação, através de sucessivas mitoses, originando as oogônias meioticamente ativas (SUH, SONNTAG e ERICKSON, 2002). Ao atingir a prófase I da meiose, as oogônias são agora denominadas oócitos primários, os quais se desenvolvem como aglomerados ou ninhos de células, unidas por pontes intercelulares (PEPLING, 2006). Durante este período, mais de um terço dos oócitos morrem por apoptose e/ou autofagia (DE FELICI et al., 2005; LOBASCIO et al., 2007). Cerca de quatro dias após o nascimento, a maioria das CGP desaparece e os oócitos sobreviventes são individualmente cercados por células da pré-granulosa, formando o folículo primordial (WANG et al., 2017).

O crescimento dos folículos primordiais é também conhecido como ativação e as características morfológicas observadas são o aumento do diâmetro oocitário e a alteração na morfologia das células da pré-granulosa, que passam de pavimentosa para cúbica. Com a ativação dos folículos primordiais, o oócito passa a ser circundado por uma camada completa de células da granulosa com morfologia cúbica, sendo então denominados de folículos primários (GOUGEON e BUSSO, 2000). Essa ativação folicular ocorre sob a influência de inúmeros fatores de crescimento como o fator de crescimento de fibroblastos básico (FGFb), KL, fator inibidor da leucemia (LIF) e fator de crescimento semelhante à insulina I (IGF-I) (NILSSON, DETZEL e SKINNER, 2006; CAVALCANTE et al., 2015; KOMATSU et al., 2015; BEZERRA et al., 2018). Posteriormente, por ação de fatores como o fator de crescimento de diferenciação-9 (GDF-9) e a proteína morfogenética óssea-15 (BMP-15) (THOMAS et al., 2005; MARTINS et al., 2008), os folículos primários se desenvolvem até o estádio de folículos secundários. Estes possuem, pelo menos, duas camadas de células de granulosa envolvendo um oócito situado centralmente (GOUGEON 1996; OKTAY et al., 1997). As células da granulosa dos folículos secundários são capazes de sofrer proliferação e começam a expressar receptores para enzimas esteroidogênicas, mas não são capazes de sofrer luteinização (OKTEM e URMAN, 2010). Esta característica os distingue dos folículos em estádios mais avançados, nos quais as células da granulosa têm capacidade de luteinização (GUZEL e OKTEM, 2017).

Em geral, a cavidade antral é formada quando o folículo atinge um diâmetro entre 200 e 300 µm. O antro cresce através do acúmulo de fluido derivado do sangue, que flui através dos capilares, além de produtos de secreção das células foliculares. Alguns deles, como

hialuranos e proteoglicanos, geram um gradiente osmótico que participa do crescimento do antro (RODGERS e IRVING-RODGERS, 2010).

Na fase final do desenvolvimento folicular, observa-se a formação do folículo pré-ovulatório. As células da granulosa são divididas em duas populações diferentes: as células da granulosa que ficam próximas ao oócito sofrem diferenciação para formar as células do cumulus, enquanto que as demais se diferenciam em células da granulosa murais. Durante esse tempo, o folículo dominante adquire receptores para o Hormônio Luiteinizante (LH) nas células da granulosa e aumenta a sua dependência aos níveis desse hormônio e do FSH e então, finalmente, resulta na ovulação induzida por um pico de LH (MATSUDA et al., 2012).

2.3. População ovariana e atresia

A hipótese mais aceita atualmente é que o número total de folículos ovarianos é determinado no início da vida da fêmea, e o esgotamento deste *pool* leva à senescência reprodutiva. No entanto, o conceito da neo-oogênese vem ganhando impulso devido à descoberta de células com atividade mitótica que podem gerar oócitos inclusos em pequenas estruturas semelhantes a folículos em ovários adultos de várias espécies de mamíferos (camundongos: JHONSON et al., 2004; suínos: DYCE, WEN e LI, 2006). Independente desta controvérsia, o destino de cada folículo é distinto e controlado por fatores endócrinos e, mais importante, por diversos fatores parácrinos (HSUE et al., 2015). Assim, a população folicular difere entre as espécies, além de ser observada uma grande variação individual (KATSKA–KSIAZKIEWICZ, 2006), sendo de aproximadamente 20.000 na cabra (BEZERRA et al., 1998) e aproximadamente 33.000 na ovelha (AMORIM et al., 2000). Apesar desta grande população folicular presente no ovário dos mamíferos, durante o desenvolvimento e amadurecimento deste órgão, ocorre um fenômeno espontâneo conhecido como atresia folicular no qual a maioria dos folículos presentes no ovário (cerca de 99,9%) morre e não chega à ovulação (MATSUDA et al., 2012; WORKU et al., 2017). Embora a perda dos folículos ovarianos através deste processo seja muito alta, este é um evento crucial para a manutenção da homeostase do ovário mamífero, o que assegura ciclicidade animal (AMSTERDAM et al., 2003).

O processo de atresia, usualmente, ocorre de forma diferenciada entre folículos pré-antrais e antrais. Em folículos pré-antrais, os primeiros sinais de morte folicular surgem no

oócito, onde se pode observar a retração da cromatina nuclear e a fragmentação oocitária (SILVA et al., 2002). Após a formação do folículo antral, ocorre uma alteração na sensibilidade do oócito e das células da granulosa. Em geral, a partir deste estádio, o oócito torna-se mais resistente e as primeiras alterações indicativas de atresia são observadas nas células da granulosa (JORIO, MARIANA e LAHLOU-KASSI, 1991). Independente do tipo folicular, a atresia pode ocorrer pela via apoptótica, pela via degenerativa (necrose ou necroptose) ou pelo processo de autofagia.

O processo de apoptose é controlado por proteínas intracelulares e é o principal responsável pela perda da reserva ovariana (GOUGEON, 2010). Segundo Barnett et al. (2006), a apoptose folicular ocorre de forma ordenada, sendo geneticamente regulada pela expressão de genes específicos tais como BAX e caspases. Além disso, o desbalanço entre fatores pró e anti-apoptóticos é determinante na sobrevivência e no desenvolvimento folicular (HSU e HSUEH, 2000). Esse desbalanço de fatores pró e anti-apoptóticos é chamado de via intrínseca, assim, membros pró-apoptóticos da família da Bcl-2 (Bax ou Bak) são ativados e atuam sobre as mitocôndrias, induzindo a permeabilização da membrana externa da mitocôndria que em última instância, resulta na libertação de fatores apoptogênicos e na autodestruição das células. Por esse motivo, a família da Bcl-2 desempenha um papel importante na apoptose celular (BRAS, QUEENAN e SUSIN, 2005).

As caspases são consideradas as executoras principais da via apoptótica e atuam ativando DNases, que são endonucleases responsáveis pela fragmentação do DNA internucleossomal a cada 180-200 pares de bases. A apoptose pode ser promovida por fatores extrínsecos, tais como citocinas, ativação de genes promotores de apoptose e proteínas virais ou pela remoção de fatores de crescimento. Além disso, pode ser induzida por fatores intrínsecos, tais como o estresse oxidativo ou irradiação. Independentemente dos tipos de fatores envolvidos, ocorre o envolvimento de uma ou mais caspases de iniciação (caspase 8 e 9) ou efetoras (caspase 3, 6 e 7) (JOHNSON e BRIDGHAM, 2002). Além destas, estudos recentes identificaram o papel regulador apoptótico dos microRNAs (miRNAs) nas células da granulosa (WORKU et al., 2017). Estas moléculas tem a capacidade de inibir os RNAm dos fatores de crescimento, como o fator de crescimento de transformação-β (TGFβ) ou ainda aumentar ou diminuir a produção da caspase-3 ativada (YANG et al., 2012; NIE et al., 2015; ZHOU et al., 2015). A via extrínseca por outro lado, é iniciada pela interação de determinados ligantes aos receptores de morte na superfície celular. Estes receptores pertencem à família dos receptores do Fator de Necrose Tumoral (TNF), contendo um domínio citoplasmático (necrose death domain – DD) que tem um papel de extrema importância na transmissão do

sinal, desencadeando uma série de eventos que resulta na cascata de ativação de enzimas pró-apoptóticas (BEERE, 2014).

A necrose é uma forma de morte celular iniciada por estímulos ambientais, como por exemplo, choque térmico, choque osmótico, estresse mecânico, isquemia, ou aumento excessivo das ERO, resultando na rápida perda da homeostase celular (BRAS, QUEENAN e SUSIN, 2005). Acreditava-se que este tipo de morte celular acontecia de forma não regulada (DE ALMAGRO e VUCIC, 2015), no entanto, recentes estudos já demonstram que esta via de morte celular ocorre por uma séria de processos morfológicos, bioquímicos e moleculares, indicando uma regulação neste processo (JENKINS, TIMMONS e McCALL, 2013). As primeiras indicações de necrose são o aumento do volume das organelas (principalmente mitocôndrias) e aparência da cromatina aglomerada no núcleo, seguida do aumento do volume celular e ruptura da membrana plasmática. Além destas características morfológicas que definem a necrose, existem vários eventos intracelulares associados, incluindo aumento de ERO, depleção do ATP e aumento de Ca^{2+} citosólico (PETERSON et al., 2015). Nos mamíferos, uma forma específica de necrose é chamada necroptose, que é iniciada pelo TNF, mas também pode ser induzida por outros membros da família TNF, além de não ser dependente das caspases, assemelhando-se à via extrínseca da apoptose (DE ALMAGRO e VUCIC, 2015).

A autofagia é um mediador da resposta celular ao estresse e ocorre também no ovário, seja devido ao desenvolvimento folicular normal através da mitofagia ou engolfamento seletivo de mitocôndrias danificadas (JIN e YOULE, 2012), ou até a resposta celular induzida pelo estresse térmico (HALE et al., 2017). Neste processo, a célula forma uma vesícula intracelular chamada de autofagossomo que se funde com o lisossomo para formar um autolisossomo, promovendo assim, a degradação celular ou de apenas algumas organelas, utilizando as enzimas hidrolases lisossômicas (KLIONSKY e ERM, 2000).

2.4. Cultivo *in vitro* de folículos ovarianos

O sucesso reprodutivo visa o completo desenvolvimento fetal no útero; no entanto, deve-se atentar que ele começa com o crescimento dos oócitos dentro de um folículo. O folículo é uma estrutura ovariana que proporciona e influencia a qualidade do oócito presente nele; portanto, muitas vezes, as tecnologias de reprodução assistida removem o oócito do seu microambiente folicular e induzem seu desenvolvimento para os estádios finais *in vitro*. Para compensar a ausência de um meio natural, o papel do folículo durante a diferenciação final

dos oócitos deve ser completamente compreendido (HENNET e COMBELLES, 2012). Para isso, o desenvolvimento de técnicas que promovam adequadamente o crescimento *in vitro* de folículos ovarianos de mamíferos tem despertado o interesse dos laboratórios que estudam a fisiologia do folículo a nível molecular e celular (MURRAY e SPEARS, 2000; NAYUDU et al., 2001; PICTON et al., 2003).

Os sistemas de cultivo *in vitro* de folículos isolados envolvem o isolamento mecânico (MACEDO et al., 2017) e/ou enzimático (GUEDES et al., 2017) dos folículos ovarianos, ou a combinação de ambos, permitindo o monitoramento individual do crescimento folicular, bem como a análise do efeito *in vitro* de diferentes substâncias sobre cada etapa do seu desenvolvimento (McLAUGHLIN et al., 2018). Outros autores mostraram a eficácia do sistema de cultivo *in situ*, que promove o crescimento inicial dos folículos primordiais (ativação folicular) inclusos em fragmentos de tecido ovariano (ovinos: BEZERRA et al., 2018; caprinos: CAVALCANTE et al., 2015; roedores: AMOUSHAHI et al., 2017; bovinos: SILVA et al., 2017; humanos: HAO et al., 2018). Esse tipo de cultivo tem a vantagem de manter a integridade estrutural folicular e as interações entre as células foliculares e do estroma (LIMA et al., 2013), possibilitando uma maior praticidade de execução do trabalho, além de permitir estudar com mais facilidade a ativação de folículos primordiais (ABIR et al., 2006). Entretanto, o tecido cortical do ovário pode agir como uma barreira à perfusão do meio de cultivo, resultando em um crescimento de folículos primordiais somente até o estádio de folículo secundário (FORTUNE, 2003). Desta forma, como o desenvolvimento de folículos antrais pode ser inibido no cultivo de tecido ovariano, a utilização do cultivo de folículos isolados pode proporcionar o estudo das duas categorias foliculares: pré-antrais e antrais.

2.4.1 Cultivo *in vitro* de folículos pré-antrais isolados

Grandes progressos já foram obtidos com o cultivo *in vitro* de folículos pré-antrais isolados em diferentes espécies animais. Em caprinos (SARAIVA et al., 2010; MAGALHÃES et al., 2011), ovinos (ARUNAKUMARI et al., 2010), bubalinos (GUPTA et al., 2008), suínos (WU, EMERY e CARRELL et al., 2001) e primatas não-humanos (XU et al., 2011), o cultivo *in vitro* de folículos secundários resultou na produção de oócitos maturos, os quais foram fecundados *in vitro*, gerando embriões. Por outro lado, nas espécies bovina (ROSSETTO et al., 2016) e canina (SERAFIM et al., 2010), folículos pré-antrais isolados foram cultivados *in vitro* e se desenvolveram somente até o estádio antral. Em humanos, os folículos pré-antrais alcançaram o estádio de folículo antral, apresentado oócitos

meioticamente competentes (em metáfase II) (XIAO et al., 2015). Desta forma, uma das limitações deste cultivo são as baixas taxas de maturação de oócitos provenientes de folículos pré-antrais isolados crescidos *in vitro*, o que consequentemente, faz com que a produção de embriões também seja baixa nas espécies domésticas.

Vale ressaltar que, até o presente momento, apenas em camundongos foi alcançado a produção de embriões e o nascimento de 59 camundongos viáveis a partir de oócitos de folículos primordiais cultivados *in vitro* (O'BRIEN, PENDOLA e EPPIG, 2003). Inicialmente, estes autores realizaram o cultivo *in vitro* dos folículos primordiais inclusos no tecido ovariano, e, em seguida, isolaram os folículos secundários obtidos e os cultivaram até a obtenção dos folículos antrais. Este estudo foi o que representou o maior avanço obtido utilizando folículos pré-antrais recuperados do ambiente ovariano.

2.4.2 Cultivo *in vitro* de folículos antrais

Os eventos de diferenciação oocitária (final do crescimento do oócito, capacitação e maturação) ocorrem poucos dias antes da ovulação. Neste momento, o folículo é composto por uma cavidade cheia de líquido, o antro folicular, que é produzido pelas células da granulosa. Cada componente do folículo antral contribui para que a diferenciação dos oócitos seja bem suscedida e subsequentemente, este oócito esteja pronto para ser fecundado (HENNET e COMBELLES, 2012). Dessa forma, é possível que os folículos secundários e antrais iniciais se comportem de forma diferente nas mesmas condições de cultivo; portanto, eles podem apresentar diferentes necessidades de suplementação *in vitro*. Esse fato poderia ser um fator chave para o desenvolvimento de um sistema de cultivo *in vitro* de folículos antrais iniciais (CADENAS et al., 2017).

Para alcançar tal objetivo, estudos utilizando hormônios e fatores de crescimento no cultivo *in vitro* de folículos antrais iniciais vêm sendo realizados. Em cabras, Cadenas et al. (2017) demonstraram que o hormônio do crescimento (GH) melhora o crescimento e a maturação dos oócitos após o cultivo *in vitro* de folículos antrais iniciais. Na espécie canina, os folículos antrais iniciais (>230 a $\leq$ 330 μm) tratados com FSH, durante 20 dias, apresentaram um maior crescimento folicular e um padrão aumentado na produção de 17β-estradiol e progesterona (NAGASHIMA et al., 2017). Em camundongos, os fitoestrógenos inibem o crescimento *in vitro* de folículos antrais, além de afetar negativamente a produção de hormônios esteroides e enzimas esteroidogênicas (MAHALINGAM et al., 2016; PATEL et al., 2016;). Outros autores confirmaram que substâncias como o Di(2-etil-hexil) phtalato e

subprodutos utilizados para desinfecção da água para torna-la potável (ácidos bromoacético, cloroacético e iodoacético), são potencialmente tóxicos para o ovário mamífero, inibindo o crescimento *in vitro* de folículos antrais (HANNON et al., 2015; JEONG et al., 2016).

Assim, devido a importância dessa categoria folicular para a aquisição da competência meiótica dos oócitos, mais estudos são necessários sobre o cultivo *in vitro* de folículos antrais iniciais, os quais ainda não existem na espécie ovina. Ademais, para um melhor rendimento referente à produção de oócitos maturos a partir do cultivo de folículos pré-antrais e antrais, ainda se faz necessário a adequação dos meios e sistemas de cultivo disponíveis.

2.5. Composição do meio de cultivo *in vitro*

Em condições apropriadas, os folículos ovarianos serão capazes de atingir seu tamanho final e liberar oócitos maturos (PAZOKI et al., 2015). Assim, a composição do meio de cultivo é imprescindível para o desenvolvimento folicular. Além das diferenças dos sistemas de cultivo *in vitro* de folículos ovarianos, diferenças como a natureza, as concentrações e os efeitos das fontes de proteínas, fatores de crescimento, hormônios e/ou antioxidantes utilizados no meio de cultivo, podem influenciar o crescimento do folículo e a produção de oócitos *in vitro*. Essas diferenças são altamente relevantes no desenvolvimento de sistemas que suportam o completo crescimento folicular *in vitro* e a maturação do oócito. No entanto, em todos os protocolos, algumas características são vitais para otimizar o crescimento *in vitro*: o fornecimento de nutrientes, proteínas, eletrólitos, antioxidantes, aminoácidos, substratos energéticos, vitaminas, hormônios e fatores de crescimento (PICTON et al., 2008).

Os meios de cultivo comerciais foram desenvolvidos com o intuito de promover um adequado crescimento *in vitro* de células. Dentre eles, o Meio Essencial Mínimo alfa (αMEM) tem sido utilizado no cultivo de folículos ovarianos em diferentes espécies, permitindo a manutenção da viabilidade folicular (camundongos: GAO et al., 2007; caprinos: SILVA et al., 2010). Esse meio é composto por 21 aminoácidos essenciais, vitaminas, sais inorgânicos e piruvato, componentes importantes para manutenção da viabilidade e o crescimento folicular *in vitro* (PICTON et al., 2008). Apesar disso, diversas pesquisas demonstraram a necessidade de se incrementar o meio de cultivo com a adição de outros componentes, como por exemplo, fontes de proteínas específicas e hormônios.

2.6. Fontes protéicas e os meios de cultivos definidos e não definidos

Fontes proteicas como por exemplo o soro fetal bovino (SFB), são utilizadas comumente nos meios de maturação de complexos cúmulos oócitos (CCOs) de diferentes espécies (camundongos: ABOUZARIPOUR et al., 2018; ratos: MESBAH et al., 2017; ovinos: GOODARZI et al., 2018; bovinos: YANG et al., 2017), bem como auxiliam no desenvolvimento folicular *in vitro* (camundongos: AMOUSHAHI et al., 2017; KIM et al., 2013; suínos: HOAI et al., 2018) e na produção de embriões (bovinos: MURILLO-RÍOS et al., 2017). O soro possui vários componentes que melhoram o desenvolvimento embrionário, como fatores de crescimento, quelantes de metais pesados, além de componentes que ajudam na expansão das células do cumulus (ABE e HOSHI 2003).

Embora o SFB possa conter todos os componentes necessários para melhorar o crescimento e desenvolvimento das células mantidas em ambientes artificiais, ele contém vários fatores que podem influenciar negativamente o resultado dos experimentos. O SFB é um produto biológico complexo de composição desconhecida, com flutuações sazonais e geográficas, e portanto, difere entre diferentes lotes, inclusive do mesmo fabricante (VAN DER VALK and GSTRAUNTHALER, 2017). Devido a estas características, quando este é adicionado aos meios de cultivo de células, o meio recebe o nome de meio de cultivo não definido. A variabilidade de lote para lote relacionada a variações nas concentrações de componentes séricos e sua atividade biológica pode levar à variabilidade experimental e limitar a reprodutibilidade inter-laboratoriais, particularmente para a cultivo, expansão e diferenciação de células primárias, representando um custo importante associado ao cultivo celular (USTA et al., 2014). Dessa forma, a reprodutibilidade dos resultados não se torna confiável (VAN DER VALK et al., 2010) e assim, meios considerados como definidos ou "livres de soro", com a utilização de uma fonte de proteína específica, com a albumina sérica bovina (BSA) (MOHAMMADZADEH et al., 2017), suplemento substituto de soro^{TM} (SSS) (MOTOHASHI et al., 2017), substituto de soro knouckout^{TM} (JIN et al., 2018) e álcool polivinílico (PVA) (MURILLO-RÍOS et al., 2017), tem apresentado efeitos positivos no desenvolvimento celular.

Especificamente sobre a BSA, fonte de proteína que foi utilizada no presente trabalho, já foi possível observar que a diminuição na quantidade de SFB e a associação com a BSA no meio de maturação *in vitro* de CCOs bovinos promove um melhor desenvolvimento embrionário e reduz o acumulo de lipídeos no embrião (DEL COLLADO et al., 2016; MURILLO et al., 2017). Ainda nesta espécie, o SFB apresentou uma redução no

desenvolvimento e na qualidade dos blastocistos produzidos *in vitro* quando comparado à presença de BSA no meio de cultivo de embrião (GARCIA et al., 2015). Em ovinos, utilizando diferentes concentrações da BSA em comparação ao SFB durante a maturação oocitária seguida da fertilização e transferência de embriões, notou-se que apenas oócitos tratados com a BSA apresentaram cordeiros mais pesados ao nascimento em comparação aqueles tratados com SFB (MARA et al., 2015). Além disso, um recente trabalho relatou que esta proteína pode manter a sobrevivência folicular após vitrificação de tecido ovariano ovino em comparação ao SFB (MOHAMMADZADEH et al., 2017).

2.7. Hormônio Folículo Estimulante (FSH)

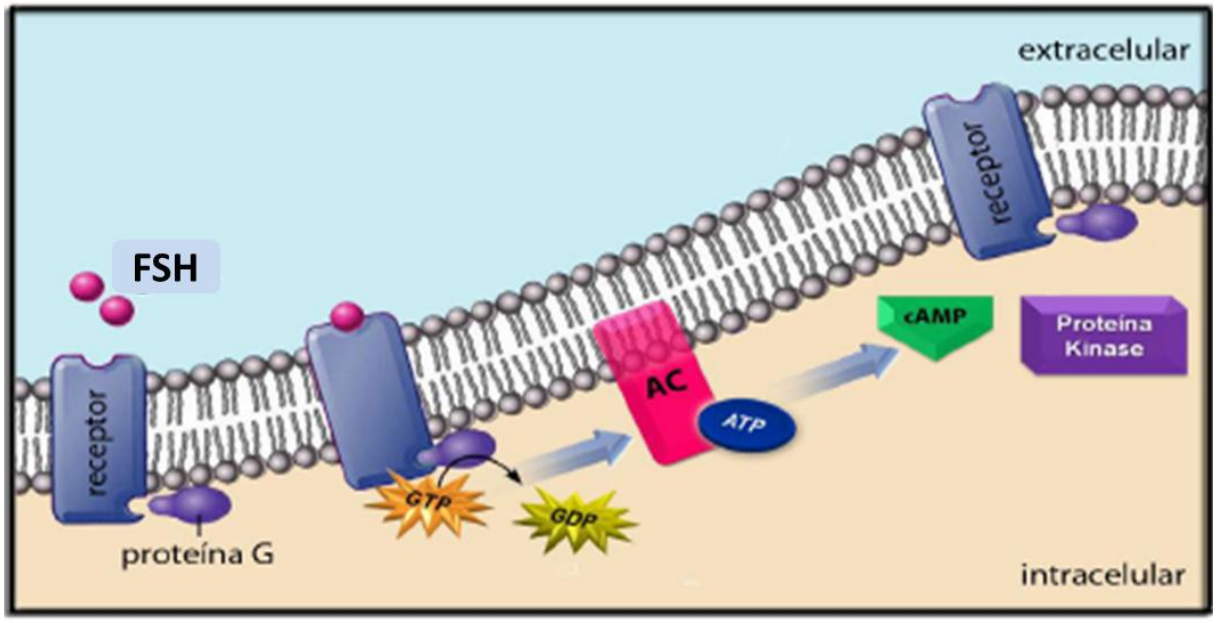
O FSH é uma glicoproteína sintetizada e secretada pelas células gonadotróficas localizadas na hipófise anterior, contendo uma subunidade α em comum e uma subunidade β hormônio específica (BROWN e McNEILLY, 1999). Este hormônio é liberado de forma constitutiva, isto é, grande parte do hormônio é liberada na velocidade em que é produzida, embora uma pequena parcela possa ser armazenada para ser liberada em resposta ao GnRH (FARNWORTH, 1995).

O receptor do FSH (FSHR) é composto de um grande domínio extracelular N-terminal, sete domínios transmembranários e um domínio C-terminal intracelular acoplado à proteína G (PAPADIMITRIOU et al., 2016). Através de testes utilizando o RNAm do FSHR foram identificados diferentes isoformas deste receptor em diferentes espécies. Pelo menos quatro isoformas foram descritas: FSHR-1 (forma acoplada à proteína G), FSHR-2 (forma negativa dominante), FSHR-3 (fator de crescimento do tipo 1) e FSHR-4 (FSHR solúvel) (MISRAHI et al., 1996; SAIRAM et al., 1996; SIMONI, NIESCHLAG e GROMOLL, 2002; BABU, DANILOVICH e SAIRAM, 2001). Apenas o FSHR-1 é expresso em células ovarianas e está envolvido no desenvolvimento de folículos e na diferenciação de células da granulosa (PAPADIMITRIOU et al., 2016).

A ligação de FSHR-1/FSH resulta na ativação da proteína heteromérica G que estimula a conversão da guanosina tri-fosfato (GTP) em guanosina di-fosfato (GDP), essa energia desencadeia a ativação de uma enzima intracelular (adenilciclase - AC). Esta enzima converte parte da Adenosina Trifosfato (ATP) intracelular em e estimula a produção de adenosina monofosfato cíclico (AMPc) (ULLOA-AGUIRRE et al., 2007). Uma das cascatas de sinalização intracelular ativada é a da via da proteína de ligação ao AMPc e proteína quinase A (PKA), que desencadeia a expressão de genes envolvidos na função da célula da

granulosa, incluindo aromatase e inibina-A (WAYNE et al., 2007) (Figura 3). A ativação do AMPc também induz a ativação da via fosfatidilinositol 3-quinase (PI3K)/proteína quinase B (AKT), que desempenha um papel fundamental nas respostas celulares à proliferação celular, apoptose, reparo do DNA e síntese protéica. Além disso, a via de sinalização PI3K/AKT está associada ao recrutamento de folículos primordiais, proliferação das células da granulosa, sobrevivência do corpo lúteo e maturação dos oócitos (LIU et al., 2010; LAI, KILLINGSWORTH e LEE, 2015) De uma forma geral, a interação do FSH com FSHR estimula a proliferação celular, a síntese de esteroides e a expressão de receptores para Fator de Crescimento Epidermal (EGF), IGF-1 e LH (VAN DEN HURK e ZHAO, 2005).

Figura 3. Esquema ilustrativo da atuação dos receptores para FSH. GTP: Guanosina tri-fosfato; GDP: Guanosina di-fosfato; AC: Adenilciclase; ATP: Adenosina tri-fosfato; cAMP: Adenosina monofosfato cíclico.



Fonte: Saraiva et al., 2010.

A expressão do RNAm para o FSHR já foi demonstrada nas células da granulosa em folículos a partir do estádio primário (humanos: OKTAY et al., 1997; búfalos: SHARMA, DUBEY e KUMAR, 2011; caprinos: SARAIVA et al., 2011) e em oócitos, células da granulosa e epitélio superficial de ovários suínos (DURLEJ et al., 2011). A proteína para o FSHR foi observada em oócitos de folículos primordiais e primários e nas células da granulosa de folículos secundários em ovários caprinos (BARROS et al., 2013). Resultado semelhante foi demonstrado na espécie ovina, sendo observada também a marcação positiva

para o FSHR nas células da granulosa de folículos antrais e no epitélio superficial do ovário (PATEL et al., 2013). Estes achados reforçam a ideia da ação do FSH sobre a foliculogênese inicial.

O FSH pode agir indiretamente, estimulando a síntese e secreção de fatores parácrinos nos grandes folículos (O'SHAUGHNESSY; DUDLEY; RAJAPAKSHA, 1996; SARAIVA et al., 2011), auxiliando assim o crescimento de pequenos folículos ou até agindo indiretamente em folículos primários. Alguns trabalhos demonstraram que o FSH regula a expressão de vários fatores de crescimento, tais como KL, GDF-9, FGF-2 e BMP-15, que têm um papel importante na ativação e no posterior crescimento folicular (THOMAS et al., 2005; TANG et al., 2012).

O FSH tem sido utilizado no cultivo *in vitro* de folículos pré-antrais, promovendo o desenvolvimento e mantendo a viabilidade folicular em camundongos (HARDY et al., 2017), ratas (KOBAYASHI et al., 2009), mulheres (DEWAILLY et al., 2016), vacas (ROSSETTO et al., 2016), cabras (SILVA et al., 2017), ovelhas (LIMA et al., 2016); porcas (MAO et al., 2002), éguas (MAX et al., 2017) e até a obtenção de embriões quando associado ao LH e EGF (SARAIVA et al., 2010). Durante a maturação oocitária em bovinos, o FSH promoveu o aumento da maturação nuclear através da ativação da via PI3K (SOUZA et al., 2018).

Apesar dos efeitos positivos causados pelo FSH em diferentes espécies, os meios de cultivo não padronizados dificultam a repetição dos resultados. Assim, na espécie ovina, embora já tenham sido testadas diferentes concentrações do FSH no cultivo *in vitro* de folículos pré-antrais (pFSH: CECCONI et al., 1999; rbFSH: RODRIGUES et al., 2010), a origem do FSH, por exemplo, (I) extraído da hipófise humana (hFSH), (II) extraído da hipófise de animais domésticos, essencialmente, suínos (pFSH) e ovinos (oFSH) seguido de purificação hormonal, ou (III) extraído através da tecnologia recombinante usando células de ovário de hamster chinês (rFSH) e a origem da proteína adicionada ao meio de cultivo (fonte de proteína) podem comprometer os resultados. A tabela 1 resume os diferentes tipos de FSH utilizados no cultivo celular *in vitro* e seus principais efeitos.

Tabela 1 – Principais tipos de FSH utilizados no cultivo de folículos ovarianos e seus efeitos *in vitro*.

Tipos de FSH	Concentração	Espécie estudada	Tipo de cultivo	Principais efeitos	Referência
			Extraído da pituitária		
hFSH	10 ng/mL	Caprina	*In situ*	Ativação de folículos primordiais e mantém a integridade ultraestrutural de folículos pré-antrais caprinos cultivados por 7 dias.	Costa et al. (2015)
oFHS		Murina (Ratos)	Folículos pré-ovulatórios isolados	Ação antiapoptótica através da estimulação da síntese de GSH folicular e supressão da produção de ERO.	Tsai-Turton e Luderer, (2006).
	Dia 0–6: 50 ng/mL; Dia 7–12: 100 ng/mL	Bovina	Folículos secundários isolados	Associado BMP-5 promove o crescimento e mantém a viabilidade e integridade ultraestrutural dos folículos.	Passos et al. (2013)
pFSH	50 ng/mL	Caprina	*In situ*	Manteve a integridade morfológica dos folículos pré-antrais e promoveu a ativação e o crescimento folicular.	Matos et al. (2007)
		Equina	*In situ*	Melhorou a integridade morfológica dos folículos pré-antrais durante 6 dias de cultivo.	Max et al. (2017)
	1µg/mL	Ovina	Folículos pré-antrais secundários isolados	Aumentou a formação da cavidade antral, crescimento folicular e as taxas de maturação oocitária.	Cecconi et al. (1999)

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rbFSH	50 ng/mL	Equina	*In situ*	Aivação dos folículos primordiais, manteve a sobrevivência folicular, aumentou a produção de estradiol e diminuiu a produção de ROS.	Aguiar et al. (2016)
	100 ng/mL	Bovina	Folículos pré-antrais secundários isolados	Em associação com a insulina, fornece altas taxas de sobrevivência, crescimento e produção de estradiol em folículos pré-antrais.	Rossetto et al. (2016)
	Dia 0–6: 100 ng/mL; Dia 6–12: 500 ng/mL; Dia 12-18: 1000 ng/mL	Caprina	Folículos pré-antrais secundários isolados	Impacto significativo no desenvolvimento *in vitro* dos folículos pré-antrais.	Saraiva et al. (2010)
	100 µg/mL	Caprina	Folículos pré-antrais secundários isolados	Associado ao hormônio do crescimento (GH) e insulina, melhora o crescimento folicular e oocitário, a recuperação meiótica de oócitos e a produção de estradiol (E2).	Ferreira et al. (2016)
	100 µg/mL	Caprina	Folículos pré-antrais secundários isolados	Associado à insulina durante um longo do período de cultivo, melhorou a taxa de retomada meiótica e produziu oócitos em MII a partir de	Silva et al. (2017)

				folículos pré-antrais caprinos cultivados *in vitro*.	
	Dia 0–6: 100 ng/mL; Dia 6–12: 500 ng/mL; Dia 12-18: 1000 ng/mL	Canina	Folículos pré-antrais secundários isolados	Manteve a sobrevivência dos folículos pré-antrais e promoveu aumento da taxa de crescimento folicular e formação de antro.	Serafim et al. (2010)
	100 ou 1000 ng/mL	Caprina e Ovina	Cultivo de folículos pré-antrais secundários isolados	Aumentou a taxa de crescimento folicular em ambas espécies.	Rodrigues et al. (2010)
	0.05 fg/mL	Ovina	*In situ*	Promoveu a sobrevivência e ativação folicular.	Lima et al. (2016)
	Dia 0–6: 100 ng/mL; Dia 6–12: 500 ng/mL; Dia 12-18: 1000 ng/mL	Ovina	Cultivo de folículos pré-antrais secundários isolados	Adicionado ao meio de cultivo alternativo, composto por extrato de *Amburana cearenses* na concentração de 0,2 mg/mL, melhorou o desenvolvimento folicular.	Gouveia et al. (2016)
rpFSH	50 ng/mL e 100 ng/mL	Caprina	*In situ*	Os fármacos Stimufol® e Folltropin®, preservaram a morfologia folicular e promoveram o crescimento folicular, respectivamente.	Magalhães et al. (2009)

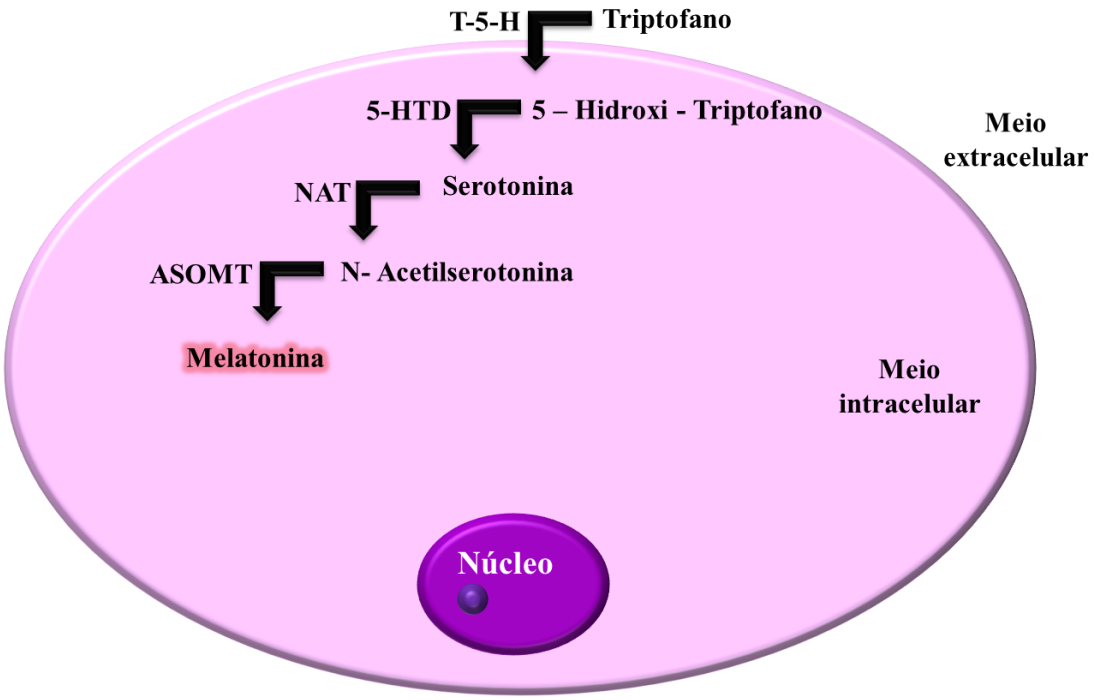
	100 ng/mL	Ovina		Promoveu o desenvolvimento folicular associado ao ácido indol-3-acético (IAA).	Costa et al. (2010)
rhFSH	100 mUI/mL	Camundongo	Cultivo de folículos secundários isolados	Promoveu melhor desenvolvimento dos folículos para a fase antral.	Mitchell, Kennedy e Hartshorne, (2002)
	100 mIU/ml	Camundongo	Cultivo de folículos secundários isolados	Promoveu melhor desenvolvimento folicular em meio isento de soro.	Park et al. (2013)
	10 mIU	Camundongo	Cultivo de folículos secundários isolados	Sistema de cultivo *in vitro* 3D em comparação a um cultivo 2D, auxilia na manutenção da morfologia espacial, taxa de crescimento e redução da expressão de genes de maturação.	Sadr et al. (2015)
	10 ng/mL	Ratos	Cultivo de folículos secundários isolados	Promoveu o crescimento folicular através de ação direta do hormônio tireoidiano.	Kobayashi et al. (2009)

hFSH: FSH humano; oFHS: FSH ovino; pFSH: FSH suíno; rbFSH: FSH recombinante bovino; rpFSH: FSH recombinante suíno; rhFSH: FSH recombinante humano.

2.8. Melatonina

A melatonina (N-acetil-5-metoxitriptamina) é uma indolamina produzida a partir do aminoácido triptofano (Figura 4), é secretada principalmente pela glândula pineal, como também em outros locais: retina, glândula lacrimal extra-orbitária, trato gastrointestinal, pele e ovário (HARDELAND et al., 1993; HUETHER, 1993; ITOH et al., 1999; REITER et al., 2013).

Figura 4. Esquema representativo das etapas envolvidas na biossíntese da melatonina na célula, mostrando os precursores e as enzimas participantes do processo. 5-HTD: 5-hidroxitriptofano descarboxilase; NAT: N-acetiltransferase; ASOMT: O-metiltransferase.



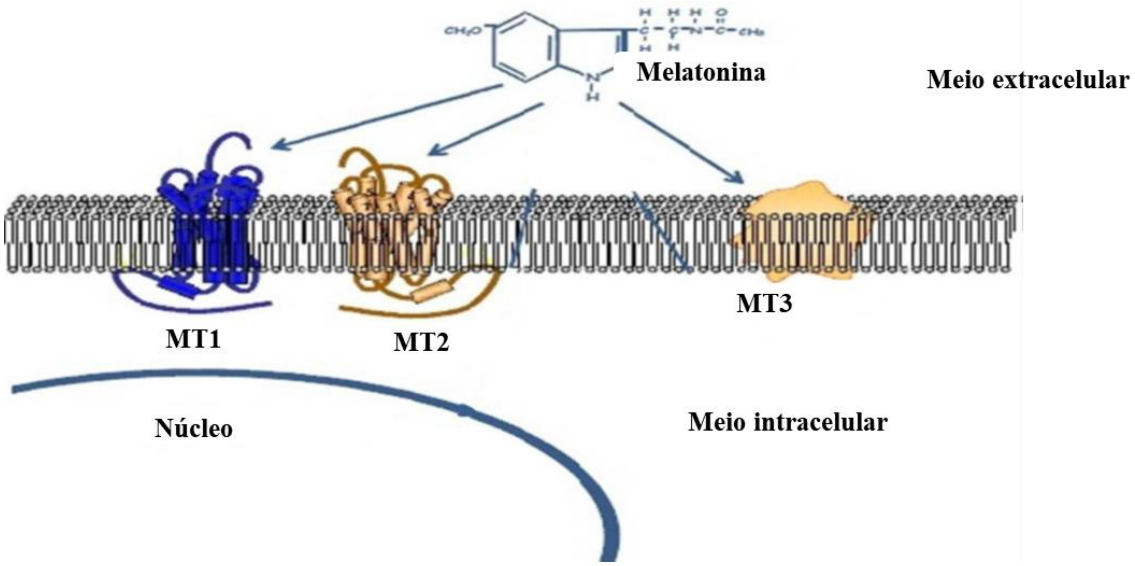
Adaptado de Rocha et al., 2011.

Este hormônio auxilia na sazonalidade, especialmente em espécies com fotoperíodo negativo, como os ovinos (DUPRÉ et al., 2008). O fotoperíodo negativo, período de dias curtos, estimula a produção de melatonina, que atua nos núcleos supraquiasmáticos do hipotálamo, estimulando a liberação do hormônio liberador de gonadotrofinas (GnRH) e a adeno-hipófise para liberação das gonadotrofinas FSH e LH (SCHAEFFER e SIROTKIN, 1997). Em ovelhas, foram identificados os genes que são regulados pela melatonina na pituitária (*Cry1* – gene que controla o relógio circadiano, *Kcnq5*, *Pbef/Nampt* – genes

reguladores da insulina, do metabolismo energético celular e tecido adiposo), um local alvo para o controle hormonal da sazonalidade (DUPRÉ et al., 2008).

Os receptores de membrana da melatonina são MT1, MT2 e MT3 (Figura 5), que pertencem à superfamília dos receptores acoplados à proteína G (GPCRs) (PAVLOS e FRIEDMAN, 2017). Estes receptores apresentam diferentes afinidades para a melatonina: o MT2 tem uma afinidade 5 vezes maior do que o MT1, tanto em seres humanos como em outras espécies (LEGROS et al., 2013; JOCKERS et al., 2016; LIU et al., 2016). Já o MT3 é um receptor com ligação de baixa afinidade e corresponde a uma quinona redutase 2, uma enzima que catalisa a redução de quinonas em quinóis com importância e implicações sobre o estresse oxidativo (BOUTIN, 2016). A expressão do RNAm para os receptores MT1 e MT2 foi demonstrada em células da granulosa de folículos pré-ovulatórios e células luteais humanas (NILES et al., 1999; WOO et al., 2001), células da granulosa de folículos secundários, terciários e corpo lúteo de ratas (SOARES Jr. et al., 2003) e células da granulosa de folículos antrais bovinos (WANG et al., 2012). Além disso, o RNAm para o MT1 foi encontrado em células da granulosa e células do cumulus de folículos ovarianos suínos (KANG et al., 2009), folículos antrais e corpo lúteo de equinos (PEDREROS, RATTO e GUERRA et al., 2011). A proteína para MT1 foi observada em corpo lúteo equino (PEDREROS, RATTO e GUERRA et al., 2011), oócitos de folículos antrais bovinos (SAMPAIO et al., 2012), oócitos de folículos primários, secundários e antrais e nas células da granulosa em camundongos (BARBERINO et al., 2017), células da granulosa de folículos secundários, células do cumulus e mural de folículos antrais iniciais e avançados em caprinos (BARROS et al., 2013). Além disso, na espécie ovina, a proteína para receptores MT1 e MT2 foi identificada através da técnica de imunofluorescência em oócitos, células do cumulus e da granulosa (TIAN et al., 2017). Utilizando a técnica de imuno-histoquímica, a proteína para o MT1 foi demonstrada nas células da granulosa de folículos pré-antrais secundários (MACEDO et al, 2012). Um recente trabalho demonstrou que o knockout do receptor MT1 nas células da granulosa de camundongos promoveu o aumento da apoptose e inibiu a proliferação celular (TALPUR et al., 2017). Estes achados demonstram que a melatonina parece exercer uma importante ação na foliculogênese em diferentes espécies.

Figura 5. Esquema representativo dos receptores da melatonina, MT1 e MT2 (receptores de membrana acoplados à proteína G) e MT3 (quinona redutase 2).



Adaptado de Tamura et al. (2009).

Em células normais, a melatonina e seus derivados (3-hidroximelatonina cíclica, N1-acetil-N2-formil-5-metoxicinuramina e a N1-acetil-5-metoxi-nuramina) são poderosos eliminadores de ERO (REITER et al., 2016; MANCHESTER et al., 2015), pois possuem a capacidade de reagir com o radical superóxido (O_2^-), radical hidroxila (OH^-), peróxido de hidrogênio (H_2O_2), ácido hipocloroso (HOCl) e óxido nítrico (NO) (HARDELAND, 2005). A melatonina pode executar suas ações diretas de eliminação das ERO através de mecanismos mediados ou não por receptores (BARBERINO et al., 2017). Em comparação com outros antioxidantes, esta indolamina tem uma capacidade igual ou superior para proteger os tecidos de lesões oxidativas, mesmo quando comparada a antioxidantes sintéticos direcionados à proteção das mitocôndrias (LOWES et al., 2011). A principal diferença entre a melatonina e outros eliminadores de ERO é a sua anfifilicidade, ou seja, sua capacidade de afinidade tanto com o meio aquoso como com os lipídeos das membranas celulares, o que permite que a melatonina se distribua ao longo do ambiente celular e organelas (ACUÑA-CASTROVIEJO et al., 2014).

A melatonina pode retardar o envelhecimento de oócitos de camundongos fêmeas por diminuir o estresse oxidativo *in vitro* e reduzir os níveis da ativação de caspases (LORD et al., 2013; TAMURA et al., 2017). De acordo com Tamura et al. (2012), a melatonina elimina as ERO produzidas nos folículos, especialmente durante o processo de ovulação e reduz o

estresse oxidativo, o qual pode estar envolvido na maturação de oócitos, no desenvolvimento embrionário e na luteinização de células da granulosa.

Estudos *in vitro* já demonstraram que a melatonina exógena diminui a taxa de apoptose após cultivo de células da granulosa de folículos antrais em camundongos e bovinos (WANG et al., 2012; TANABE et al., 2015), bem como reduz a atresia após cultivo *in vitro* de células ovarianas de hamster, diminuindo os níveis de ERO e aumentando o RNAm para enzimas antioxidades (TAMURA et al., 2012). Mais recentemente, foi demonstrado que a melatonina pode proteger os folículos ovarianos das reações causadas por quimioterápicos. Em camundongos, o pré-tratamento com 20 mg/Kg de melatonina antes da administração de cisplatina preservou a morfologia folicular normal e a taxa de proliferação celular, reduziu a apoptose e os danos mitocondriais (BARBERINO et al., 2017). Ademais, foi observado que camundongos neonatos expostos à nicotina apresentaram diminuição na reserva de folículos ovarianos, no entanto, quando 1 µM de melatonina foi injetada antes da exposição, o número de folículos ovarianos foi semelhante ao grupo controle (WANG et al., 2018).

A melatonina também está envolvida com formação do hormônio indutor de maturação, que promove a quebra da vesícula germinal (CHATTORAJ et al., 2005), além de ativar a expressão de genes ligados à maturação como o GDF9, MARF1 (gene essencial regulador da progressão meiótica durante a oogênese) e DNMT1a (gene responsável pela metilação do DNA) (MARQUES et al., 2018). Yang, et al. (2017) utilizaram 10^{-9} M de melatonina no meio de maturação *in vitro* de oócitos bovinos e obtiveram maiores percentuais de maturação e produção embrionária. Além disso, a melatonina pode afetar diretamente a qualidade embrionária através do aumento na atividade mitocondrial, da enzima glutationa peroxidase (GSH) e da redução de ERO (PANG et al., 2017; MARQUES et al., 2018). Em ovinos, esta indolamina utilizada em altas concentrações (700 e 800 µM) durante o armazenamento dos ovários na temperatura de 24° C, resulta na melhora significativa das taxas de fertilização e qualidade de blastocistos (GOODARZI et al., 2017). A melatonina também pode aumentar o grau de expansão das células do cumulus durante a maturação oocitária *in vitro*, efeito que foi associado à regulação da metilação do DNA (FANG et al., 2018). A expansão das células do cumulus é considerado um importante marco durante a maturação oocitária e é essencial para a subsequente fertilização, clivagem e desenvolvimento embrionário (GUTNISKY et al., 2007).

Há poucos estudos sobre a utilização da melatonina em sistemas de cultivo *in vitro* de folículos pré-antrais de pequenos ruminantes. Rocha et al. (2013), observaram um aumento do diâmetro folicular após 7 dias de cultivo *in vitro* de tecido ovariano caprino em meio contendo

melatonina (1000 pg/mL) e FSH (50 ng/mL). Outro estudo demonstrou que a adição de concentrações crescentes de melatonina ao meio de cultivo (meio sequencial) mantém a sobrevivência de folículos secundários caprinos isolados e promove o crescimento folicular e oocitário *in vitro* (BARROS et al., 2013). Em folículos antrais, a melatonina estimulou o aumento na produção de progesterona e androstenediona durante cultivo *in vitro* por 30 horas (TANAVDE e MAITRA, 2003).

Apesar destes resultados promissores com a melatonina, ainda não há nenhum estudo sobre os efeitos deste hormônio no cultivo e na maturação *in vitro* de oócitos ovinos oriundos de folículos pré-antrais secundários ou de folículos antrais iniciais.

2.9 Maturação *in vitro* (MIV)

Apenas uma fração dos milhares de oócitos presentes nos ovários ao nascer será ovulado durante a vida de uma fêmea mamífera. Isso acontece porque as ovulações ocorrem somente quando a puberdade é atingida e sofre uma pausa durante o período de gestação, devido aos efeitos supressores da progesterona na pulsatilidade do hormônio luteinizante (LH). Além disso, a duração da vida reprodutiva da fêmea é finito, limitado pela menopausa em humanos e pelo tempo de vida relativamente curto da maioria dos animais presentes nos sistemas de produção animal (LONERGAN e FAIR, 2015).

A maturação *in vitro* é uma versão modificada da Fertilização *in vitro* (FIV) tradicional, no qual os oócitos são coletados de folículos ovarianos pequenos após pequena ou nenhuma estimulação de gonadotrofinas exógenas. Estes oócitos coletados são imaturos, e os estádios finais da maturação são concluídos *in vitro* sob a influência da adição de hormônios aos meios de cultivo incluindo, mas não se limitando a, gonadotrofina coriônica humana (hCG), FSH e/ou LH (WALLS e HART, 2018). Para fins de pesquisa, os oócitos geralmente são recuperados dos ovários de fêmeas abatidas em matadouros licenciados. Em contraste, para transferência comercial de embriões, os oócitos são tipicamente recuperados de animais vivos por punção folículo-transvaginal. Em termos de eficiência, aproximadamente 90% dos oócitos imaturos sofrem maturação nuclear e progridem da prófase I para metafase II (o estádio no qual eles seriam ovulados *in vivo)*; aproximadamente 80% sofrem fertilização e clivagem pelo menos uma vez para o estágio de duas células (LONERGAN e FAIR, 2016).

A MIV de oócitos provenientes de folículos pré-antrais crescidos *in vitro* é uma importante técnica de avaliação do cultivo folicular, pois permite a avaliação da qualidade dos folículos e oócitos (SARAIVA, 2010). No entanto, em animais domésticos, grandes esforços foram feitos para produzir embriões viáveis a partir de oócitos oriundos de folículos secundários cultivados *in vitro* em várias espécies (caprino: SARAIVA et al., 2011, MAGALHÃES-PADILHA et al., 2011; ovino: ARUNAKUMARI et al., 2010; BARBONI et al., 2011; bubalina: GUPTA et al., 2008; e suína: HIRAO et al., 19994; WU, et al., 2001). Contudo, exceto para as espécies bubalina, a percentagem de embriões produzidos *in vitro* utilizando estes oócitos é muito baixa (1%), e os embriões não são capazes de se desenvolver para o estádio de blastocisto. Embora vários resultados positivos tenham sido descritos estudos utilizando folículos secundários cultivados de camundongos, a produção de embriões em espécies domésticas ainda é extremamente baixa e variável, sendo inadequado seu uso em um ambiente comercial.

3. JUSTIFICATIVA

A espécie ovina pode ser utilizada como modelo experimental por apresentar semelhanças morfofisiológicas com o ovário humano, além de contribuir para o aperfeiçoamento de biotécnicas reprodutivas, visando maximizar o potencial reprodutivo de animais domésticos de alto valor zootécnico ou em processo de extinção, bem como auxiliar no tratamento de mulheres com problemas de infertilidade. Nesse sentido, torna-se de fundamental importância o emprego de biotécnicas que permitam um melhor entendimento do processo da foliculogênese ovina, e que otimizem ou maximizem a utilização dos milhares de oócitos presentes no ovário.

Para alcançar este objetivo, o cultivo *in vitro* de folículos ovarianos é uma técnica que vem sendo empregada com o intuito de avaliar o efeito de diferentes substâncias, em diferentes concentrações e em diferentes estádios do desenvolvimento folicular, podendo auxiliar no aumento da produção de descendentes de animais de alto valor zootécnico ou em perigo de extinção, formação de bancos de germoplasma animal (oócitos), redução do intervalo entre gerações em animais por meio da utilização de ovários de animais jovens, melhora e padronização dos resultados de biotécnicas como a fertilização *in vitro*, clonagem, transgênese e transferência de embriões e também na pesquisa fundamental: fonte abundante de informações a respeito da foliculogênese na fase pré-antral. Mas, para que os resultados utilizando esta técnica sejam positivos, a composição do meio é essencial para assegurar a sobrevivência e o crescimento folicular, dada a complexa interação entre concentrações, associações e tempos de adição dos fatores adicionados ao meio de base.

Dentre as principais substâncias adicionadas ao meio de cultivo, merecem destaque, o FSH e a melatonina. Apesar de estes hormônios já terem auxiliado na manutenção da viabilidade folicular e no crescimento oocitário em várias espécies, a necessidade da padronização dos meios de cultivo para melhor desempenho e obtenção de resultados confiáveis ainda é necessária na espécie ovina. Diante disso, a investigação de diferentes concentrações do FSH e da melatonina no cultivo *in vitro* de folículos ovarianos faz-se necessária, constituindo, portanto, a originalidade da presente tese.

4. OBJETIVOS

4.1. Objetivo Geral

- Estudar o efeito do FSH e da melatonina sobre o desenvolvimento folicular e a maturação *in vitro* de oócitos ovinos.

4.2. Objetivos Específicos

- Avaliar o efeito de diferentes concentrações de FSH e melatonina sobre o cultivo *in vitro* de folículos secundários ovinos isolados, tendo como parâmetros:
  - a morfologia, a formação de antro e o crescimento folicular;
  - os níveis de ERO, glutationa e mitocôndrias ativas no folículo;
  - as taxas de maturação *in vitro* dos oócitos ovinos.
- Avaliar o efeito de diferentes concentrações da melatonina sobre o cultivo *in vitro* de folículos antrais iniciais isolados, observando-se os mesmos parâmetros descritos acima.
- Avaliar a utilização de um meio sequencial no cultivo *in vitro* de folículos ovarianos ovinos.

Capítulo I.

In vitro survival, growth and maturation of sheep oocytes from secondary follicles cultured in serum-free conditions: impact of a constant or a sequential medium containing recombinant human FSH

(Sobrevivência, crescimento e maturação *in vitro* de oócitos oriundos de folículos secundários ovinos cultivados em meio livre de soro: impacto do meio constante ou sequencial contendo FSH recombinante humano)

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In vitro survival, growth and maturation of sheep oocytes from secondary follicles cultured in serum-free conditions: impact of a constant or a sequential medium containing recombinant human FSH

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Running head: Recombinant human FSH in ovine follicle culture

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Abstract

This study evaluated the *in vitro* development and maturation of ovine oocytes from secondary follicles cultured in serum-free medium containing fixed or sequential concentrations of rhFSH. Follicles were cultured in α-MEM$^+$ alone or with constant (500, 750 or 1000 ng/mL) or sequential concentrations of rhFSH (Seq. 1: Day 6=500; Day 12=750; Day 18=1000 ng/mL and Seq. 2: Day 6=100; Day 12=500; Day 18=1000 ng/mL). At the end of experiment, follicular survival was higher ($P<0.05$) in 750 ng/mL rhFSH than the control and 1000 ng/mL. As early as day 6 of culture, antral cavity formation was observed in all treatments. Follicular diameter increased progressively and significantly in all treatments throughout 18 days of culture. Furthermore, addition of rhFSH to the medium promoted a significant increase in the percentage of fully grown oocytes in all treatments compared to α-MEM$^+$. Mitochondrial activity was higher in rhFSH treatments than the control, except rhFSH Seq. 2 ($P<0.05$). Maturation rates increased in oocytes from intact follicles cultured in 750 ng/mL rhFSH compared to the control ($P<0.05$). In conclusion, rhFSH at 750 ng/mL maintained the survival of secondary follicles cultured in serum-free medium, improved oocyte growth, mitochondrial activity, and oocyte maturation.

Additional keywords: Preantral follicle, Gonadotropin, Development, Meiosis, Mitochondria.

1. Introduction

Only a few of the thousands of oocytes present in the ovary develop into competent oocytes or offspring. The rest of the oocytes gradually become atretic [1]. Therefore, the development of *in vitro* culture systems has become an important tool for supporting the growth of follicles and oocytes, maximizing the utilization of female gametes, as well as for understanding early follicle development, and it also has clinical relevance for the field of fertility preservation [2, 3]. Follicles of some domestic animals (cow, sheep and goat) can resemble those of humans in terms of growth rate and size, which makes the use of nonhuman models important to improve the follicular culture systems [4]. However, in farm animals, supporting follicle growth has been a significant challenge since only a few studies have shown embryo development using oocytes from *in vitro* cultured preantral follicles (swine: [5]; bubaline: [6]; caprine: [7]; ovine:[8, 9].

It is commonly agreed that follicular growth in the preantral phase is largely independent of the gonadotrophic stimulus [10]. However, the presence of FSH receptors in follicles in the early stage (human: [11]; swine: [12], bubaline: [13]; caprine: [14]) may explain the fact that this hormone maintained viability (caprine: [15]; ovine: [16]; bovine: [17]), and promoted the *in vitro* growth of preantral follicles (bovine: [18]; canine: [19]; murine: [20]; caprine: [14]; equine: [21]). Nevertheless, some authors did not observe any beneficial effect of FSH on the initial follicular development (bovine: [22, 23]; caprine: [24]). These differences may be due to the origin of FSH, the other supplements added to the culture medium (eg, the protein source), as well as the species studied.

In the ovine species, isolated secondary follicles cultured for 6 days in medium containing 1 μg/mL porcine pituitary FSH (pFSH) developed to the antral stage and had higher rates of healthy cumulus-oocyte complexes than the lowest concentrations of pFSH

(0.01 and 0.1 μg/mL) [25]. In addition, the same concentration of recombinant bovine FSH (rbFSH) increased daily growth rates after 18 days of culture of isolated ovine secondary follicles [26]. However, rbFSH is not easily available for purchase or commercialization. Nevertheless, recombinant human FSH (rhFSH) is commercially available and has been successfully used in ovine oocyte maturation and embryo production [27]. Given the growing concerns surrounding the efficacy of recombinant FSH versus pituitary FSH [28, 15], it would be important to evaluate the effects of rhFSH on the *in vitro* culture of isolated ovine preantral follicles. In addition, the use of increasing concentrations of FSH throughout the culture allowed the meiosis resumption of oocytes from caprine secondary follicles grown *in vitro* [29], probably due to the increase in FSH receptors along folliculogenesis [29, 14]. Therefore, we hypothesized that growth rate of ovine preantral follicles would be enhanced with sequential addition of FSH to the *in vitro* culture medium.

It is important to note that the two studies that have tested FSH, without interaction with other hormones or growth factors, on the culture of isolated ovine preantral follicles used fetal bovine serum (FBS) as a source of protein in the base medium [25, 26]. FBS quality varies a lot from batch to batch. It may contain different concentrations as well as undefined components (fatty acids, growth factors, amino acids, proteins and vitamins), which may interact with other components added to the medium, making it difficult to reproduce results [30, 31]. Therefore, the variability associated with sera is undesirable, and less complex alternatives such as albumin or synthetic replacements should be added to the medium [32].

The aim of this study was to evaluate the survival, follicular development and maturation of ovine oocytes from isolated secondary follicles cultured in serum-free medium containing fixed or sequential concentrations of rhFSH.

2. Material and methods

2.1. Chemicals

Unless noted otherwise, supplements, hormones and chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Source of ovaries

Ovaries (n = 30) were collected at a local abattoir from 15 adults (1–3 years old) mixed-breed sheep (*Ovis aries*). Immediately postmortem, the pairs of ovaries were washed once in 70% alcohol followed by two rinses in minimum essential medium (MEM) buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100 mg/mL penicillin and 100 mg/mL streptomycin). Thereafter, the ovaries were transported within 1 hour to the laboratory in tubes containing MEM-HEPES with antibiotics at 4°C [33].

2.3. Isolation and selection of ovine secondary follicles

Isolation, selection, culture and follicular evaluation were performed according to [34]. In the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries. The ovarian cortical slices (1 to 2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in fragmentation medium consisting of MEM-HEPES with antibiotics. Ovine secondary follicles with ≥ 200 μm in diameter and without antral cavities were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and mechanically isolated by microdissection from the strips of the ovarian cortex using 26-gauge (26 G) needles. Thereafter, the follicles were transferred to 100

µL droplets (one follicle per droplet) containing base culture medium (α-MEM) for evaluation of the quality. Only secondary follicles that displayed the following characteristics were selected for *in vitro* culture: an intact basement membrane, two or more layers of granulosa cells and a visible, healthy, round oocyte that was centrally located within the follicle, without any dark cytoplasm.

2.4. In vitro culture of secondary follicles

After selection, the follicles were individually cultured (one follicle per droplet) in 100 µl droplets of the culture medium under mineral oil in petri dishes (60 x 15 mm; Corning, Sarstedt, Newton, NC, USA), at 39°C and 5% CO_2 in the air for 18 days. The base control medium (α-MEM$^+$) consisted of α-MEM (pH 7.2-7.4) supplemented with 3 mg/mL bovine serum albumin (BSA), 10 ng/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine and 50 µg/mL ascorbic acid. To verify the effect of constant or increasing concentrations (sequential) of rhFSH (Gonal-F®; Merk Serono S.p.A. Bari – Italy), secondary follicles were randomly distributed in the following treatments: α-MEM$^+$ alone (control) or in the presence of rhFSH in fixed (500, 750 or 1000 ng/mL) or increasing concentrations: Sequential 1 (from day 0 to day 6 = 500 ng/mL rhFSH; from day 6 to day 12 = 750 ng/mL rhFSH; from day 12 to day 18 = 1000 ng/mL rhFSH) or Sequential 2 (from day 0 to day 6 = 100 ng/mL rhFSH; from day 6 to day 12 = 500 ng/mL rhFSH; from day 12 to day 18 = 1000 ng/mL rhFSH – according to Saraiva *et al.*, 2011). The concentrations of FSH were chosen based on previous studies [25, 15, 14, 35] and initially tested in a pilot experiment from our laboratory. In all fixed rhFSH treatments, 60 µL of the culture medium was replaced with fresh medium in each droplet every 2 days. For all sequential medium treatments, a complete replacement of the medium (100 µl) was performed every 6 days for a change in the

rhFSH concentration. Six replicates of each treatment were performed with approximately 60 follicles per treatment.

The morphological aspects of all preantral follicles were assessed every 6 days using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) at 100x magnification. Two types of follicles were considered as surviving follicles: (i) intact follicles, characterized as translucent with an intact basement membrane and surrounded by homogeneous and bright granulosa cells throughout the culture, and (ii) extruded follicles, characterized by rupture of the basement membrane during culture and a bright and intact oocyte (with no signs of an irregular contour, a darkened oocyte and/or granulosa cells) [36]. Follicle atresia was recognized when a darkening of the oocytes and/or surrounding cumulus cells or misshapen oocytes was noted. The following characteristics were analyzed in the morphologically normal follicles: (i) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers and (ii) the diameter of intact follicles, measured from the basement membrane, which included two perpendicular measures of each follicle. After 18 days of culture, all oocytes from intact (recovered with 26 G needles) and extruded follicles were recovered under a stereomicroscope. The oocyte diameter (zona pellucida not included) was measured using a pre-calibrated ocular micrometer in a stereomicroscope. The percentage of fully grown oocytes, i.e., oocyte greater than 110 μm, was calculated as the number of acceptable quality oocytes (≥110 μm) recovered out of the total number of cultured follicles (x100). Furthermore, healthy oocytes retrieved were evaluated for mitochondrial activity and *in vitro* maturation.

2.5. Measurement of metabolically active mitochondria

After 18 days of culture, the oocytes were recovered and mitochondrial activity was measured using MitoTracker Red (MitoTracker Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) as red fluorescence [34]. Approximately 30 oocytes per treatment were incubated in the dark for 30 minutes in PBS supplemented with 100 nM MitoTracker Red at 39 ºC. Thereafter, the oocytes were washed with PBS and the fluorescence was observed under an epifluorescence microscope with UV filters (579–599 nm). Fluorescent image intensities of the oocytes were analyzed by using the Image J software (National Institute of Health, Bethesda, MD, USA).

2.6. Maturation of ovine oocytes from in vitro cultured secondary follicles

In vitro maturation (IVM) was performed in oocytes derived from *in vitro* grown follicles after 18 days of culture in α-MEM$^+$ (control medium) or in the treatment that obtained the best results (750 ng/mL rhFSH). For this, additional pairs of ovine ovaries (n=10) were collected, washed and transported to the laboratory as described above. After 18 days of culture, cumulus oocyte complexes (COCs) from intact (mechanically collected with 26 G needles) or extruded follicles were selected for IVM. Only oocytes ≥ 110 µm of diameter with a homogeneous cytoplasm and surrounded by at least 1 compact layer of cumulus cells were selected for IVM as previously described [9]. COCs from intact or extruded follicles were separately cultured in drops of 100 µL of maturation medium composed of TCM 199 supplemented with 10% fetal calf serum (FCS), 1 µg/mL rhFSH and 1 µg/mL ovine pituitary LH under oil, and incubated for 24 h under 5% CO_2 in the air [25]. Oocytes were washed three times in drops of PBS/polyvinylpyrrolidone (PVP) and incubated in drops of PBS containing 10 mM Hoechst 33342 for 15 minutes at room temperature in the dark. Thereafter, oocytes were washed in drops of PBS/PVP, and slides were prepared for

evaluation using an epifluorescence microscope (Nikon E200, Tokyo, Japan) at a magnification of x 400. The oocyte meiotic stage labeled with Hoechst was analyzed for intact germinal vesicle (GV), meiotic resumption (germinal vesicle breakdown [GVBD], metaphase I [MI], anaphase I [AI] and telophase I [TI]) or nuclear maturation (metaphase II [MII]).

2.7. Detection of DNA fragmentation using TUNEL assay

At the end of IVM, oocytes were subjected to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay to assess DNA fragmentation as previously described [37]. Briefly, oocytes were fixed in 4% paraformaldehyde solution for 1 hour at room temperature. Thereafter, approximately 20 oocytes per treatment were washed three times in solution of PBS/PVP and stored at 4 °C in eppendorf with PBS/PVP until the beginning of TUNEL procedure. Next, oocytes were incubated in droplets of 100 µL of permeabilizing solution (0.1% [v:v] Triton X-100 in 10 mM PBS) for 3 hours at room temperature. Positive and negative controls were incubated in drops of 100 µL containing DNase free RNase (Invitrogen Corporation, Carlsbad, CA, USA) at 37 °C for 1 hour and washed three times in drops of PBS/PVP. The TUNEL assay was prepared as indicated by the manufacturer (In Situ Cell Detection Kit, Fluorescein: Boehringer Mannheim/Roche Diagnostics Ltd., Indianapolis, USA). To this end, 12.5 µL terminal deoxynucleotidyl transferase enzyme and 112.5 µL of marker solution of 2-deoxyuridine triphosphate 5-FITC were made to obtain 125 µL of TUNEL mixture for reaction. The experimental groups and the positive control were incubated with 15 µL of this solution for 1 hour at 37 °C in a moist chamber in the dark. The negative control was incubated with 15 µL of the marker solution. Oocytes were washed three times in drops of PBS/PVP and incubated in drops of PBS

containing 10 mM Hoechst 33342 for 15 minutes at room temperature in the dark. Thereafter, oocytes were washed in drops of PBS/PVP, and slides were prepared for evaluation using an epifluorescence microscope (Nikon E200, Tokyo, Japan) at a magnification of x 400. TUNEL-positive oocytes (with DNA fragmentation) were those with marked chromatin with green fluorescence.

2.8. Statistical analysis

All statistical analyses were performed using Bioestat 5.0® [38]. Data from surviving follicles, antrum formation and retrieval of *in vitro* grown oocytes after culture were expressed as percentages and compared by the Chi squared test. Data from mitochondrial activity, follicular diameter and growth rate were submitted to the Shapiro–Wilk test to verify normal distribution of residues and homogeneity of variances. Thereafter, the Kruskal–Wallis nonparametric test was used for comparisons. When main effects or interactions were significant, means were compared by the Student Newman Keuls test. DNA fragmentation and meiotic resumption were expressed as percentages and compared by the Fisher Exact test. These results were expressed as the means ± standard error of the mean and differences were considered significant when $P < 0.05$.

3. Results

3.1. Follicular morphology and development after in vitro culture

The figure 1 shows a secondary follicle before culture (Fig. 1A; α-MEM^{+}), an antral follicle after 18 days of culture (Fig. 1B), with extruded oocytes in 750 ng/mL rhFSH (Fig. 1C), and an atretic follicle in the control (Fig. 1D).

Fig. 1. Morphologically normal secondary follicle at Day 0 (A), antral (B), extruded (C) and atretic (D) follicles. GC, granulosa cell; O, oocyte. Arrow: antral cavity, *: rupture of the basement membrane. Scale bar: 100 μm (100 x magnification).

Follicular survival decreased significantly from day 6 to 18 in all treatments, except in the concentration of 750 ng/mL FSH ($P>0.05$; Fig. 2A). This latter treatment also had more ($P<0.05$) surviving follicles than the control (α-MEM^{+}) and 1000 ng/mL rhFSH after 18 days of culture. There was no difference ($P>0.05$) in the rates of follicle extrusion among the treatments (α-MEM^{+}: 8,8%; rhFSH 500: 11,9%; rhFSH 750: 14,0%; rhFSH 1000: 7,3%; rhFSH Sequential 1: 13,2% and rhFSH Sequential 2: 9,1%).

As early as day 6 of culture, antral cavity formation was observed in all treatments (Fig. 2B). However, after 18 days, there was no difference ($P>0.05$) in the antrum formation among the groups. Follicular diameter increased progressively and significantly in all treatments throughout 18 days of culture (Fig. 2C). Furthermore, addition of rhFSH to the medium promoted a significant increase in the percentage of fully grown oocytes in all treatments compared to α-MEM^{+} (Fig. 2D).

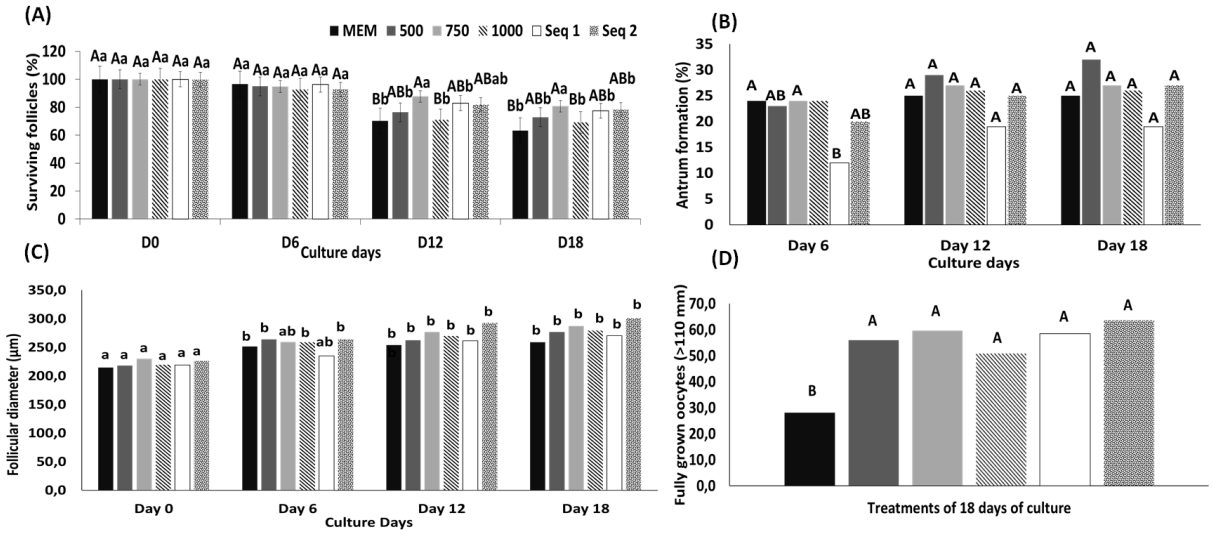

Fig. 2. Percentage of surviving follicles (A), percentage of antrum formation (B), mean ± SEM of follicular diameter (C) and percentage of fully grown oocytes (≥110 µm) (D) in α-MEM+ or fixed concentrations of rhFSH (500, 750 or 1000 ng/ml) or sequential medium containing rhFSH (Seq. 1 or Seq. 2).

[A,B,C] Different letters denote significant differences among treatments in the same period of culture ($P < 0.05$);

[a,b,c] Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$).

3.2. Mitochondrial activity after culture, chromatin configuration and DNA fragmentation after IVM

Oocytes cultured in all treatments containing rhFSH showed greater levels of mitochondrial activity ($P<0.05$) than α-MEM^{+}, except the Sequential 2 medium ($P>0.05$; Fig. 3).

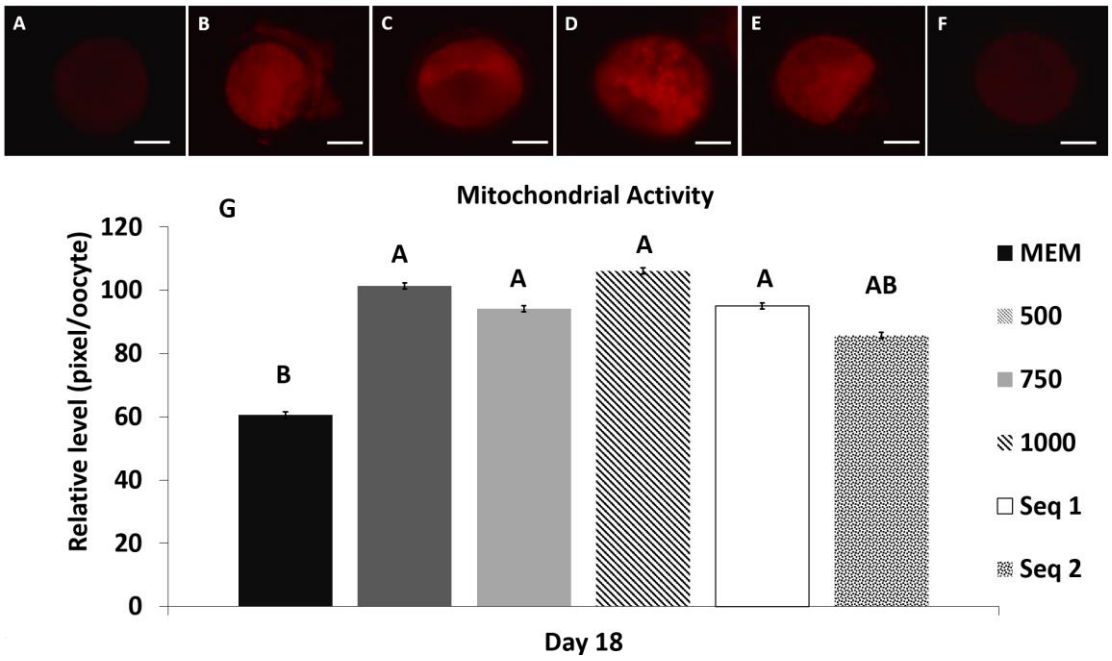

Fig. 3. Epifluorescent photomicrographic images of ovine oocytes stained with MitoTracker Red (A–F) to detect intracellular levels of active mitochondria: Oocytes cultured in α-MEM+ (A) or 500 (B), 750 (C), 1000 ng/mL rhFSH (D), Sequential 1 (E), Sequential 2 (F), and (G) relative level (pixel/oocyte) of intracellular mitochondrial activity in ovine oocytes after 18 days of *in vitro* culture. Scale bars: 100 μm.

[A,B,C] Different letters denote significant differences among treatments ($P < 0.05$).

In addition to the control medium (α-MEM$^+$), IVM was performed in the oocytes from the medium containing 750 ng/mL rhFSH because it was the only that had more surviving follicles than α-MEM$^+$ and 1000 ng/mL. For IVM, about 68% of the oocytes from the α-MEM$^+$ treatments were obtained from intact follicles and 32% from extruded follicles. In the 750 ng/mL rhFSH treatment, this number was 59.3% and 40.6%, respectively (Table 1).

Table 1. Meiotic stages (%) of sheep oocytes from *in vitro* grown secondary follicles after 18 days of culture in α-MEM$^+$ or 750 ng/mL rhFSH.

Treatments	No. Oocytes	% GV (n)	% GVBD (n)	% MI (n)	% MII (n)
α-MEM$^+$					
Intact	17	40.0 (8/17)	35.2 (6/17)	11.7 (2/17)	0 (0/17)[b]
Extruded	8	37.5 (3/8)	50.0 (4/8)	0 (0/8)	12.5 (1/8)
750 rhFSH					
Intact	19	42.1 (8/19)	15.7 (3/19)	10.5 (2/19)	31.5 (6/19)[a]
Extruded	13	53.8 (7/13)	23.0 (3/13)	15.3 (2/13)	0 (0/13)

[a,b] Different letters denote significant differences among treatments and source of the oocytes (from intact or extruded follicle) ($P<0.05$).

However, there were not significant differences between treatments (α-MEM$^+$ and 750 ng/mL rhFSH; Table 1) with respect to oocytes in GV (Fig. 4A), GVBD (Fig. 4B), AI, TI (data not shown) and MI (Fig. 4C). Nevertheless, there was a significant increase in MII rates after IVM of oocytes recovered from intact follicles cultured in 750 ng/mL rhFSH (31.5%; Table 1; Fig. 4D) compared to those recovered from intact follicles cultured in α-MEM$^+$ (0%; Table 1). Regarding the oocytes recovered from follicles with early extrusion, there was no difference between treatments ($P> 0.05$).

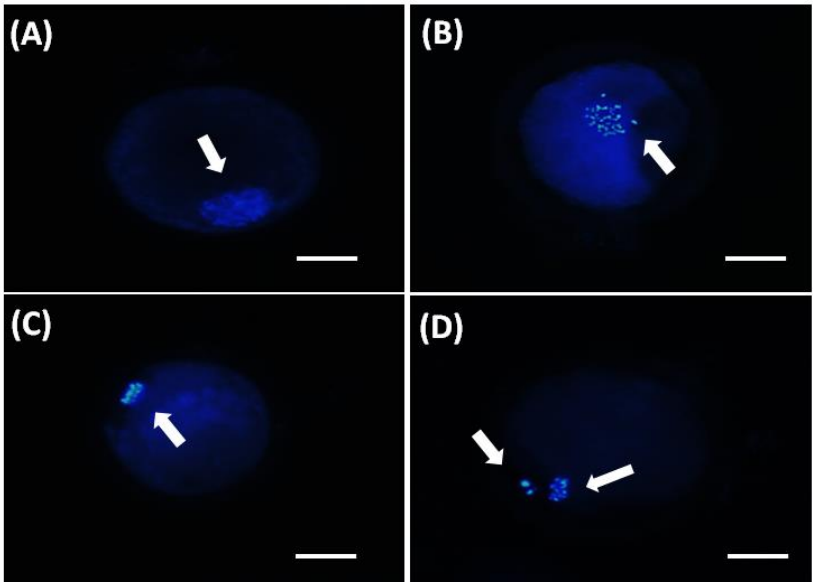

Fig. 4. Chromatin configuration of ovine oocytes stained with Hoechst 33342 after IVM. Oocytes in GV (A) and GVBD (B) cultured in α-MEM^{+}; oocytes in MI (C) and MII (D) cultured in medium containing 750 ng/mL rhFSH.

The TUNEL assay revealed absence or few oocytes with DNA fragmentation after IVM (Fig. 5). No significant differences (P> 0.05) were observed between treatments for DNA fragmentation (data not shown).

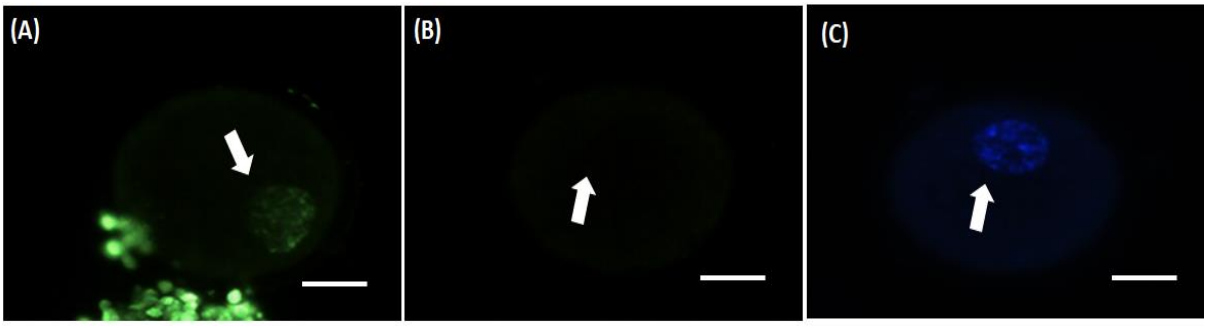

Fig. 5. DNA fragmentation of ovine oocytes after IVM. Oocyte with DNA fragmentation in the positive control (A), TUNEL negative oocyte cultured in α-MEM+ (B) and the same oocyte stained with Hoechst 33342 (C). White arrow: nuclear chromatin. Scale bars: 50 µm.

4. Discussion

This is the first study to test different concentrations of rhFSH or sequential media with rhFSH in the culture of ovine secondary follicles in serum-free medium. Other studies have shown that fixed concentrations of FSH can promote growth and antrum formation after *in vitro* culture of ovine secondary follicles (pFSH: [25]; rbFSH: [26]), but in medium supplemented with FBS as a source protein.

Although some good results have been reported with serum supplementation in *in vitro* growth media [39 - 41], serum quality varies from batch to batch [42], and serum used for follicle culture contains numerous known and unknown compounds (proteins, amino acids, carbohydrates, trace elements, hormones, growth factors and extracellular matrix components - [30]), which could interact with the other components added in the medium [31]. Furthermore, the use of serum-containing media hampers the further optimization and standardization of follicle culture systems. Therefore, the removal or replacement of serum and its components represents the starting point for developing a defined culture system [43]. Comparisons of the efficacy of *in vitro* growth serum-based versus serum-free culture systems for ovarian follicles (mouse: [43]; feline: [44]; caprine: [26]; equine: [45]; human: [46]) and embryos (bovine: [47, 48]; feline: [49]; ovine: [50]) have been conducted previously. For example, in caprine species, Rodrigues *et al.* (2010) compared isolated secondary follicle growth in culture medium supplemented with either fetal calf serum (FCS) or BSA, showing that supplementation with 3.0 mg/mL BSA improved follicular development and provided a meiotically competent oocyte. Therefore, in an attempt to establish a serum-free defined system for the culture of sheep ovarian follicles, we replaced FCS with a commercially available protein source (BSA). One of the BSA properties is related to the antioxidant activity because it can scavenge metal ions that can act as a source of reactive oxygen species

(ROS) [51]. Moreover, it provides protection against lipid peroxidation propagated by ROS generated in aerobic metabolism both *in vivo* and *in vitro* [52]. In addition to the concern about the use of BSA in culture media, it is important to note that rbFSH is not easily available for purchase or commercialization, which directs us to use other sources of FSH, such as recombinant human FSH.

Using rhFSH, the concentration of 750 ng/mL was the only one that promoted maintenance of follicular survival throughout the 18 days of culture. Similar to our study, secondary follicles of monkey reshus cultured in the presence of 500 mIU/mL rhFSH showed an increase in follicular survival for two weeks compared to those cultured in the absence of rhFSH [53]. These results can be explained by the presence of receptors for FSH in secondary follicular granulosa cells [14]. Some authors distinguish groups of secondary follicles as FSH dependent versus FSH responsive, as the smaller follicles still exhibited dose-dependent increases in growth, lactate production, and steroid secretion [53]. Moreover, through its receptors, FSH may enhance the activity of the G protein through the inhibition of adenyl cyclase activity (cAMP), which is directly involved in the survival and proliferation of granulosa cells [54].

In this experiment, addition of rhFSH to the culture medium increased the rate of fully grown oocytes compared to the control group. The oocytes obtained at the end of the culture had an average diameter greater than those obtained by Cecconi *et al.* (1999) (160 µm x 115 µm, respectively). Moreover, oocytes from intact follicles grown in medium containing 750 ng/mL rhFSH showed 31.5% metaphase II rate, while no oocytes recovered from follicles grown in the control medium reached this stage. Therefore, these results represent a significant advantage over those previously reported by Cecconi *et al.* (1999), in which only 5% of the oocytes reached the MII stage and Rodrigues *et al.* (2010), in which all oocytes were immature (GV) after IVM. Moreover, it is important to highlight that our results of

maturation were the highest achieved so far for caprine (29% - [55]) and ovine species (29.4% - [9]). In bovine, FSH stimulated the production of oocyte-derived factors by cumulus cells, like epidermal growth factor (EGF), in addition to inducing the expression of multiple genes involved in the acquisition of meiotic competence, such as the cumulus expansion gene (HAS2) and the gene for progesterone production (PGR) [56]. Therefore, we suggest that FSH may have an indirect action, stimulating the production of other factors and promoting oocyte maturation.

Additionally, the highest mitochondrial activity observed in oocytes from follicles cultured in medium containing rhFSH, when compared to control, may have positively influenced the *in vitro* growth of ovine oocytes and consequently oocyte maturation in medium containing 750 ng/mL rhFSH. The accumulation of the mitochondria close to the perinuclear region during maturation of the oocytes contributes to increase the ATP supply and promote maturation [57, 58]. Moreover, the low mitochondrial activity seems to be a characteristic of bovine oocytes that did not undergo adequate maturation [59].

In this study, although there was no difference in the maturation stage of oocytes from follicles with early extrusion, these cells are able to resume meiosis, as demonstrated by oocytes in GVBD, MI and TI. It has been suggested that the presence of cumulus cells is not essential to the meiotic maturation if the oocytes have already acquired developmental competence at the moment of oocyte removal from follicular environment. However, when oocytes still need to acquire competence, the presence of cumulus cells may contribute to their acquisition of developmental capacity during IVM. Therefore, in our study, the oocytes from extruded follicles would require longer time in IVM [60].

In conclusion, the concentration of 750 ng/mL rhFSH maintained the survival of ovine secondary follicles cultured in serum-free medium for 18 days, improved oocyte growth and mitochondrial activity and promoted oocyte maturation *in vitro*. These results represent an

important step towards the goal of achieving full oocyte growth and development *in vitro* in the sheep model, which has been validated for the purposes of understanding ovarian function in many other species, including human.

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Conflicts of interest

The authors declare no conflicts of interest.

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Capítulo II.

Melatonin improves development, mitochondrial function and promotes the meiotic resumption of sheep oocytes from *in vitro* grown secondary follicles

(Melatonina melhora o desenvolvimento, função mitocondrial e promove a retomada da meiose de oócitos ovinos oriundos de folículos secundários crescidos *in vitro*)

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Melatonin improves development, mitochondrial function and promotes the meiotic resumption of sheep oocytes from *in vitro* grown secondary follicles

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Running head: Melatonin on sheep follicle culture

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Abstract

This study aimed to evaluate the effect of melatonin concentrations on the *in vitro* culture of isolated sheep secondary follicles. Isolated secondary follicles were cultured for 18 days in α-MEM^{+} alone (control) or with different concentrations of melatonin (100, 500 or 1000 pg/mL) or sequential concentrations (Mel Seq. Day 6= 100; Day 12= 500; Day 18=1000 pg/mL). The percentage of morphologically normal follicles and the rate of antral cavity formation increased significantly in 1000 pg/mL melatonin compared to the other treatments. After 18 days, Mel 1000 showed a similar follicular diameter to Mel Seq ($P>0.05$) and greater ($P<0.05$) than other treatments. Mel 500 showed a similar rate of fully grown oocytes to Mel 1000 ($P>0.05$) and was significantly higher than other treatments. After oocyte maturation, the levels of ROS were lower ($P<0.05$) in 1000 pg/mL melatonin compared to the other treatments. There were no significant differences between Mel 500 and 1000 regarding to meiotic stages ($P>0.05$). In conclusion, the concentration of 1000 pg/mL of melatonin maintains follicular survival, promotes the development of ovarian follicles and increases the levels of active mitochondria after *in vitro* culture of sheep secondary follicles. Moreover, this concentration promotes the meiotic competence of oocytes and decreases the production of ROS during oocyte maturation.

Keywords: Ovine. Mitochondrial function. Meiosis. ROS. Pinel hormone.

Introduction

Folliculogenesis and oogenesis are complex reproductive processes controlled by both paracrine and endocrine factors (Matsuda *et al.*, 2012). However, due to their unique developmental and regulatory mechanisms, the majority of the mammalian oocyte resource is not fully utilized (Lonergan and Fair, 2016). Therefore, *in vitro* production of mature oocytes is not only an important target of reproductive research, aiming to identify the underlying regulatory mechanisms of oogenesis, but also a way to optimize the use of the female germ cell pool to improve current assisted reproductive technologies (Wang *et al.*, 2017). The events that ultimately lead to the release of a mature fertilizable oocyte from a preovulatory follicle occurs by an endocrine control through the hypothalamic-pituitary-gonadal axis plays (Walters *et al.*, 2018). One of the possible candidates for this role is melatonin.

Melatonin (N-acetyl-5- methoxytryptamine) is a neurohormone secreted from the vertebrate pineal gland and partially by other peripheral organs such as gut, gonads, retina, and immune-competent cells (Acuña-Castroviejo *et al.*, 2014). The expression of melatonin and its two membrane receptors (MT1 and MT2) in the ovaries of various species including human (Niles *et al.,* 1999), rat (Soares *et al.,* 2003), bovine (Wang *et al.,* 2012), porcine (He *et al.,* 2016); mice (Lee *et al.,* 2001; Barberino *et al.*, 2017), caprine (Barros *et al.,* 2013) and ovine (Tian *et al.,* 2017) confirm the importance of this hormone in reproduction. Moreover, evidences indicated that melatonin present in follicular fluid is synthesized by the oocyte, except that derived from blood circulation (Sakaguchi *et al.*, 2013).

In vitro studies have demonstrated that melatonin is a highly effective antioxidant and anti-apoptotic agent. Due to its direct scavenging of toxic oxygen derivatives and its ability to reduce reactive oxygen species (ROS) and reactive nitrogen species (RNS), melatonin prevents DNA damage in granulosa cells (mouse: Tanabe *et al.,* 2015) and decreased the production of ROS during oocyte maturation (mice: Keshavarzi *et al.,* 2018; bovine: Yang *et al.,* 2017). Moreover, supplementation of sheep ovarian tissue transport medium with melatonin improved the oocyte competence for development to the blastocyst stage as compared to control (Goodarzi *et al.,* 2017).

In association with FSH, melatonin increased preantral follicle diameter after *in vitro* culture of goat ovarian tissue (Rocha *et al.,* 2013). Melatonin was also used to promote oocyte maturation (ovine: Tian *et al.,* 2017) and embryo development (porcine: Do *et al.,* 2015; bovine: Wang *et al.,* 2014). In addition, melatonin improved secondary follicle growth (caprine: Barros *et al.,* 2013; mouse: Ganji *et al.,* 2015). However, it has never been used in the culture medium for ovine secondary follicles. Therefore, we hypothesized that the use of melatonin in the *in vitro* culture medium could improve follicular development and further oocyte maturation in sheep.

The aim of this study was to evaluate the survival, follicular development and maturation of ovine oocytes from isolated secondary follicles cultured in medium containing fixed or sequential concentrations of melatonin.

Material and methods

Unless noted otherwise supplements, hormones, and chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Source of ovaries

Ovaries (n = 100) were collected at a local abattoir from 50 adults (1–3 years old) mixed-breed sheep (*Ovis aries*). Immediately postmortem, pairs of ovaries were washed once in 70% alcohol and twice in minimum essential medium (MEM) buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). Thereafter, the ovaries were transported within 1 hour to the laboratory in tubes containing MEM-HEPES with antibiotics at 4°C (Chaves *et al.,* 2008).

The experiments reported herein were approved by the Committee on Ethics and Deontology in Studies and Research (CEDEP) from the Federal University of San Francisco Valley, according to the guidelines of the Research Ethics Committees and the National Commission for Research Ethics.

2.2. Isolation and selection of ovine secondary follicles

Isolation, selection, culture and follicular evaluation were performed according to Barros *et al.* (2013). In the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries. The ovarian cortical slices (1 to 2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in fragmentation medium consisting of MEM-HEPES with antibiotics. Secondary follicles with ≥ 300 µm in diameter and without antral cavities were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and mechanically isolated by microdissection from the strips of the ovarian cortex using 26-gauge (26 G) needles**.** Therefore, the follicles were transferred to 100 µL droplets (one follicle per droplet) containing base culture medium (α-MEM) for evaluation of the quality. Only secondary follicles that displayed the following characteristics were selected for *in vitro* culture: an intact basement membrane, two or more layers of granulosa cells and a visible, healthy oocyte that was round and centrally located within the follicle, without any dark cytoplasm. Isolated follicles were randomly allocated to the treatment groups with approximately 50 follicles per group.

***In vitro* culture of secondary follicle**

After selection, the follicles were individually cultured (one follicle per droplet) in 100 µL droplets of the culture medium under mineral oil in petri dishes (60 x 15 mm; Corning,

Sarstedt, Newton, NC, USA) at 39°C and 5% CO_2 in the air for 18 days. The base control medium (α-MEM$^+$) consisted of α-MEM (pH 7.2-7.4) supplemented with 3 mg/mL bovine serum albumin (BSA), 10 ng/mL insulin, 5,5 μg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine and 50 μg/mL ascorbic acid. To verify the effect of fixed or sequential concentrations of melatonin (Mel), secondary follicles were randomly distributed into five treatment groups: α-MEM$^+$ alone (MEM or control); α-MEM$^+$ supplemented with 100, 500 or 1000 pg/mL melatonin (Mel 100, Mel 500 and Mel 1000, respectively) or α-MEM$^+$ supplemented with increasing concentrations of melatonin (MelSeq: from day 0 to day 6 = 100 pg/mL; from day 6 to day 12 = 500 pg/mL Mel; from day 12 to day 18 = 1000 pg/mL Mel), called as MelSeq. The concentrations of melatonin were chosen based on previous studies (Barros *et al.*, 2013; Rocha *et al.*, 2013). In the control and treatments with fixed concentration of melatonin, 60 μL of the culture medium was replaced with fresh medium every 2 days. However, in the sequential treatment, a complete replacement of the medium (100 μL) was performed every 6 days.

Morphological evaluation of follicle development

The morphological aspects of all preantral follicles were assessed every 6 days using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) at 100x magnification. Two types of follicles were considered as surviving follicles: (i) intact follicles, characterized as translucent with an intact basement membrane and surrounded by homogeneous and bright granulosa cells throughout the culture, and (ii) extruded follicles, characterized by rupture of the basement membrane during culture and a bright and intact oocyte (with no signs of an irregular contour, a darkened oocyte and/or granulosa cells) (PESSOA et al. 2014). Follicle atresia was recognized when a darkening of the oocytes and/or surrounding cumulus cells or misshapen oocytes was noted. The following characteristics were analyzed in the morphologically normal follicles: (i) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers and (ii) the diameter of intact follicles, measured from the basement membrane, which included two perpendicular measures of each follicle. In addition, after 18 days of culture, all oocytes were recovered with 26 G needles under a stereomicroscope. The percentage of fully grown oocytes, i.e., oocyte greater than 110 μm, was calculated as the number of acceptable quality oocytes (≥110 μm) recovered out of the total number of cultured follicles (x100).

Furthermore, healthy oocytes retrieved from this experiment were evaluated for intracellular glutathione (GSH) levels, reactive oxygen species (ROS) levels and mitochondrial activity.

Assessment of reactive oxygen species levels, glutathione and active mitochondria

After *in vitro* culture, the oocytes were recovered to evaluate the levels of intracellular reactive oxygen species (ROS), glutathione (GSH) and mitochondrial activity (Tanabe *et al.*, 2015). Briefly, 2', 7'- diacetate dichlorodihydrofluorescein (H2DCFDA; Invitrogen Corporation, Carlsbad, CA, USA), 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker® Blue; CMF2HC; Invitrogen Corporation) and Mitotracker Red (Mitotracker® Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) were used to detect intracellular levels of ROS, GSH and mitochondrial activity as green, blue and red fluorescence, respectively. Approximately 7-27 oocytes per treatment group were incubated in the dark for 30 min in PBS supplemented with 10 μM H2DCFDA, 10 μM of CellTracker Blue® and 100 nM Mitotracker® Red at 39°C. After incubation, the follicles were washed with PBS for 30 minutes and the fluorescence was observed under an epifluorescence microscope with UV filters (460 nm for ROS, 370 nm for GSH and 579 nm for mitochondrial activity). The fluorescence intensities of the oocytes were analyzed by the Image J software (National Institute of Health, Bethesda, MD, USA).

Maturation of ovine oocytes from *in vitro* grown secondary follicles

In vitro maturation (IVM) was performed in the oocytes derived from *in vitro* grown secondary follicles after 18 days of culture in the treatments that obtained the best results of follicular development to verify the ability of these oocytes to resume meiosis. For this, additional pairs of ovine ovaries (n = 20 ovaries) were collected, washed and transported to the laboratory as described above. The follicles were cultured in treatments Mel 500 and Mel 1000 and after 18 days of culture, cumulus oocyte complexes (COCs) enclosed in healthy follicles were carefully and mechanically collected with 26-G needles under a stereomicroscope. Only oocytes ≥110 μm of diameter with a homogeneous cytoplasm were selected for IVM. The COCs were transferred to drops of 100 μL of maturation medium composed of TCM 199 supplemented with 10% fetal calf serum (FCS), 1 μg/mL human recombinant FSH and 1 μg/mL LH (ovine pituitary) under oil (Cecconi *et al.*, 1999) and incubated for 32-48 h under 5% CO_2 in the air (Kim *et al.*, 2005). After IVM, the oocytes

were incubated in drops of PBS containing 10 mM Hoechst 33342 for 15 min at room temperature in the dark and visualized under fluorescence microscopy. The chromatin configuration was analyzed through observation of the intact germinal vesicle (GV), meiotic resumption (germinal vesicle breakdown - GVBD), metaphase I (MI) or nuclear maturation (metaphase II [MII]). Moreover, after IVM, the levels of ROS and active mitochondria in the oocytes were measured as described above.

Statistical analysis

Data from follicular survival, antrum formation, retrieval of fully grown oocytes and maturation rates after *in vitro* culture were submitted to chi-squared test. Data from follicular diameter, growth rate, GSH, mitochondrial activity and ROS levels were submitted to the Shapiro-Wilk test to verify normal distribution of residues and homogeneity of variances. Thereafter, Kruskal-Wallis non-parametric test was used for comparisons. When main effects or interactions were significant, means were compared by test Student Newman Keuls. The results were expressed as the means ± standard error of the mean (SEM), and the differences were considered significant when $P< 0.05$.

Results

Follicular morphology and development after *in vitro* culture

Morphologically normal secondary follicles showed centrally located oocytes and normal granulosa cells, which were enclosed by an intact basement membrane (Fig. 1A). At day 6 of culture, antral (Fig. 1B) and atretic follicles (Fig. 1C) could be observed, in α-MEM and Mel 1000, respectively.

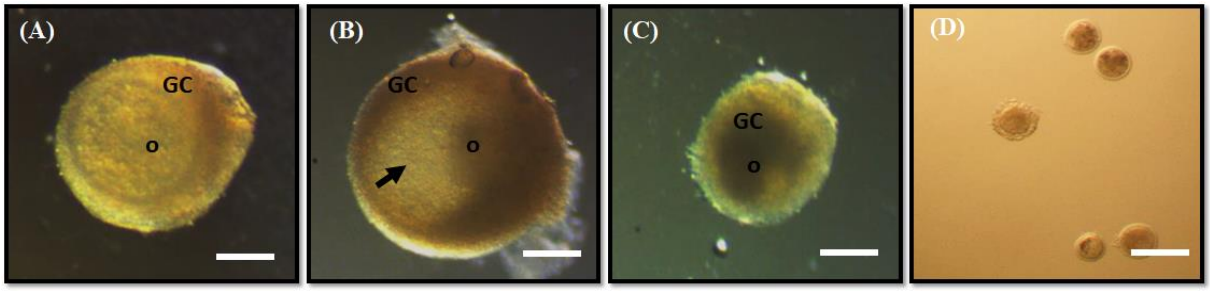

Figure 1. Morphologically normal secondary follicle at Day 0 (A), antral (B) and atretic (C) follicles, and fully grown oocytes after 18 days of culture (D).
GC: granulosa cell; O: oocyte. Arrow: antral cavity. Scale bar: 200 µm (100 x magnification).

The percentage of morphologically intact follicles decreased significantly throughout the culture period in all treatments. However, at the end of culture period, Mel 1000 (70%) showed a higher ($P<0.05$) percentage of intact follicles than other treatments (MEM: 28%, Mel 100: 46%, Mel 500: 34% and MelSeq: 28%; Fig. 2A). The percentage of extruded follicles was similar ($P>0.05$) among all treatments (data not shown).

The rate of antral cavity formation increased in all treatments ($P>0.05$) from day 6 to day 12 the culture (Fig. 2B). However, at days 12 or 18 of culture, there were more ($P<0.05$) antral follicles in Mel 1000 than other treatments (22%, 30%, 28%, 70% and 50% for MEM, Mel 100, Mel 500, Mel 1000 and MelSeq, respectively) (Fig. 2B).

At day 6, follicles in the control medium (MEM) had lower ($P<0.05$) diameter (283.7 µm) than other treatments (Mel 100: 317 µm, Mel 500: 332.8 µm, Mel 1000: 320.8 µm and MelSeq: 332.13 µm) (Fig. 2C). It was also observed an increase of follicular diameter throughout the culture period in both Mel 1000 and MelSeq ($P<0.05$). Moreover, after 18 days, follicles cultured in Mel 1000 (388.52 µM) showed a larger diameter ($P<0.05$) than other treatments (289.06 µM, 348.4 µM and 310.85 µM for MEM, Mel 100 and Mel 500, respectively), except for MelSeq (364.42 µM; $P>0.05$) (Fig. 2C). At the end of culture, Mel 500 (56%) and Mel 1000 (40%) treatments showed similar ($P>0.05$) rates of fully grown oocytes. In addition, significantly more fully grown oocytes were observed in medium containing 500 pg/mL melatonin (Mel 500) compared to MEM (12%), Mel 100 (28%) and MelSeq (10%), being similar ($P>0.05$) to MEL1000 (Fig. 2D).

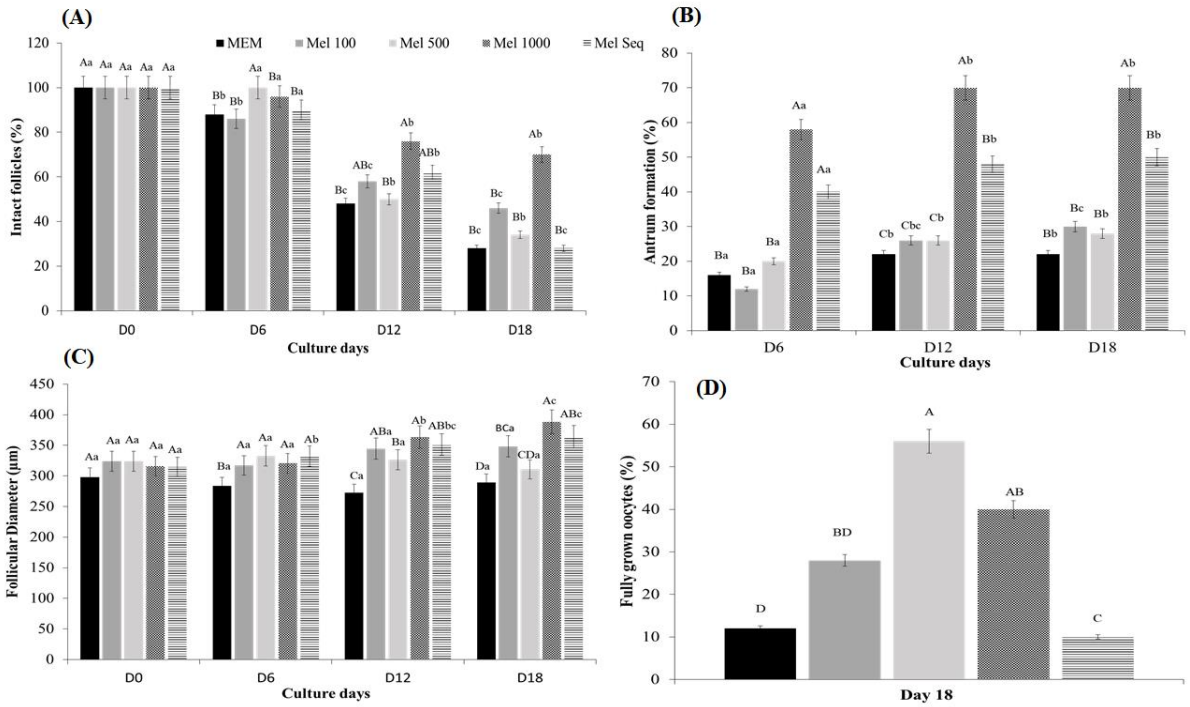

Figure 2. Percentages of normal follicles (A), antrum formation (B), follicular diameter (C) and fully grown oocytes (≥110 µm) (D) in α-MEM^{+} or fixed concentrations of melatonin (100, 500 or 1000 pg/ml) or sequential melatonin.

^{A,B,C} Different letters denote significant differences among treatments in the same period of culture ($P<0.05$);

^{a,b,c} Different letters denote significant differences among culture periods in the same treatment ($P<0.05$).

Intracellular levels of ROS, GSH and metabolically active mitochondria

The intracellular levels of ROS were similar ($P>0.05$) among all treatments (data not shown). The oocytes cultured in Mel 500 treatment showed a higher GSH level than all other treatments ($P<0.05$; Fig. 3A-E; 3K). Moreover, Mel 1000 and MelSeq treatments showed a similar ($P>0.05$) level of mitochondrial activity, being both significantly higher than other treatments (Fig. 3F-J; 3L).

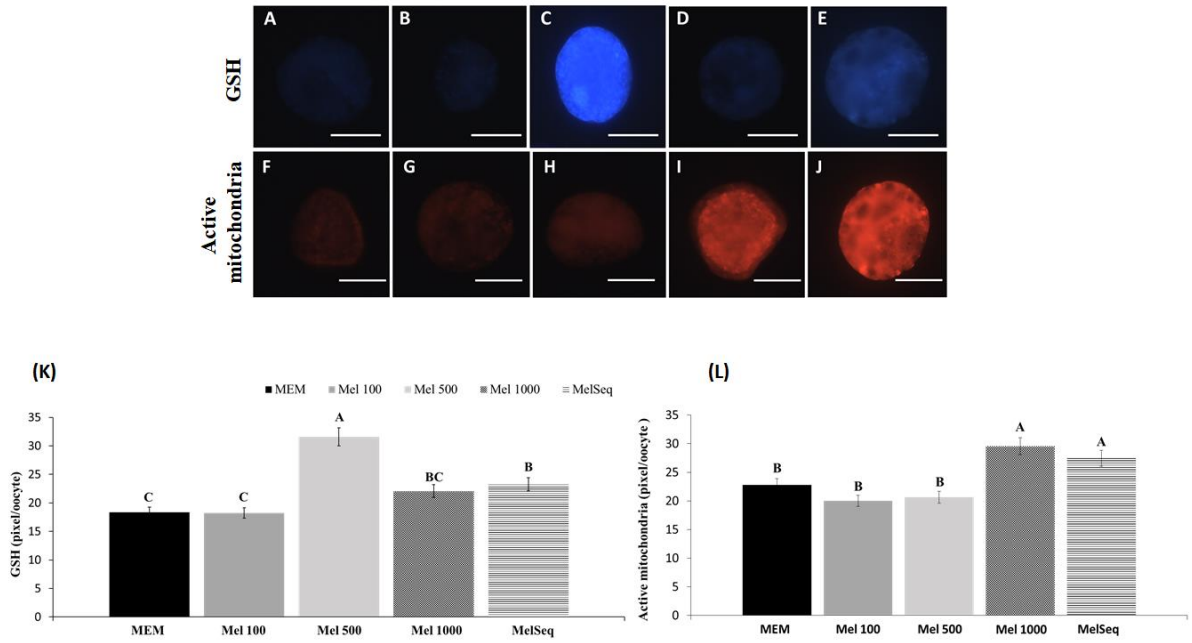

Figure 3. Epifluorescent photomicrographic images of ovine oocytes showing intracellular levels of GSH (A-E) and active mitochondria (F-J). Oocytes after 18 days of *in vitro* culture in α-MEM^{+} (A and F), 100 (B and G), 500 (C and H) or 1000 pg/mL melatonin (D and I) and melatonin sequential (E and J). Intracellular levels of GSH (K) and mitochondrial activity (L) in oocytes from different experimental groups. Scale bars: 200 µm.

^{A,B,C} Different letters denote significant differences among treatments ($P < 0.05$).

In vitro maturation (IVM) and levels of ROS and metabolically active mitochondria after IVM

Oocyte meiotic competence was assessed by IVM of COCs obtained from secondary follicles that had been cultured in Mel 500 and Mel 1000. These treatments were chosen because they have presented the highest rates of fully grown oocytes (Mel 500 and Mel 1000), GSH levels (Mel 500) and mitochondrial activity (Mel 1000). When evaluating chromatin configuration, no significant differences ($P>0.05$) regarding to meiotic stages were observed between treatments (Table 1; Fig 4A-C). However, MII oocytes were obtained only after IVM of oocytes cultured in 1000 pg/mL melatonin (Table 1; Fig 4D).

Table 1. Meiotic stages of sheep oocytes from *in vitro* grown secondary follicles after 18 days of culture in 500 or 1000 pg/mL melatonin.

Treatments	No. Oocytes	% GV (n)	% GVBD (n)	% MI (n)	% MII (n)
Mel 500	22	45.45% (10/22)	36.36% (8/22)	18.19% (4/22)	0% (0/22)
Mel 1000	19	57.89% (11/19)	26.32% (5/19)	5.26% (1/19)	10.53% (2/19)

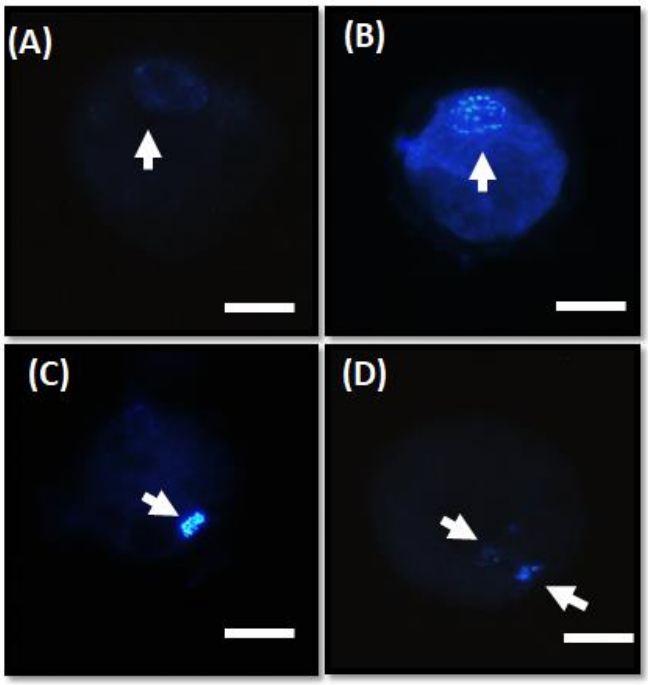

Figure 4. Chromatin configuration of ovine oocytes stained with Hoechst 33342 after IVM. Oocytes in GV (A), GVBD (B) and MI (C) cultured in Mel 500; and oocytes in MII (D) cultured in Mel 1000 pg/mL. Scale bars: 50 µm.

After IVM, the intracellular levels of ROS were lower ($P<0.05$) in Mel 1000 (70.89 pixel/oocyte) than Mel 500 (99.46 pixel/oocyte) (Fig. 5). All treatments showed a similar ($P>0.05$) level of mitochondrial activity (data not shown).

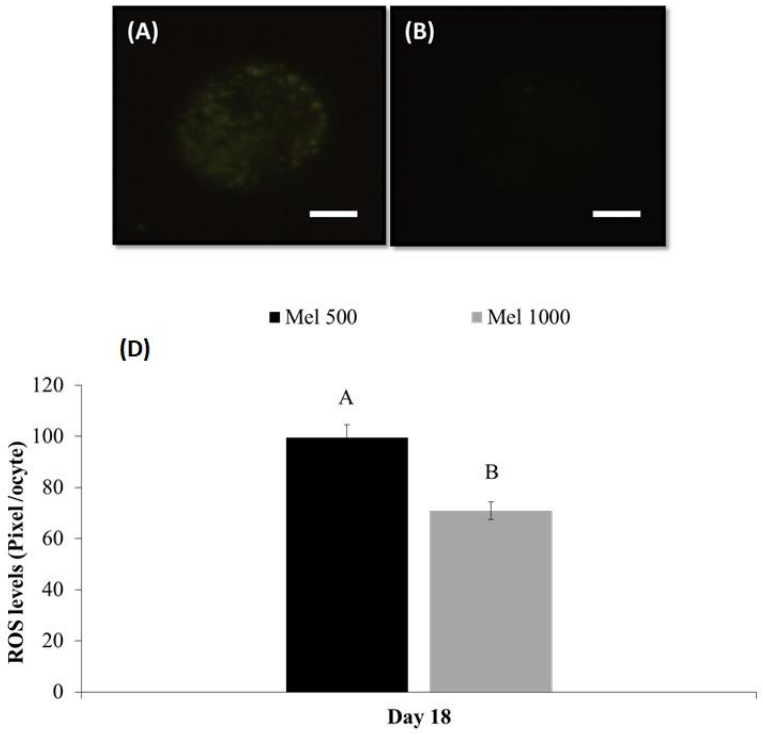

Figure 5. Epifluorescent photomicrographic images of ovine oocytes showing intracellular levels of ROS. Oocytes after *in vitro* culture and maturation in 500 (A) and 1000 (B) pg/mL melatonin. Intracellular levels of ROS (C) in oocytes from different experimental groups. Scale bars: 100 µm.

[A,B,C] Different letters denote significant differences among treatments ($P < 0.05$).

Discussion

To our knowledge, this study showed for the first time that melatonin maintains follicular survival, stimulates antral cavity formation and subsequent follicular (1000 pg/mL) and oocyte (500 and 1000 pg/mL) growth, as well as increases GSH (500 pg/mL) and metabolically active mitochondria (1000 pg/mL) levels after *in vitro* culture of sheep secondary follicles. Antioxidants such as melatonin are used in culture media and oocyte maturation to avoid the harmful effects of oxidative stress *in vitro* (Zhang *et al.*, 2016; Pang *et al.*, 2017). Melatonin is considered as a highly efficient antioxidant because it is amphiphilic, i.e., it easily crosses all the morphophysiological barriers (Hardeland *et al.*, 2011; Reiter *et al.*, 2013). In addition, one of the most appealing properties of melatonin, which distinguishes it from most antioxidants, is that it also has the ability to scavenge ROS and RNS (Galano, et al., 2013).

After 18 days of culture, the concentration of 1000 pg/mL of melatonin showed more morphologically normal follicles and stimulated antrum formation compared to all other treatments. Moreover, this concentration also increased follicular growth and decreased mitochondrial damage, except when compared to sequential melatonin treatment. Mitochondria are the principal font of the superoxide anion radical and the other intracellular ROS and RNS (Reiter *et al.,* 2018). Under high stress conditions, ROS and RNS generation becomes elevated inducing damage in the mitochondrial DNA. Mammalian oocytes and zygotes subjected to mild to intense oxidative stress have demonstrated significant mitochondrial dysfunction and ultrastructural changes. Mitochondrial dysfunction has a clear impact on both mitochondrial ATP synthesis and the activation of its apoptotic mechanisms (Liu *et al.*, 2000; Zhang *et al.,* 2006). Melatonin is a direct ROS scavenger (Reiter *et al.*, 2017) and may reduce O_2^- production by preventing the formation of their precursors or by scavenging them at the level of the mitochondrial electron transport chain by a process referred to as radical avoidance (Hardeland, 2009). In mouse, melatonin protects the integrity of granulosa cells by reducing oxidative stress, avoiding DNA damage, mitochondrial dysfunction, and lipid peroxidation of plasma membranes (Tanabe *et al.*, 2015). Therefore, in the present study, the preservation of mitochondria function may have supported follicular health (morphologically normal follicles) after *in vitro* culture in 1000 pg/mL melatonin.

Furthermore, it is possible that these melatonin actions prevented oxidative stress, thereby stimulating subsequent follicular development (antrum formation and growth). A previous study also observed an increase of follicular diameter after *in vitro* culture of goat ovarian cortex in medium containing 1000 pg/mL melatonin associated with FSH (Rocha *et al.*, 2013). In addition, melatonin maintained survival and increased follicular diameter during *in vitro* culture of secondary follicles (mouse: Ganji *et al.,* 2015; goat: Barros *et al.,* 2013). A recent study has shown that through its receptors, melatonin may enhance the activity of the G protein through the inhibition of adenyl cyclase activity (cAMP), which is directly involved in the proliferation of granulosa cells (Talpur *et al.,* 2018). Moreover, melatonin can induce the proliferation of bovine theca cells through the inhibition of active caspase-3 (Feng *et al.,* 2017) or acting on ovarian angiogenesis stimulating vascular growth through endothelial cell proliferation (Basini *et al.,* 2017), which is strictly necessary for the follicular development (Basini *et al.*, 2016).

In the present study, follicles cultured in medium containing 500 or 1000 pg/mL melatonin showed a similar rate of fully grown oocytes and some of these oocytes resumed meiosis (54.5% for Mel 500 and 42.1% for Mel 1000). One possible explanation for these

results is that the concentration of 500 pg/mL of melatonin presented the highest level of GSH after *in vitro* culture (before IVM), which may have contributed to further protect the oocyte against oxidative damage during IVM. This increase in GSH levels in response to 500 pg/mL melatonin might be an attempt to protect the follicles from oxidative damage since melatonin may act as a pro-oxidant as well as an antioxidant agent depending on the concentration administered and cell type (Clapp-Lilly *et al.,* 2001; Albertini *et al.,* 2006).

Nevertheless, after IVM, it is important to highlight that ROS production decreased in the oocytes cultured in medium containing 1000 pg/mL melatonin, which was the only concentration that presented MII oocytes. The intracellular levels of ROS and GSH are known to be important factors that affect oocyte maturation. The physiological concentration of ROS has an essential role in follicular rupture and acts by modulating the expression of the genes that govern the processes of oocyte maturation (Kala *et al.,* 2016). Glutathione, a ubiquitous intracellular free thiol compound, is involved in various cellular processes, including the synthesis of DNA and proteins, the metabolism of chemicals, cellular protection, and amino acid transport (Nunes and Serpa, 2018). Melatonin can significantly reduce ROS formation and inhibit the apoptosis index in oocytes compared to the untreated samples (Marques *et al.,* 2018; Remiao et al. 2016). Moreover, Han et al. (2017) demonstrated that melatonin improved oocyte quality both *in vivo* and *in vitro* by reducing the oxidative stress of these cells in mice. Additionally, supplementation of the culture medium with melatonin significantly reduced ROS and increased GSH levels of bovine oocytes (Yang *et al.,* 2017). Melatonin also enhances oocyte maturation in many species (bovine: Marques *et al.,* 2018; porcine: Park *et al.,* 2018; mouse: Keshavarzi *et al.,* 2018; caprine: Soto-Heras *et al.,* 2018; ovine: Succu *et al.,* 2014), perhaps by protecting cumulus cells from nuclear fragmentation, increasing the expression of antioxidant enzymes, and decreasing ROS levels in the oocytes (Rodrigues-Cunha et al. 2016). The fact that only two MII oocytes were obtained is a clear indication that an optimization of the IVM conditions (for example, use of antioxidants including melatonin, and/or additional energy substrates in the medium) may be required to achieve high developmental competence.

In conclusion, the concentration of 1000 pg/mL of melatonin maintains follicular survival, promotes ovarian follicle development and increases mitochondrial function after *in vitro* culture of sheep secondary follicles. In addition, this concentration promotes the meiotic competence of oocytes and decreases the production of ROS during oocyte maturation. New studies focused on the role of melatonin in IVM of ovine oocytes are also important to better understand its mechanisms of action.

Conflicts of interest

The authors declare no conflicts of interests.

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Capítulo III.

Melatonin reduces ROS production and promotes the meiotic resumption of sheep oocytes from *in vitro* grown early antral follicles

(Melatonina reduz a produção de ERO e promove a retomada da meiose de oócitos ovinos oriundos de folículos antrais iniciais crescidos *in vitro*)

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Melatonin reduces ROS production and promotes the meiotic resumption of sheep oocytes from *in vitro* grown early antral follicles

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Running head: Melatonin on sheep antral follicle culture

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Abstract

This study aimed to evaluate the effect of melatonin concentrations on the *in vitro* culture and maturation of isolated sheep early antral follicles. Isolated early antral follicles were cultured for 12 days in α-MEM+ alone (control) or α-MEM+ added with fixed different concentrations (100, 500 or 1000 pg/mL) or a sequential concentration of melatonin (MelSeq. Day 6=100; Day 12=500 pg/mL). The percentage of morphologically normal follicles was higher ($P<0.05$) in 500 pg/mL melatonin when compared to the other treatments at 6 days. Mel 500 also showed a higher rate of fully grown ($P<0.05$) than other treatments. After *in vitro* culture, the levels of ROS were similar ($P>0.05$) between Mel 500 and MelSeq, being both lower ($P<0.05$) than other treatments. Oocytes cultured in both Mel 500 and Mel 1000 showed a similar ($P>0.05$) level of GSH with the control group and was higher ($P<0.05$) than other treatments. The mitochondrial activity was similar ($P>0.05$) among control, Mel 500 and Mel 1000 treatments. However, MelSeq presented the lower ($P<0.05$) mitochondrial activity than other treatments. Mel 500 treatment presented a higher percentage of GVBD oocyte than control group and similar percentages than other. Oocytes cultured for 12 days in melatonin followed by maturation with the addition of 500 pg/mL melatonin *in vitro* maturation medium showed increased ($P<0.05$) level of mitochondrial activity than α-MEM^{+}. In conclusion, the concentration of 500 pg/mL of melatonin, promotes ovarian follicle development, and decrease ROS levels after *in vitro* culture of sheep early antral follicles. In addition, this concentration promotes the meiotic competence of oocytes.

Additional keywords: *In vitro* culture. Meiosis. Mitochondrial active. Fully grown oocyte. Antioxidant.

1. Introduction

The majority of domestic embryos *in vitro* produced is derived from oocytes of a limited number of large antral follicles [1,2,3]. Nevertheless, in the ovary, there is a greater number of early antral follicles than follicles at more advanced growth stages. Thus, an alternative option is to recover smaller follicles and carry out *in vitro* culture (IVC) of these follicles to provide an extra source of oocytes for biotechnological purposes [4,5]. Current improvements in IVC technology have made it possible to develop oocytes of domestic animals from early antral follicles that can be used for further *in vitro* maturation (IVM) (swine: [6,7]; caprine: [5]) and subsequently embryo production (swine: [8]). However, standard IVC of ovarian follicles and IVM conditions usually increase reactive oxygen species (ROS), which have been implicated as one of the major causes for reduced embryonic development. Then, the optimization of the culture and maturation media with antioxidant supplementation could improve the survival and development of the oocytes [9,10]. Among these antioxidant agents, the hormone melatonin has frequently been investigated in recent years.

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized by the pineal gland, and its major function is to modulate circadian rhythm [11]. It is a highly lipophilic and somewhat hydrophilic molecule that easily crosses cell membranes, reaching intracellular organelles including the mitochondria and nucleus [12]. This hormone acts directly on ROS, stimulates the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH) and catalase, inhibiting the activity of prooxidant enzymes like cyclooxygenase [13,14]. These effects may be exerted through interactions with the G protein-coupled transmembrane melatonin receptors MT1 and/or MT2 [15], which are present in several ovarian cell compartments (rat: [16]; mice: [17]; caprine: [18]; ovine: [19]).

Studies in caprine species demonstrated that supplementation of IVC medium of secondary follicles with melatonin enhanced the antrum formation, follicular growth and the

percentage of fully grown oocytes [18] as well as in association with FSH, increased preantral follicle diameter after *in vitro* culture of ovarian tissue [20]. Moreover, during IVM, melatonin has shown beneficial effects on developmental competence of oocytes, decreasing the production of ROS and increasing the enzyme antioxidant activity (swine: [21] bovine [22]; mice: [23]). Furthermore, melatonin improved the cytoplasmic maturation of bovine oocytes by improving the normal distribution of organelles (for example, mitochondria, endoplasmic reticulum), increasing intracellular GSH and ATP levels and enhancing antioxidant gene expression levels [24]. It is important to note that mitochondrial distribution and function are necessary features of oocyte cytoplasmic maturation, affecting the subsequent development of the oocyte after fertilization [25].

Although the importance of melatonin in ovarian follicle development is well known, it has never been used in the culture and maturation media for ovine oocytes from early antral follicles. Thus, the aim of this study was to evaluate the follicular development and maturation of ovine oocytes from isolated early antral follicles cultured in medium containing fixed or sequential concentrations of melatonin.

2. Material and methods

Unless noted otherwise supplements, hormones, and chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Source of ovaries

Ovaries (n = 100) were collected at a local abattoir from 50 adults (1–3 years old) mixed-breed sheep (*Ovis aries*). Immediately postmortem, pairs of ovaries were washed once in 70% alcohol and twice in minimum essential medium (MEM) buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). Thereafter, the ovaries were transported within 1 hour to the laboratory in tubes containing MEM-HEPES with antibiotics at 4°C [26].

2.2. Isolation and selection of ovine early antral follicles

In the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries. The ovarian cortical slices (1 to 2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in fragmentation medium consisting of MEM-HEPES with antibiotics. Early antral follicles with between 400 and 500 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; 100x) and mechanically isolated by microdissection from the strips of the ovarian cortex using 26-gauge (26 G) needles**.** Thereafter, the follicles were transferred to 100 µL droplets (one follicle per droplet) containing base culture medium (α-MEM) for evaluation of the quality. Only antral follicles that displayed the following characteristics were selected for *in vitro* culture: an intact basement membrane, multiple layers of granulosa cells, a visible translucent cavity within the granulosa cell layers and healthy round oocyte without any dark cytoplasm. Isolated follicles were pooled and then randomly allocated to the treatment groups with approximately 47-50 follicles per group.

2.3. *In vitro* culture of early antral follicle

After selection, the follicles were individually cultured (one follicle per droplet) in 100 µl droplets of the culture medium under mineral oil in petri dishes (60 x 15 mm; Corning, Sarstedt, Newton, NC, USA) at 39°C and 5% CO_2 in the air for 12 days. The base control medium (α-MEM$^+$) consisted of α-MEM (pH 7.2-7.4) supplemented with 3 mg/mL bovine serum albumin (BSA), 10 ng/mL insulin, 5,5 µg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine and 50 µg/mL ascorbic acid. To verify the effect of fixed or sequential concentrations of melatonin (Mel), early antral follicles were randomly distributed into five treatments: α-MEM$^+$ alone (control medium); α-MEM$^+$ supplemented with 100, 500 or 1000 pg/mL melatonin (Mel 100, Mel 500 and Mel 1000, respectively) or α-MEM$^+$ supplemented with increasing or sequential concentrations of melatonin (MelSeq: from day 0 to day 6 = 100 pg/mL; from day 6 to day 12 = 500 pg/mL Mel). The concentrations of melatonin were chosen based on previous studies [18,20]. In all treatments, 60 µL of the culture medium was replaced with fresh medium every 2 days. However, on day 6, a complete replacement of the medium (100 µL) was performed.

2.4. Morphological evaluation of follicular development

The morphological aspects of all early antral follicles were assessed at the beginning, during and end of culture (day 0, 6 and 12) using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon) at 100x magnification. Two types of follicles were considered as normal follicles: (i) intact follicles, characterized by translucent and with an intact basement membrane surrounded by homogeneous and bright granulosa cells throughout the culture, and (ii) extruded follicles, characterized by rupture of the basement membrane during culture and a bright and intact oocyte (with no signs of an irregular contour, a darkened

oocyte and/or granulosa cells) [27]. Follicle atresia was recognized when a darkening of the oocytes and/or surrounding cumulus cells or misshapen oocytes was noted.

The diameter of intact follicles was measured from the basement membrane, which included two perpendicular measures of each follicle. In addition, after 12 days of culture, all healthy oocytes were recovered with 26 G needles under a stereomicroscope. The percentage of fully grown oocytes, i.e., oocyte greater than 110 μm, was calculated as the number of acceptable quality oocytes (≥110 μm) recovered out of the total number of cultured follicles (x100). Furthermore, healthy oocytes retrieved from this experiment were evaluated for intracellular levels of ROS, GSH, and active mitochondria.

2.5. Assessment of intracellular levels of ROS, GSH and metabolically active mitochondria

After *in vitro* culture, the oocytes were recovered to evaluate the intracellular levels of ROS, GSH and mitochondrial activity [28]. Briefly, 2', 7'- diacetate dichlorodihydrofluorescein (H2DCFDA; Invitrogen Corporation, Carlsbad, CA, USA), 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker® Blue; CMF2HC; Invitrogen Corporation) and Mitotracker Red (Mitotracker® Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) were used to detect intracellular levels of ROS, GSH and mitochondrial activity as green, blue and red fluorescence, respectively. The oocytes were incubated in the dark for 30 min in PBS supplemented with 10 μM H2DCFDA, 10 μM of CellTracker Blue® and 100 nM Mitotracker® Red at 39°C. After incubation, the follicles were washed with PBS for 30 min and the fluorescence was observed under an epifluorescence microscope with UV filters (460 nm for ROS, 370 nm for GSH and 579 nm

for active mitochondria). The fluorescence intensities of the oocytes were analyzed by the Image J software (National Institute of Health, Bethesda, MD, USA).

2.6. Maturation of ovine oocytes from *in vitro* cultured early antral follicles

In vitro maturation (IVM) was performed in the oocytes derived from *in vitro* grown early antral follicles after 12 days of culture in α-MEM$^+$ and Mel 500. This treatment was chosen because they are presented the highest rates of normal follicles and fully grown oocytes and to verify the ability of these oocytes to resume meiosis. For IVM, additional pairs of ovine ovaries (n = 20 ovaries) were collected, washed and transported to the laboratory as described above. The follicles were cultured in treatments α-MEM$^+$ (control medium) and Mel 500 and after 12 days of culture, the cumulus oocyte complexes (COCs) enclosed in healthy follicles were carefully and mechanically collected with 26-G needles under a stereomicroscope. Only oocytes ≥110 μm of diameter with a homogeneous cytoplasm were selected for IVM. The COCs were transferred to drops of 100 μl of IVM medium composed of tissue culture medium 199 (TCM 199) supplemented with 10% fetal calf serum (FCS), 1 μg/mL follicle stimulating hormone (human recombinant FSH; Gonal-F; Serono Laboratórios, São Paulo, Brazil) and 1 μg/mL luteinizing hormone (LH; ovine pituitary) (TCM 199$^+$) [29] or to this medium (TCM 199$^+$) supplemented with 500 pg/mL melatonin: (i) oocytes cultured in α-MEM$^+$ followed by maturation in TCM 199$^+$ (α-MEM$^+$) or (ii) in TCM 199$^+$ supplemented with 500 pg/mL melatonin (α-MEM$^+$ + Mel 500); (iii) oocytes cultured in Mel 500 followed by maturation in TCM 199$^+$ (Mel 500) or (iv) in TCM 199$^+$ added with 500 pg/mL melatonin (Mel 500 + Mel 500). IVM was performed under oil and incubated for 36 h under 5% CO_2 in the air. After IVM, the oocytes were incubated in drops of PBS containing 10 mM Hoechst 33342 for 15 min at room temperature in the dark and visualized under

fluorescence microscopy. The chromatin configuration was analyzed through observation of the intact germinal vesicle (GV), meiotic resumption (germinal vesicle breakdown - GVBD), metaphase I (MI) or nuclear maturation (metaphase II - MII). Moreover, after IVM, the levels of ROS and active mitochondria in the oocytes were measured as described above.

2.7. Statistical analysis

Data from follicle survival, extrusion, recovery of fully grown oocytes and maturation rates after *in vitro* culture were compared by the chi-squared test. Data from ROS and GSH levels, mitochondrial activity and daily growth rates were submitted to the Shapiro-Wilk teste to verify normal distribution of residues and homogeneity of variances. Thereafter, Kruskal-Wallis non-parametric test was used for comparisons. When main effects or interactions were significant, means were compared by test Student–Newman–Keuls. Data from follicular diameter were submitted to the ANOVA parametric test and, when main effects or interactions were significant, means were compared by test Tukey. The results were expressed as the means ± standard error of the mean (SEM), and differences were considered significant when $P<0.05$.

3. Results

3.1. Follicular morphology and development after *in vitro* culture

Morphologically normal early antral follicles with centrally located oocytes, a visible translucent cavity within the granulosa cell layers and normal granulosa cells were found in

Mel 500 (Fig. 1A), while extruded and atretic follicles could be observed in Mel 1000 (Fig. 1B) and in α-MEM$^+$ (Fig. 1C), respectively.

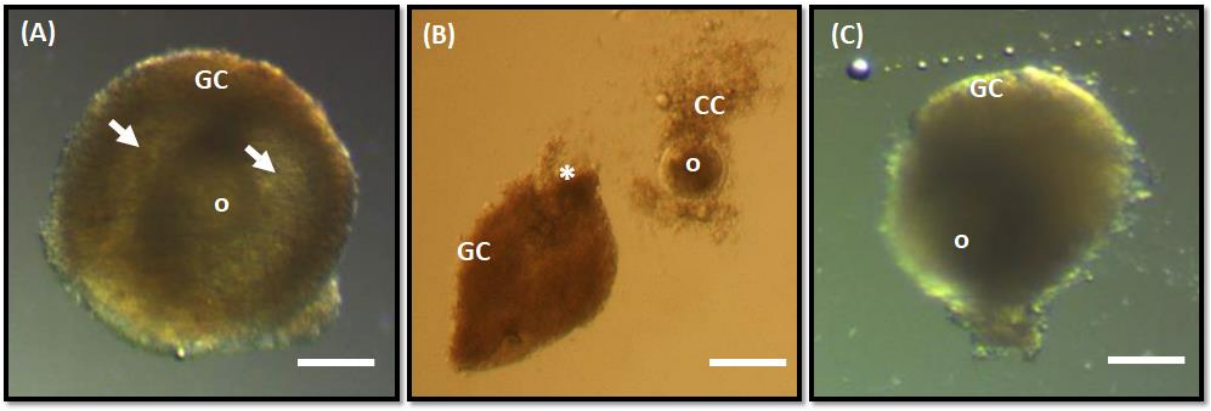

Figure 1. Morphologically normal early antral follicle (A), extruded (B) and atretic (C) follicles after 6 days if culture in 500 pg/mL melatonin, 1000 pg/mL melatonin and in α-MEM$^+$, respectively. GC: granulosa cell; O: oocyte. Arrow: antral cavity. Scale bar: 200 µm (100 x magnification).

The percentage of morphologically normal follicles (intact follicles) decreased significantly throughout the culture period in all treatments, except Mel 500 group that maintained the normal morphology of the follicles from day 0 to day 6 ($P>0.05$), decreasing ($P<0.05$) only on the day 12. At day 6 of culture, Mel 500 (95.7%) showed a higher ($P<0.05$) percentage of normal follicles than other treatments (α-MEM$^+$: 74%, Mel 100: 81.6%, Mel 1000: 61.2% and MelSeq: 76%). However, at the end of culture period, more normal follicles ($P<0.05$) were found in Mel 500 (48.9%) compared to Mel 1000 (30.6%) and MelSeq (42%), but similar percentages were observed compared to α-MEM$^+$ and Mel 100 (40% and 42.9%, respectively; Fig 2A). The percentage of extruded follicles was similar ($P>0.05$) among all treatments (data not shown).

At day 6, follicles in the control medium (α-MEM^{+}: 457.9 µm) and Mel 100 (463.2 µm) showed lower (P<0.05) diameters than Mel 500 (507.7 µm) (Fig. 2B). Moreover, after 12 days, Mel 500 (493.8 µm) showed higher (P<0.05) follicular diameter than Mel 100 (432.1 µm) and similar (P>0.05) to the other treatments (444.7 µm, 452.0 µm and 453.0 µm for α-MEM^{+}, Mel 1000 and MelSeq, respectively). It was also observed a maintenance (P>0.05) of follicular diameter throughout the culture period in Mel 500 (Fig. 2B). Additionally, Mel 500 (82.97%) showed a higher (P>0.05) rate of fully grown oocytes than other treatments (α-MEM^{+}: 44%; Mel 100: 53.06%; Mel 1000: 55.1% and MelSeq: 50%; Fig. 2C).

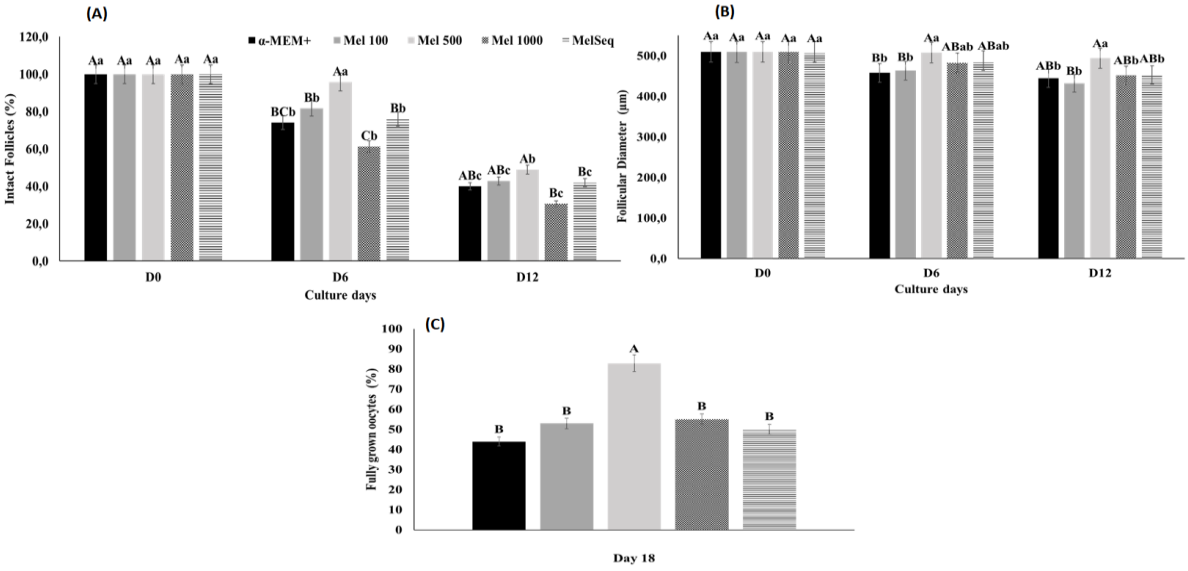

Figure 2. Percentages of morphologically normal follicles (A), follicular diameter (B) and fully grown oocytes (≥110 µm) rates (C) in α-MEM^{+} or different fixed concentrations of melatonin (100, 500 or 1000 pg/ml) or sequential melatonin.

^{A,B,C} Different letters denote significant differences among treatments in the same period of culture (P<0.05);

^{a,b,c} Different letters denote significant differences among culture periods in the same treatment (P<0.05).

3.2. Intracellular levels of ROS, GSH and metabolically active mitochondria

The intracellular levels of ROS were similar (P>0.05) in Mel 500 and MelSeq treatments and lower (P<0.05) than other treatments (Fig. 3A-E; 3P). Nonetheless, oocytes from early antral follicles cultured in Mel 500 and Mel 1000 showed similar levels of GSH and higher (P<0.05) levels than Mel 100 and MelSeq (Fig. 3F-J; 3Q). In addition, the mitochondrial activity increased (P<0.05) in Mel 1000 treatment compared to Mel 100 and MelSeq, but was similar (P>0.05) to control medium and Mel 500 (Fig. 3K-O; 3R).

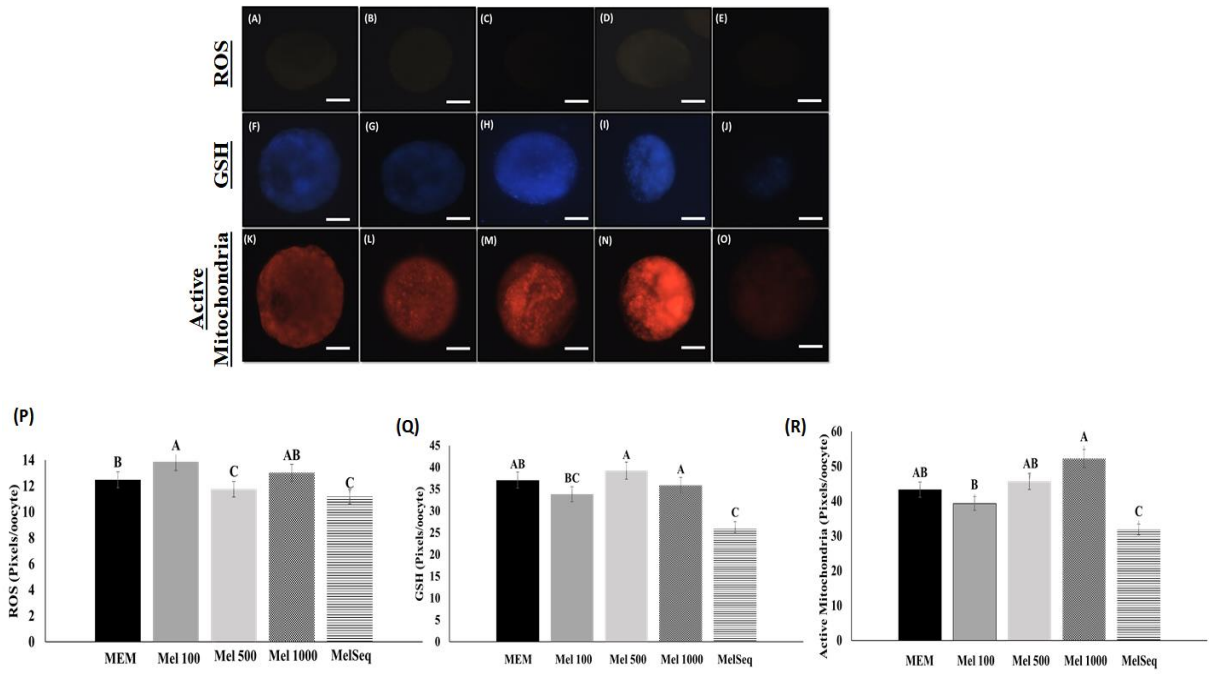

Figure 3. Epifluorescent photomicrographic images of ovine oocytes showing intracellular levels of ROS (A-E), GSH (F-J) and active mitochondria (K–O). Oocytes after culture in α-MEM^{+} (A, F and K) or in control medium containing 100 (B, G and L), 500 (C, H and N) or 1000 pg/mL melatonin (D, I and N) and melatonin sequential (E, J and O). Scale bars: 50 µm (100x). Intracellular levels of ROS (P), GSH (Q) and mitochondrial activity (R) in oocytes after culture of ovine early antral follicles.

[A,B,C] Different letters denote significant differences among treatments (P<0.05).

3.3. Chromatin configuration and levels of ROS and metabolically active mitochondria after IVM

When evaluating chromatin configuration, follicles cultured in Mel 500 treatment (with standard IVM) and those cultured in Mel 500 associated with further IVM in Mel 500 showed a lower percentage of GV oocytes (41.18% and 35.3%, respectively) than control group (α-MEM$^+$: 71.43%; Fig. 4A) (Table 1). In addition, Mel 500 treatment presented a higher percentage (58.82%; Fig 4B) of GVBD oocytes than control group (28.57%) and similar percentages to other treatments (α-MEM$^+$ + Mel 500: 40% and Mel 500 + Mel 500: 52.94%). Interestingly, MI and MII oocytes were obtained only in oocytes that had been cultured in Mel 500 with further IVM in Mel 500 (Table 1; Fig 4C).

Table 1. Meiotic stages (%) of sheep oocytes from *in vitro* grown early antral follicles after 12 days of culture in α-MEM$^+$ or 500 pg/mL melatonin following the IVM in TCM 199 or TCM 199 added of 500 pg/mL of melatonin.

Treatments	No. Oocytes	% GV (n)	% GVBD (n)	% MI (n)	% MII (n)
α-MEM$^+$	14	71. 43%(10/14)A	28.57 % (4/14)B	0% (0/14)A	0% (0/14)A
α-MEM$^+$ + Mel 500	15	60% (9/15)AB	40 % (6/15)AB	0 % (0/15)A	0% (0/15)A
Mel 500	17	41.18% (7/17)B	58.82% (10/17) A	0% (0/17)A	0% (0/17)A
Mel 500 + Mel 500	17	35.3% (6/17)B	52.94% (9/17)AB	5.88% (1/17)A	5.88% (1/17)A

A, B Different letters denote significant differences among treatments (P<0.05).

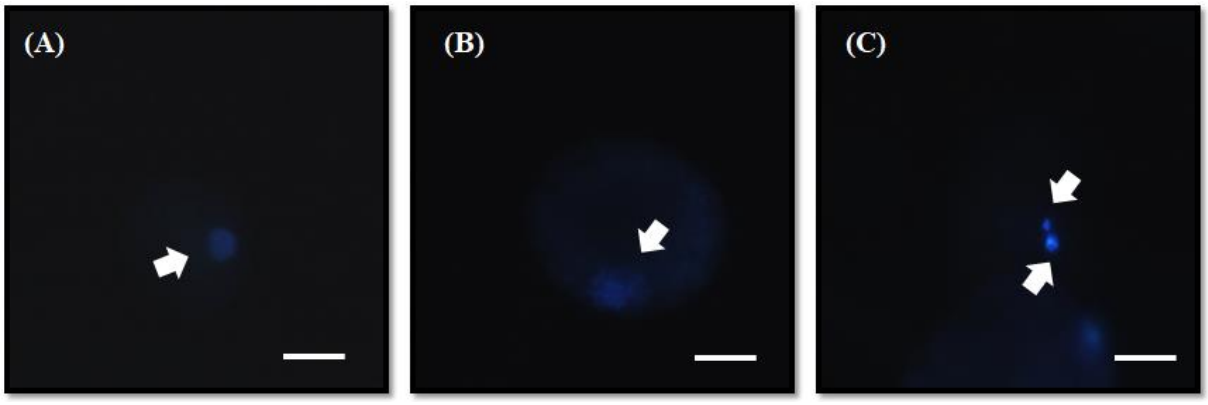

Figure 4. Epifluorescent photomicrographic images of ovine oocytes from *in vitro* cultured early antral follicles stained with Hoeschst 33342 after IVM. Oocytes in GV (A) cultured in α-MEM^{+}; oocytes in GVBD (B) cultured in Mel 500; and oocytes in MII (D) cultured in Mel 500 + Mel 500. Scale bars: 50 µm (100x).

After IVM, oocytes were evaluated for ROS levels and mitochondrial activity. The intracellular levels of ROS were similar among all treatments (data not shown). Oocytes cultured for 12 days in 500 pg/mL melatonin followed by maturation with 500 pg/mL melatonin increased ($P<0.05$) the levels of mitochondrial activity compared to α-MEM^{+} (Fig. 5).

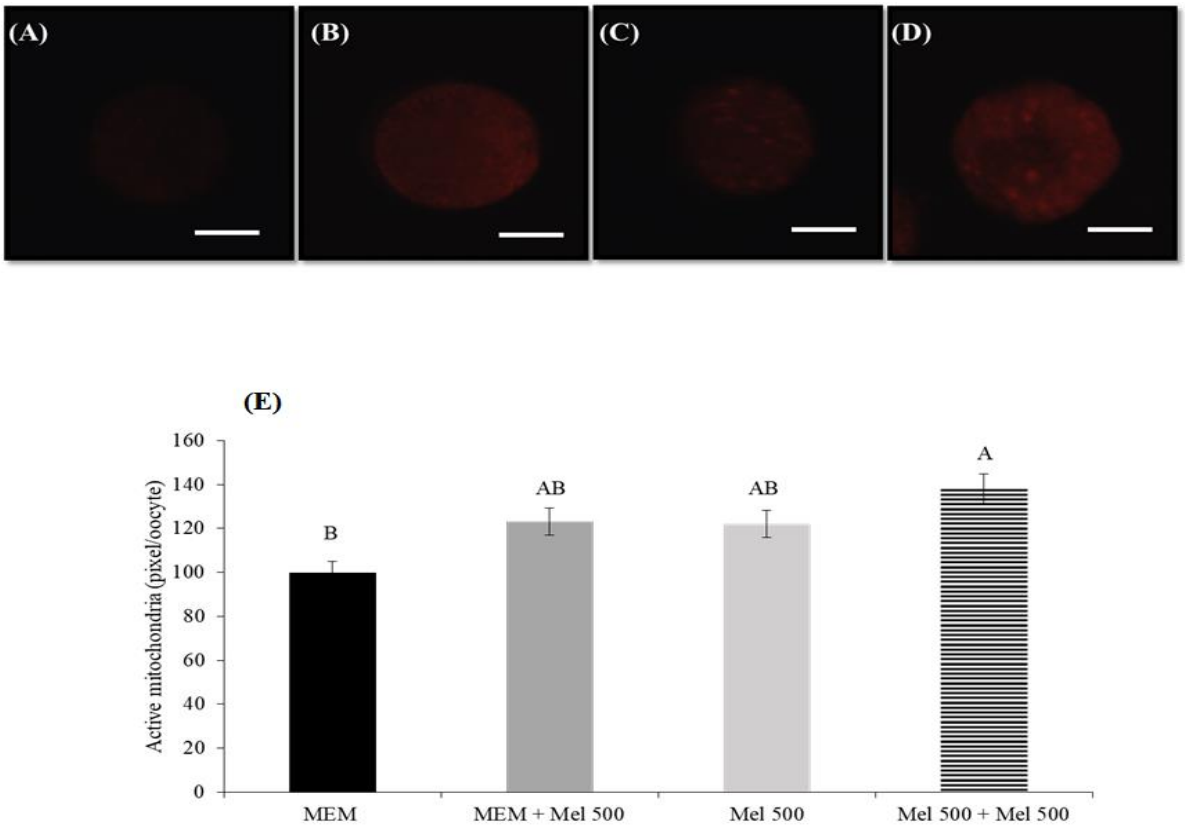

Figure 5. Epifluorescent photomicrographic images of ovine oocytes showing intracellular levels of active mitochondria (A-D). Oocytes after culture and 32 h of maturation in α-MEM^{+} (A), α-MEM^{+} + Mel 500 (B), Mel 500 (C) and (D) Mel 500 + Mel 500 pg/mL melatonin. Scale bars: 75 µm (100x). Intracellular levels of active mitochondria (E) in oocytes after IVM of ovine oocytes.

^{A,B,C} Different letters denote significant differences among treatments ($P<0.05$).

4. Discussion

To our knowledge, this study showed for the first time that melatonin (500 pg/mL) promotes oocyte growth and decreases ROS production after *in vitro* culture of sheep early antral follicles. In addition, supplementation of the IVC medium with melatonin followed by the addition of this hormone to the IVM medium increased mitochondrial activity and improved the meiotic competence of the oocytes. Melatonin is an antioxidant used in culture

media and oocyte maturation to avoid the harmful effects of oxidative stress *in vitro* by reducing ROS levels [11,30,31].

In this study, after 12 days of culture, melatonin at the concentration of 500 pg/mL showed higher rate of fully grown oocytes and reduced ROS production. Previous reports showed that the addition of melatonin to the IVC maintained survival and increased follicular diameter of secondary follicles (caprine: [18]; mouse: [32]), as well as increased the rates of caprine fully grown oocytes [18]. In addition, melatonin is able to scavenge toxic oxygen derivatives and reduce the formation of ROS [33,34,35]. Therefore, in the present study, it is possible that the decrease in ROS production has contributed to protect the follicle against oxidative damage, promoting oocyte growth during IVC in 500 pg/mL melatonin. Other study also indicated that melatonin modulates GSH synthesis [36]; however, in the present study, the fixed concentrations of melatonin did not affect GSH levels in relation to control medium after culture. Similar results were observed after IVM of bovine [37] and murine [38] oocytes. We suggest that the high GSH levels observed in the control medium may represent a compensatory response of the follicular cells in an attempt to protect against the *in vitro* oxidative stress. However, such compensation was not effective as evidenced by a better oocyte development in 500 pg/mL melatonin.

Addition of 500 pg/mL melatonin in both culture of early antral follicles and oocyte maturation media increased the levels of mitochondrial activity compared to α-MEM^{+} and was the only treatment that presented MII oocyte. It is known that mitochondrial activity influences the quality of oocytes and is important for oocyte maturation, fertilization and subsequent embryo development [39,40]. Previous findings have shown that supplementation of the IVM medium with melatonin enhances oocyte maturation in many species (human: [41]; ovine: [42]; caprine: [43]; bovine: [35]; swine: [44]; mouse: [23]), perhaps by improving oocyte quality through increased mitochondrial activity [45,46] as observed in our study. The

fact that only one MII oocyte were obtained is a clear indication that an optimization of the IVM conditions may be required to achieve high developmental competence, as, for example, increase of the culture period and use of additional energy substrates in the IVM medium.

In conclusion, the concentration of 500 pg/mL of melatonin promotes oocyte growth and decreases ROS levels after *in vitro* culture of sheep early antral follicles and promotes the meiotic competence of oocytes. This provides an encouraging step toward increasing the number of *in vitro* mature oocytes from early antral follicles. Thus, new studies focusing on the action of melatonin in IVC and IVM of ovine oocytes from *in vitro* grown antral follicles are important to improve embryo production.

Conflicts of interest

The authors declare no conflicts of interests.

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5. Considerações finais

- Este trabalho demonstrou a importância do FSH e da melatonina sobre o desenvolvimento *in vitro* de folículos ovarianos ovinos.
- Após os resultados obtidos, pode-se inferir que utilizando um meio definido, o FSH na concentração de 750 ng/mL mantém a sobrevivência folicular e promove o aumento dos níveis de mitocôndrias ativas, do crescimento e melhora a maturação oocitária.
- Além disso, foi possível demonstrar que a melatonina no cultivo de folículos pré-antrais e antrais iniciais pode aumentar os níveis energéticos dos oócitos, reduzir a produção de espécies reativas de oxigênio e favorecer a obtenção de oócitos com tamanho apropriado para a maturação *in vitro*.

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Anexos

1) Confirmação de submissão

Manuscrito: "*In vitro* survival, growth and maturation of sheep oocytes from secondary follicles cultured in serum-free conditions: impact of a constant or a sequential medium containing recombinant human FSH"

From: Domestic Animal Endocrinology <eesserver@eesmail.elsevier.com>
To: "helena.matos@univasf.edu.br" <helena.matos@univasf.edu.br>; "htmatos@yahoo.com" <htmatos@yahoo.com>

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2) Confirmação de submissão

Manuscrito: Melatonin improves development, mitochondrial function and promotes the meiotic resumption of sheep oocytes from *in vitro* grown secondary follicles.

01-Jul-2018

Dear Dr Matos

Your manuscript entitled 'Melatonin improves development, mitochondrial function and promotes the meiotic resumption of sheep oocytes from *in vitro* grown secondary follicles' has been successfully submitted online and will be given full consideration for publication in Reproduction, Fertility and Development.

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3) Confirmação de submissão

Manuscrito: Melatonin reduces ROS production and promotes the meiotic resumption of sheep oocytes from *in vitro* grown early antral follicles

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