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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

EFEITO DO FATOR DE CRESCIMENTO SEMELHANTE À INSULINA-I (IGF-I) E DO FATOR DE CRESCIMENTO E DIFERENCIAÇÃO-9 (GDF-9) SOBRE O DESENVOLVIMENTO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS OVINOS

ALANE PAINS OLIVEIRA DO MONTE

Petrolina – 2018

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade Federal Rural de Pernambuco, como requisito para defesa de tese.

Orientadora: Profa. Dra. Maria Helena Tavares de Matos

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Aos meus pais, irmãos, minha filha, meu marido, e todas as pessoas que me amam e valorizam o meu sorriso, Dedico.

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RESUMO

O objetivo deste trabalho foi estudar o efeito do fator de crescimento semelhante à insulina-I (IGF-I) e do fator de crescimento e diferenciação-9 (GDF-9) sobre o desenvolvimento *in vitro* de folículos pré-antrais ovinos. Para isto, foram desenvolvidas três fases experimentais. A primeira fase avaliou o efeito do IGF-I sobre o cultivo *in vitro* de folículos secundários isolados de ovelhas. Parte dos ovários foram destinados à imunolocalização da proteína para o IGF-I. Os folículos secundários isolados dos demais ovários foram cultivados por 18 dias no meio controle (MEM^+) ou no MEM^+ associado de diferentes concentrações de IGF-I (10, 50 e 100 ng/mL), ou nos mesmos tratamentos adicionados de 750 ng/mL de FSH. Ao final do cultivo, foram realizadas análises de fluorescência, *maturação in vitro* (MIV) ou expressão do receptor de LH (LHR). A fase 2 avaliou a influência do GDF-9 no desenvolvimento de folículos pré-antrais cultivados *in situ*. Para isso, fragmentos ovarianos foram cultivados por 7 dias em MEM ou em MEM adicionado de GDF-9 (1, 50, 100, 200 ou 400 ng/mL). Além disso, foi realizado um segundo cultivo em MEM ou em MEM + 50 ng/mL de GDF-9, na presença ou ausência do inbidor da via PI3K. Ao final, os fragmentos foram avaliados quanto à sobrevivência, ativação, diâmetro e imunolocalização das proteínas PCNA, caspase-3 ativada e pAKT. Na fase três, folículos secundários isolados foram cultivados por 12 dias em MEM ou MEM + GDF-9 (1, 10, 50 ou 100 ng/mL). Ao final do cultivo, os oócitos foram submetidos à MIV e, posteriormente, à análise de atividade mitocondrial e ERO. Os resultados da fase 1 mostraram que 50 ng/mL de IGF-I + FSH apresentou mais oócitos totalmente crescidos ($P<0,05$). Os níveis de ERO foram maiores em 100 ng/mL de IGF-I + FSH comparado aos outros grupos, com concomitante diminuição do nível de GSH e atividade mitocondrial ($P<0,05$). Na fase 2, a sobrevivência folicular foi maior no tratamento 50 ng/mL de GFD-9 ($P<0,05$) comparado aos demais tratamentos (exceto 1 ng/mL), promoveu maior proliferação celular e reduziu a apoptose quando comparado ao controle, o que ocorreu através da via PI3K/AKT. Na fase 3, os folículos cultivados em 100 ng/mL de GDF-9 apresentaram maior percentagem de oócitos totalmente crescidos, MI e maior atividade mitocondrial comparado aos demais grupos ($P<0,05$). Em conclusão, este estudo demonstrou a presença da proteína para o IGF-I em ovários ovinos. Além disso, 50 ng/mL de IGF-I + FSH promove o desenvolvimento de folículos e de oócitos meioticamente competentes. A concentração de 50 ng/mL de GDF-9 mantém a sobrevivência de folículos primordiais cultivados *in situ,* aumenta a proliferação celular e diminui a apoptose através da via PI3K/AKT. Em folículos secundários isolados, o GDF-9 aumenta a percentagem de oócitos totalmente crescidos, aptos a retomar a meiose, e aumenta a atividade mitocondrial após a MIV.

Palavras-chaves: GDF-9, folículos pré-antrais, IGF-I, ovinos

ABSTRACT

The objective of this work was to study the effect of the insulin like growth factor-1(IGF-1) and growth differentiation factor-9 (GDF-9) on the *in vitro* development of sheep preantral follicles. For this, three experimental phases were developed. The first phase evaluated the effect of IGF-I on the *in vitro* culture of sheep isolated secondary follicles. Sixteen ovaries were allocated for the immunolocalization of IGF-I protein. Isolated secondary follicles of other ovaries were cultured for 18 days in control medium (MEM^+) or MEM^+ supplemented with different concentrations of IGF-I (10, 50 and 100 ng/mL) or the same treatments with the addition of 750 ng/ml of FSH. At the end of the culture, fluorescence, *in vitro* maturation (IVM) or LH receptor (LHR) expression analyzes were performed. Phase 2 evaluated the influence of GDF-9 on the development of preantral follicles cultured *in situ*. For this, ovarian fragments were cultured for 7 days in MEM or in MEM supplemented with of GDF-9 (1, 50, 100, 200 or 400 ng/mL). In addition, a second culture in MEM or MEM + 50 ng/mL of GDF-9 was performed in the presence or absence of the PI3K pathway inhibitor. At the end of culture, follicular survival, activation and diameter, immunolocalization of PCNA, activated caspase-3 and pAKT proteins were evaluated. In the phase three, isolated secondary follicles were cultured for 12 days in MEM or MEM + GDF-9 (1, 10, 50 or 100 ng/mL). At the end of culture, oocytes were submitted to IVM, follow by mitochondrial activity and ROS analysis. The results of phase 1 showed that 50 ng/mL of IGF-I + FSH showed more fully-grown oocytes ($P < 0.05$). ROS levels were higher in 100 ng/mL IGF-I + FSH compared to the other groups, with a concomitant decrease of GSH level and mitochondrial activity ($P < 0.05$). In the second phase, follicular survival was higher with the concentration of 50 ng/ml of GFD-9 ($P < 0.05$) compared to other treatments (except 1 ng/mL) concomitant with a greater proliferation and lower apoptosis compared to the control, through of PI3K/AKT pathway. In the last phase, the follicles cultured in 100 ng/ml GDF-9 showed a higher percentage of fully-grown oocytes, MI and higher mitochondrial activity compared to other groups ($P < 0.05$). In conclusion, this study demonstrated the presence of IGF-I protein in sheep ovary. In addition, 50 ng/mL of IGF-I + FSH promotes follicular development and meiotically competent oocytes. The concentration of 50 ng/ml of GDF-9 maintains the survival of primordial follicles cultured in situ, increases cell proliferation and decreases apoptosis via the PI3K/AKT pathway. In isolated secondary follicles, GDF-9 increases the percentage of fully-grown oocytes able to resume meiosis, and enhances mitochondrial activity after IVM.

Key words: GDF-9, preantral follicles, IGF-I, sheep

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LISTA DE ABREVIAÇÕES

A: Antro

AKT: Proteína quinase B

ALK5: Receptor de ativina tipo quinase 5

APAF-1: Protease associada à apoptose-1

ATP: Adenosina tri-fosfato

BMP-15: Proteína morfogenética óssea-15

BMPR: Receptor de proteína morfogenética óssea

BSA: Albumina sérica bovina

Ca^{+}: Íon de Cálcio

CCO ou COC: Complexo cumulus-oócito

CGP: Células germinativas primárias

CO_2: Dióxido de Carbono

DAB: Diaminobenzidina

DNA: Ácido desoxorribonucleico

EGF: Fator de crescimento epidermal

ERO ou ROS: Espécie reativa de oxigênio

FGF-2: Fator de crescimento fibroblástico-2

FOXO: Forkhead transcription factors

FSH: Hormônio folículo estimulante

GC: Células da granulosa

GDF-9: Fator de crescimento e diferenciação-9

GH: Hormônio do crescimento

GSH: Glutationa

GV: vesícula germinativa

GVBD: quebra da vesícula germinativa

IGF-I: Fator de crescimento semelhante à insulina-I

IGFBP: Proteína de ligação do fator de crescimento semelhante à insulina

KL: Kit ligante

LH: Hormônio luteinizante

LHR: Receptor do hormônio luteinizante

MI: Metáfase I

MII: Metáfase II

MAPK: proteína ativadora mitogênica quinase

MEM: meio essencial mínimo

MIV ou IMV: Maturação in vitro

mTOR: via da rapamicina mamífera

Na^{+}: Íon de sódio

O: Oócito

pAKT: Proteína quinase B fosforilada

PBS: Solução fosfato salina tamponada

PCNA: Antígeno nuclear de proliferação celular

PI3K: Fosfatidil inositol 3 quinase

PIP-2: Fosfatidil inositol-bi-fosfato

PIP-3: Fosfatidil inositol-tri-fosfato

RNAm: Ácido ribonucleico

T_4: Tiroxina

TC: Células da teca

TCM-199: Meio de cultivo de tecido

TGF-β: Fator de crescimento transformador-β

TNF: Fator de necrose tumoral

VEGF: Fator de crescimento endotelial vascular

SUMÁRIO

1. INTRODUÇÃO

O cultivo *in vitro* de folículos ovarianos pré-antrais é uma ferramenta de estudo eficiente para a elucidação de fatores que controlam o processo de foliculogênese. Esses estudos podem contribuir de forma efetiva no desenvolvimento de alternativas para diagnóstico e, consequentemente, prevenção da falha ovariana prematura em mulheres, para aumentar a eficiência de outras biotecnologias reprodutivas, ou ainda, para estudar as substâncias e as vias metabólicas que são ativadas em cada fase de desenvolvimento folicular (GINTHER et al., 2011; GASTAL et al., 2011, BAERWALD, 2009; HSUEH et al., 2015).

Apesar do progresso alcançado até o momento no cultivo de folículos pré-antrais, as taxas de maturação e de produção *in vitro* de embriões a partir desses folículos em ovinos permanecem insatisfatórias (ARUNAKUMARI et al., 2010). Nesse sentido, o desenvolvimento de um sistema de cultivo *in vitro* eficiente que promova o total crescimento folicular e a produção de oócitos competentes para posterior fecundação e produção de embriões é necessário (FIGUEIREDO et al., 2008). Diversos estudos *in vitro* demonstram que o desenvolvimento folicular é influenciado por vários fatores, como por exemplo, o sistema de cultivo (ARAÚJO et al., 2014, 2015; PESSOA et al., 2014), o meio básico (ROSSETTO et al., 2013; CASTRO et al., 2014) e a suplementação adicionada a este meio (FERREIRA et al., 2016).

O cultivo de folículos pré-antrais inclusos em tecido ovariano (cultivo *in situ*) permite que um grande número de folículos primordiais saia do *pool* de reserva do ovário e inicie seu crescimento *in vitro*, processo denominado de ativação. Desta forma, uma das vantagens deste tipo de cultivo é permitir um melhor entendimento dos fatores que influenciam a ativação folicular (YANG et al., 2007; TANG et al., 2012). Já o cultivo de folículos secundários isolados apresenta melhores resultados quanto às taxas de recuperação de oócitos competentes e posterior maturação *in vitro* (ARAÚJO et al., 2014).

Quanto à composição do meio de cultivo, este deve garantir o fornecimento adequado de eletrólitos, antioxidantes, aminoácidos, substratos energéticos e vitaminas (PICTON et al., 2008), além de hormônios e/ou fatores de crescimento (ARAÚJO et al., 2014), como por exemplo, o fator de crescimento semelhante à insulina-I (IGF-I). Na espécie ovina, o RNAm do IGF-I foi expresso nas células da granulosa e teca de folículos antrais iniciais (LEEUWENBERG et al., 1995) e também em oócitos e células da granulosa de folículos pré-antrais (BONNET et al., 2013).Estudos *in vitro* mostraram que o IGF-I estimulou o crescimento folicular em ratas (ZHAO et al., 2001), bovinos (GUTIERREZ et al., 2000; JIMENES et al., 2016) e humanos (LOUHIO et al., 2000),

manteve a sobrevivência folicular e promoveu a ativação de folículos primordiais caprinos (MARTINS et al., 2010), e aumentou o desenvolvimento e a produção de oócitos capazes de retomar a meiose em folículos caprinos secundários cultivados *in vitro*. Em ovinos, a associação do IGF-I e Hormônio Folículo Estimulante (FSH) ao Hormônio do Crescimento (GH) e Tiroxina (T4) promoveram o desenvolvimento de embriões a partir de folículos preantrais cultivados *in vitro* até o estádio de mórula (ARUNAKUMARI et al., 2010).

Além do IGF-I, pode-se destacar o fator de crescimento e diferenciação (GDF-9), que já promoveu o crescimento de folículos primários e a proliferação de células da teca de ratas (NILSON e SKINNER, 2002), ativação e crescimento até folículos secundários após cultivo *in situ* (MARTINS et al., 2008), além de crescimento folicular e produção de oócitos competentes a retomar a meiose (ALMEIDA et al., 2011) em caprinos. O GDF-9 também induziu a expansão das células do cúmulus e promoveu a maturação oocitária em folículos de camundongos (ELVIN et al., 1999). Em associação com o FSH, o GDF-9 estimulou o crescimento e a diferenciação de folículos pré-antrais de ratas (HAYASHI et al., 1999).

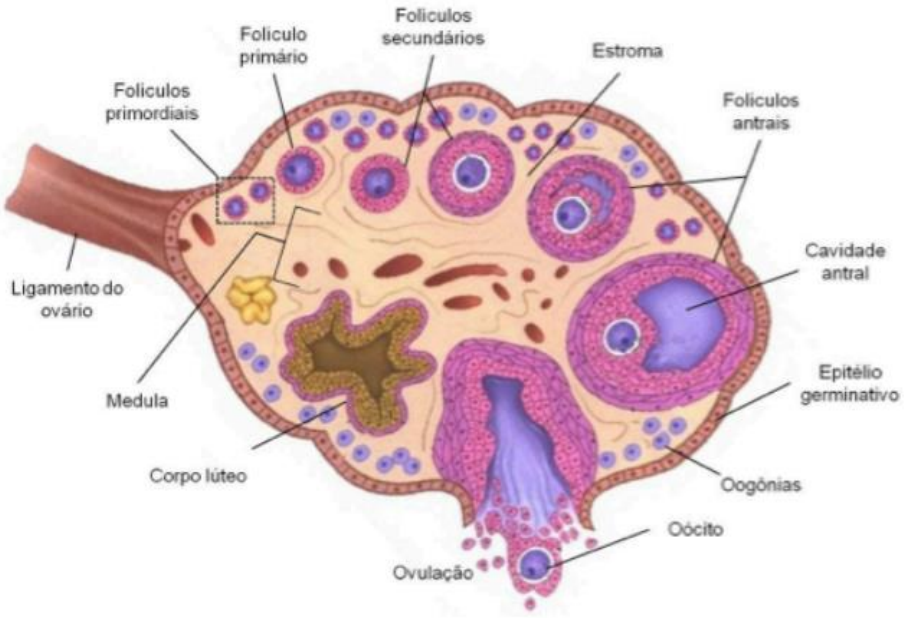
Embora muitos estudos já tenham sido realizados, os efeitos de diferentes concentrações de IGF-I e GDF-9 não foram avaliados no cultivo *in vitro* de folículos pré-antrais inclusos em tecido ovariano ou isolados na espécie ovina. Dessa forma, faz-se necessário a realização de mais estudos para explorar a função desses fatores e os possíveis efeitos sobre a foliculogênese em ovinos.

2. REVISÃO DE LITERATURA

2.1. Ovário

O ovário mamífero além de conter o suprimento de células germinativas para a produção da próxima geração, é também uma glândula reprodutiva que controla diversos aspectos fisiológicos e do desenvolvimento das fêmeas (EDSON et al., 2009; JAGARLAMUDI e RAJKOVIC, 2012). Este órgão é dividido em duas regiões bem delimitadas: cortical e medular. O córtex, localizado mais externamente e circundado pelo epitélio germinativo, é a região funcional do órgão, formado por tecido conjuntivo (fibroblastos, colágeno e fibras reticulares), folículos ovarianos e corpos lúteos em vários estágios de desenvolvimento ou em regressão, corpos hemorrágicos e corpo albicans (LIU et al., 2006). A região medular é localizada mais internamente e é constituída por tecido conjuntivo fibroelástico, células musculares lisas, nervos, vasos sanguíneos e linfáticos, responsáveis pela nutrição e estruturação do ovário (HAFEZ e HAFEZ, 2004) (Figura 1). Na espécie equina, há uma inversão corticomedular e o ovário apresenta uma depressão denominada fossa ovulatória onde o oócito é liberado (HAFEZ e HAFEZ, 2004), ou seja, o córtex é a região mais interna do ovário.

Figura 1. Desenho esquemático do ovário mamífero, mostrando a região do córtex ovariano (mais externa) com folículos em vários estágios de desenvolvimento, ovulação e corpo lúteo; e região medular (mais interna) com presença de vasos sanguíneos (ROCHA et al., 2017).



Esta gônada desempenha função exócrina que consiste no desenvolvimento, maturação, e liberação de um oócito maduro para a fecundação, e endócrina, sendo responsável pela síntese e secreção de fatores e hormônios que são essenciais para o desenvolvimento folicular, ciclo estral e manutenção da função do trato reprodutivo (GOUGEON et al., 2004). Essas duas funções (exócrina e endócrina) são exercidas sincronicamente, resultando em dois fenômenos que ocorrem no ovário, a oogênese e a foliculogênese (HAFEZ e HAFEZ, 2004).

2.2 Oogênese

Nas fêmeas mamíferas, um complexo mecanismo intercelular regula a oogênese (MATZUK et al., 2002), processo que engloba desde a formação e diferenciação das células germinativas primordiais (CGP) até a formação do oócito haploide fecundado (BRISTOL-GOULD e WOODRUFF, 2006).

Em ruminantes, as CGP oriundas do endoderma do saco vitelínico, migram até a região das gônadas primitivas e após um processo de crescimento celular e redistribuição de organelas citoplasmáticas, as CGP multiplicam-se ativamente e transformam-se em oogônias, as quais possuem alta atividade mitótica. Em seguida, as oogônias sofrem mitose e se transformam em oócitos primários (PAN et al., 2012; JAGARLAMUDI e RAJKOVIC, 2012), os quais iniciam a primeira divisão meiótica, passando pelo estádio de prófase I (VAN DEN HURK e ZHAO, 2005), em que ocorre a primeira interrupção da divisão meiótica no estádio diplóteno I ou vesícula germinativa da prófase I. Os oócitos permanecem neste estádio até serem recrutados ao crescimento, o que pode ocorrer por estímulo do pico do FSH e do hormônio luteinizante (LH) durante a puberdade. Então, os oócitos retomam a meiose e o núcleo prossegue a divisão meiótica até a ocorrência do pico pré-ovulatório de LH, formação dos oócitos secundários e outra interrupção da meiose na fase de metáfase II (FIGUEIREDO et al., 2008). A meiose será retomada somente após a fecundação do oócito pelo espermatozoide, quando finalmente, o oócito completa a meiose e expulsa o segundo corpúsculo polar, dando origem ao oócito haploide fecundado, concluindo o desenvolvimento oocitário (FIGUEIREDO et al., 2008).

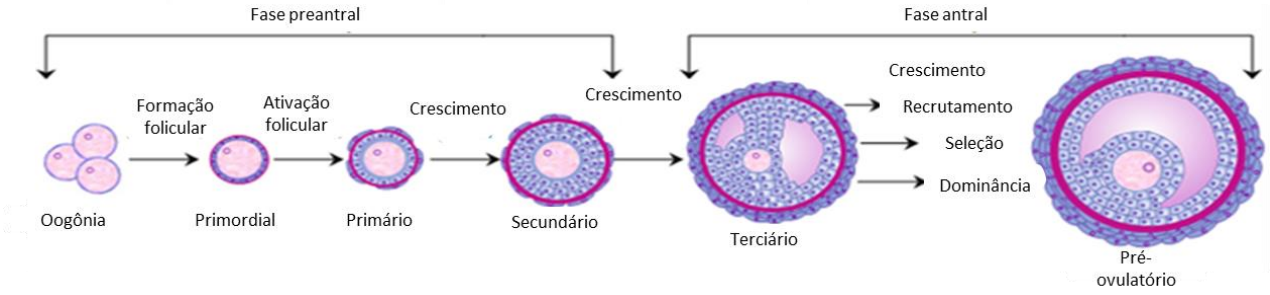
Logo no início da oogênese, os oócitos primários são circundados por uma camada de células somáticas pavimentosas, conhecidas como células da pré-granulosa, formando os folículos primordiais (PÉPIN et al., 2007), dando início ao processo denominado de foliculogênese (SILVA et al., 2009).

2.3. Foliculogênese

A foliculogênese compreende o processo de formação, crescimento e maturação dos folículos ovarianos. Esse processo se inicia ainda na vida pré-natal, na maioria das espécies, primeiramente com a formação do folículo primordial, que se desenvolve para o *pool* de folículos em crescimento, e culmina na formação do folículo pré-ovulatório ou de De Graaf (VAN DEN HURK e ZHAO, 2005) e consequente ovulação ou morte folicular (atresia) (WILLIAMS e ERICKSON, 2012).

O folículo é considerado a unidade morfofuncional do ovário, sendo formado por um oócito circundado por células foliculares e demarcado por uma membrana basal que o separa do estroma ovariano. Sua função é proporcionar um ambiente ideal para o desenvolvimento e a maturação do oócito, bem como para produção de hormônios e peptídeos que regulam o crescimento folicular (FIGUEIREDO et al., 2008). Os folículos ovarianos podem ser classificados de acordo com o grau de evolução em: 1) folículos pré-antrais, que incluem os folículos primordiais, transição, primários e secundários e 2) folículos antrais, compreendendo os folículos terciários e De Graaf ou pré-ovulatório (SILVA et al., 2004).

Figura 2: Desenho esquemático das fases de desenvolvimento folicular (ARAÚJO et al., 2014).



Os folículos primordiais permanecem em quiescência por longos períodos até serem recrutados a retomar seu desenvolvimento (PAN et al., 2012). A ativação ou início do crescimento folicular consiste na mudança na morfologia das células da granulosa de pavimentosas para cúbicas, formando o folículo de transição (folículo que contém células com morfologia pavimentosa e cúbica) (SILVA et al., 2004), e em seguida, o folículo primário (oócito circundado por camada única de células cúbicas) (BRAW-TAL e YOSSEFI, 1997). A ativação de folículos primordiais inclui ainda a retomada da proliferação das células da granulosa (VAN DEN HURK et al., 1997) e o crescimento do oócito, acompanhado por intensa síntese de RNA (VAN DEN HURK e ZHAO, 2005). No entanto, os fatores e mecanismos responsáveis pela ativação de folículos primordiais,

bem como as vias envolvidas no início do crescimento folicular, são ainda pouco conhecidos e representam uma das maiores lacunas relacionadas com a biologia ovariana.

Alguns autores têm sugerido que fatores endócrinos e parácrinos podem influenciar a ativação de folículos primordiais (HIRSHFIELD, 1991; NAYUDU et al., 2001), tais como kit-ligand (KL) (CAVALCANTE et al., 2016), fator de crescimento epidermal (EGF) (SANTOS et al., 2017), IGF-I (BEZERRA et al., 2018), GDF-9 (MARTINS et al., 2008), fator de crescimento endotelial vascular (VEGF) (ARAÚJO et al., 2011), fator de crescimento fibroblástico-2 (FGF-2) (SANTOS et al., 2014), hormônio do crescimento (GH), (MAGALHÃES et al., 2011) e a proteína morfogenética-15 BMP-15 (CELESTINO et al., 2011). Nesta etapa de crescimento, uma rede de juncões do tipo gap, que são canais intercelulares da membrana, começam a aparecer na camada granulosa, o que permite que nutrientes, íons inorgânicos, segundos mensageiros e pequenos metabólitos passem de célula para célula (GOUGEON, 2010).

A multiplicação das células da granulosa dos folículos primários leva ao aumento do número de camadas destas células ao redor do oócito, formando os folículos secundários (PAN et al., 2012). Simultaneamente com a divisão das células da granulosa, há secreção de outra substância de delimitação, a zona pelúcida, que se localiza entre as células da granulosa e o oócito. Além disso, a camada da teca é formada ao redor da membrana própria para completar as camadas do folículo (FIGUEIREDO et al., 2008).

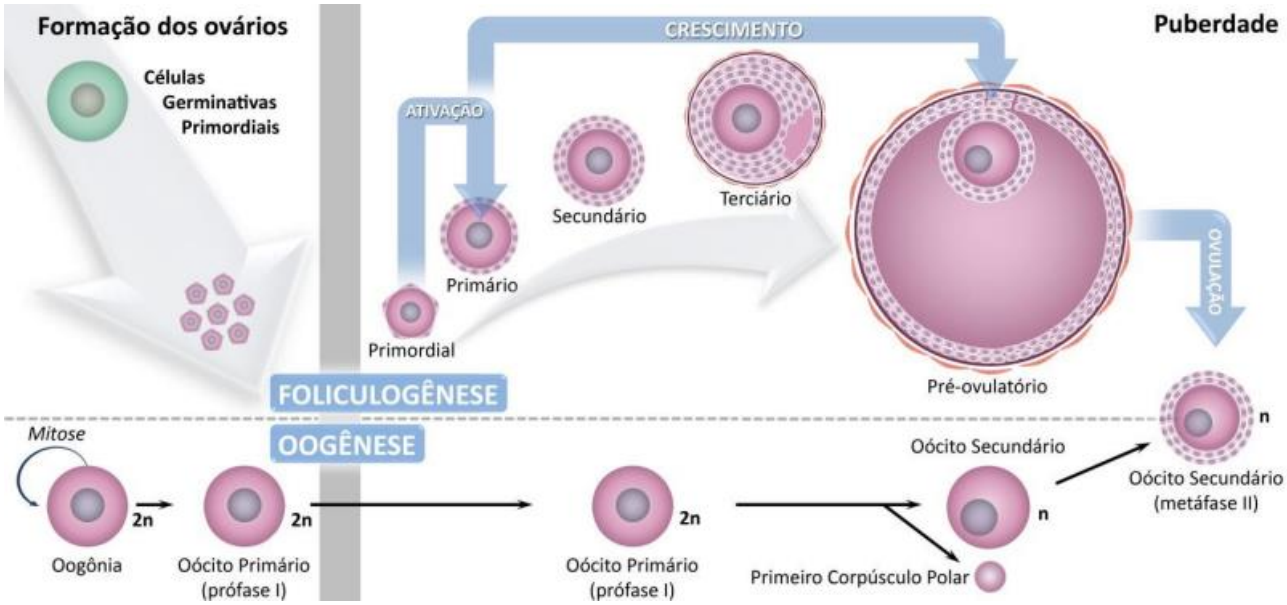
Com o crescimento dos folículos secundários e multiplicação das camadas de células da granulosa, forma-se uma cavidade repleta de líquido folicular, denominada antro, o que caracteriza os folículos antrais (FORTUNE, 2003). Diversos fatores e hormônios contribuem com o crescimento de folículos secundários *in vitro* em ovinos, como por exemplo, a insulina (10 ng/mL) (ARUNAKUMARI et al. 2010) e o IGF-I estimularam o crescimento folicular em humanos (LOUHIO et al., 2000), bovinos (GUTIERREZ et al., 2000), caprinos (MAGALHÃES-PADILHA et al., 2012), ratas (ZHAO et al., 2001) e camundongos (LIU et al., 1998) em sinergia com o FSH.

O diâmetro dos folículos antrais aumenta consideravelmente devido ao crescimento do oócito, que está intimamente relacionado com a proliferação de células da granulosa e vice-versa, multiplicação das camadas das células da granulosa e da teca (SANCHEZ, SMITZ, 2012), aumento da cavidade antral e da vascularização da camada da teca (SANCHEZ et al., 2010). Este aumento de diâmetro e volume é também resultado do acúmulo de água, íons, carboidratos, lipídios, proteínas e RNAm (FAIR et al., 1997).

O fluido antral torna-se uma importante fonte de substâncias reguladoras das células foliculares, por exemplo, gonadotrofinas, esteroides, fatores de crescimento, enzimas, proteoglicanos e lipoproteínas (WU et al., 2007). A formação do antro promove a diferenciação das células da granulosa em dois tipos celulares: células murais, que se encontram em contato com a membrana basal e apresentam função endócrina; e as células do cumulus, que estão em contato com o oócito, colaborando com seu metabolismo e maturação. As células do cumulus são a principal fonte de energia do oócito (MUNAKATA et al., 2016; ITAMI et al., 2017). O conjunto oócito e células do cumulus, forma o complexo cumulus-oócito (CCO) (COLLADO-FERNANDEZ; et al., 2012).

Nessa etapa, a maioria dos folículos entram em atresia e apenas um número reduzido continua crescendo até alcançar o estágio pré-ovulatório ou folículo de Graaf (MCGEE e HSUEH, 2000). Este é o ultimo estádio de desenvolvimento e caracteriza-se por conter uma grande proporção de fluido e possivelmente conter um oócito secundário (ARAÚJO et al., 2014).

Figura 3. Desenho esquemático representando a oogênese e foliculogênese (ROSSETTO, 2013).



Durante todo o crescimento folicular, o diâmetro oocitário aumenta significativamente, passando de aproximadamente 30 µm na fase primordial a mais de 120 µm na fase antral (BEZERRA et al., 1998; MCNATTY et al., 2000). No entanto, o núcleo continua em estádio de

prófase I e só retoma a meiose após o pico pré-ovulatório de LH, quando são os oócitos considerados maduros. Após a maturação, o oócito permanece no estádio de metáfase II até o momento da fecundação (PAN et al., 2012).

2.4. População e atresia folicular

A população folicular é formada ainda na vida fetal em ruminantes e primatas (BETTERIDGE et al., 1989) ou logo após o nascimento em roedores (HIRSHFIELD, 1991). Esta população difere entre as espécies e indivíduos (KATSKA-KSIAZKIEWICZ, 2006), sendo de aproximadamente 160.000 na ovelha (DRIANCOURT et al., 1991), 35.000 na cabra (LUCCI et al., 1999), 235.000 em vacas (BETTERIDGE et al., 1989), 1.500 no camundongo fêmea (SHAW et al., 2000), e aproximadamente 2.000.000 na mulher (ERICKSON, 1986). No entanto, a grande maioria dos folículos é eliminada durante a vida reprodutiva (MATSUDA et al, 2012). Desta forma, apesar deste grande número de folículos presentes no ovário, cerca de 99,9%, não chega à ovulação, mas morrem por um processo fisiológico denominado atresia. Embora esse processo leve à perda de muitos folículos, este evento é crucial para a manutenção da homeostase dos ovários de mamíferos, assegurando a ciclicidade reprodutiva (AMSTERDAM et al., 2003; FIGUEIREDO et al., 2007). Os processos de morte celular podem ser classificados de acordo com suas características morfológicas e bioquímicas em: apoptose, autofagia, necrose e necroptose (GRIVICICH et al., 2007).

A apoptose depende do equilíbrio na expressão de genes pró e anti-apoptóticos e tem como principal característica culminar na fragmentação do DNA a cada 180 a 200 pares de bases (HUSSEIN, 2005). Este processo pode ser reconhecido por características morfológicas marcantes e coordenadas: ocorre retração da célula, que causa perda da aderência com a matriz extracelular e células vizinhas; manutenção da morfologia das organelas celulares, com exceção das mitocôndrias, que podem apresentar ruptura da membrana externa; condensação da cromatina que se concentra junto à membrana nuclear, a qual se mantém intacta; a membrana celular forma prolongamentos (blebs) e o núcleo se desintegra em fragmentos envoltos pela membrana nuclear; os prolongamentos da membrana celular aumentam de número e tamanho e rompem, originando estruturas contendo o conteúdo celular. Estas porções celulares envoltas pela membrana celular são denominadas corpos apoptóticos, que por sua vez são rapidamente fagocitados por macrófagos e removidos sem causar um processo inflamatório (GRIVICICH et al, 2007).

As caspases são responsáveis por promover a apoptose pela clivagem de substratos que ativam uma cascata de reações. Estas culminam com a condensação, fragmentação nuclear e

externalização de fosfolipídios de membrana, eventos que marcam estas células para serem fagocitadas por macrófagos (NICHOLSON e THORNBERRY, 1997; BOATRIGHT e SALVESEN, 2003). Existem 14 caspases conhecidas e destas, seis (caspases -3, -6, -7, - 8, -9, -10) participam da apoptose e as demais estão envolvidas na maturação de citocinas e não se conhece a contribuição na apoptose (DENAULT e SALVESEN, 2002). As caspases podem ser consideradas iniciadoras (caspase-8, caspase-9) quando clivam pro-formas inativas de caspases, caracterizadas por possuírem pró-domínios longos, envolvidas na iniciação da cascata proteolítica ou caspases efetoras, (caspase-3, caspase-7) que clivam outros substratos proteicos da célula resultando no processo apoptótico, estas caspases apresentam pró-domínios curtos ou inexistentes e são responsáveis pela clivagem de substratos que executam a apoptose. A iniciação da reação em cascata é regulada por inibidores de caspases (RUPNARAIN et al., 2004). Entre os diversos substratos que interagem com as caspases, pode-se citar a mdm-2 (murine double minute), essa proteína se liga à proteína p53, mantendo-a no citoplasma. Ao ser clivada pelas caspases, essa proteína libera a p53, que se transloca para o núcleo, ativando a transcrição de genes pró-apoptóticos como o Bax (SCHULER et al, 2003).

A ativação da apoptose pode ser iniciada por duas diferentes vias: pela via intrínseca (mitocondrial) ou pela via extrínseca (citoplasmática) (GRIVICICH et al., 2007; JOHSTONE et al., 2002). Dentre os fatores extrínsecos, destacam-se o estresse oxidativo, irradiação, ativação de genes pro-apoptóticos, citocinas, proteínas virais e a deficiência de fatores de sobrevivência da célula (JOHNSON, 2003). A via extrínseca é desencadeada pela ligação de ligantes específicos a um grupo de receptores de membrana da superfamília dos receptores do fator de necrose tumoral (TNF). Esta ligação é capaz de ativar a cascata das caspases (BUDIHARDJO et al., 1999), que executará a apoptose.

A via intrínseca é ativada por estresse intra ou extracelular, como a ausência de fatores de crescimento, danos ao DNA, hipóxia ou ativação de oncogenes (HENGARTNER, 2000). Estudos sobre apoptose mostraram que a mitocôndria é o principal mediador desse tipo de morte. Essa organela integra os estímulos de morte celular, induzindo a permeabilização mitocondrial e conseqüente liberação de moléculas pró-apoptóticas (DESAGHER; MARTINOU, 2000). Os diferentes sinais indutores de apoptose são detectados pela mitocôndria, fazendo com que ocorra um desacoplamento da cadeia respiratória e consequente liberação de citocromo c e proteínas ativadoras da apoptose no citosol (GUPTA, 2003). No citoplasma, o citocromo c forma um complexo com o fator de ativação de protease associada à apoptose 1 (APAF-1) e a caspase-9, denominado apoptossomo, que por sua vez promove a clivagem da pró-caspase-9, liberando a

caspase-9 ativa (BUDIHARDJO et al., 1999), que é precursora da caspase-3, ativando a caspase-3, que vai executar a apoptose (RUPNARAIN et al., 2004; PETROS et al., 2004).

Já o processo degenerativo de necrose ocorre devido a estímulos tóxicos, isquêmicos, degenerativos e imunológicos. Tais fatores também podem induzir a apoptose. Geralmente, a necrose é iniciada por mecanismos não celulares como isquemia, deficiência dos níveis de ATP (BHATIA, 2004), trauma, levando a danos irreversíveis na célula (McCULLY et al., 2004). Alguns trabalhos têm sugerido que mecanismos "ativos" como uma sobrecarga de Na^{+}, acúmulo de Ca^{+2} e mudanças na permeabilidade da mitocôndria podem também participar e levar ao processo necrótico (PADANILAM, 2003). As características morfológicas encontradas nas células que sofreram necrose são: turgidez, rompimento da membrana plasmática e eventual extravasamento do conteúdo citoplasmático dentro do espaço extracelular (MATOS et al., 2011).

Na autofagia, inicialmente, as proteínas/organelas são sequestradas por lipídios chamados 'membranas de isolamento', que encapsulam os alvos com uma estrutura de membrana dupla chamada autofagosoma. Esses autofagosomas então se fundem com lisossomos para degradar os componentes internos (GAWRILUK et al., 2011). Em folículos ovarianos, a autofagia parece ser modulada por uma via denominada mTORC e pode estar associada ao envelhecimento celular (WULLSCHLEGER et al., 2006).

A necroptose é um tipo de necrose programada distinta da apoptose dependente da caspase (LEE et al., 2014). Uma infinidade de moléculas e processos foram caracterizados como iniciadores, moduladores ou efetores da necroptose. Estes incluem: proteína 1 que interage com o receptor (RIP1, também conhecida como RIPK1), RIP3 (também conhecida como RIPK3) (HSU et al, 1996; ZHANG et al., 2009), inibidores da caspase (VERCAMMEN et al., 1998), e ainda espécies reativas de oxigênio (ERO) geradas por mitocôndrias ou NADPH oxidase 1 (NOX1) (GOOSSENS et al., 1999; KIM et al., 2007; YAZDANPANAH et al., 2009). Esta forma de morte celular é morfologicamente distinta da apoptose, pois envolve ruptura de membrana plasmática e liberação do conteúdo citoplasmático (MOCARSKI et al., 2015).

No sentido de verificar os diversos fatores que podem promover o desenvolvimento ou prevenir a atresia folicular, bem como na tentativa de otimizar a utilização dos folículos presentes no ovário, vários estudos sobre o cultivo folicular *in vitro* vêm sendo desenvolvidos.

2.5. Cultivo *in vitro de folículo ovariano*

O cultivo *in vitro* de folículos tem como principal objetivo resgatar oócitos oriundos de folículos pré-antrais a partir do ambiente ovariano, e posteriormente cultivá-los *in vitro* até a maturação, prevenindo-os da atresia e possibilitando sua utilização em outras biotécnicas como fecundação *in vitro*, transgenia e clonagem (SILVA et al., 2016). Esta biotécnica apresenta-se como uma excelente ferramenta para o acompanhamento do crescimento de folículos ovarianos desde a fase pré-antral até a fase antral, contribuindo dessa forma para um melhor entendimento acerca do funcionamento do ovário, podendo ser utilizada para elucidar quais os fatores que interferem em cada fase do desenvolvimento folicular, no processo de atresia, e na prevenção da perda ovariana prematura em mulheres tratadas contra o câncer (FIGUEIREDO et al., 2008).

O desenvolvimento de sistemas de cultivo *in vitro* de folículos ovarianos pré-antrais que possibilitem obtenção de oócitos maduros fertilizáveis pode otimizar as diferentes biotécnicas de reprodução assistida, pois a disponibilidade de oócitos ainda representa um entrave para a maioria destas técnicas (TELFER et al., 2008). Além disso, oócitos obtidos a partir do cultivo *in vitro* de folículos pré-antrais podem ser utilizados para aumentar o potencial reprodutivo de animais geneticamente superiores ou em vias de extinção, bem como auxiliar no tratamento de infertilidade em humanos (FIGUEIREDO et al., 2008).

Nas últimas duas décadas, vários sistemas de cultivo foram desenvolvidos e os resultados são dependentes do meio básico (α-MEM, TCM-199 etc.), suplementação (hormônios, fatores de crescimento, antioxidantes, etc.), espécie animal estudada (FORTUNE, 2003, XU et al., 2011, MAGALHÃES-PADILHA et al., 2012, MENEZES et al., 2017), bem como do sistema de cultivo utilizado, dentre os quais, destacam-se o cultivo de fragmentos do córtex ovariano (SILVA et al., 2004) e o cultivo de folículos isolados (SARAIVA et al., 2010).

2.5.1 Cultivo *in situ* e ativação de folículos primordiais

Os folículos podem ser cultivados "*in situ"*, ou seja, inseridos em fragmentos do córtex ovariano ou "isolados". O principal objetivo do cultivo de pequenos fragmentos de córtex ovariano (cultivo *in situ*), rico em folículos primordiais, é estudar a ativação *in vitro* destes folículos e o

posterior crescimento de folículos primários de diferentes espécies (ovinos: BERTOLDO et al., 2014; BEZERRA et al., 2018; SANTOS et al; 2017; bovinos: BRAW-TAL e YOSSEFI, 1997; TANG et al., 2012; PAES et al., 2016) babuínos: WANDJI et al., 1997; caprinos: MATOS et al., 2007ab; MAGALHAES-PADILHA et al., 2012; ALMEIDA et al., 2015; humana: ZHANG et al., 2004).

Neste sistema de cultivo, os folículos são cultivados com o tecido ovariano circundante, incluindo outros folículos de diferentes estádios de desenvolvimento e células do estroma, e a interação entre os folículos e as células adjacentes pode influenciar seu crescimento (PELUSO e HIRSCHEL, 1988). Outra vantagem desse método é a manipulação do tecido por um curto intervalo, o que previne que as células sejam expostas por longos períodos ao ambiente externo (ARAÚJO et al., 2014).

A ativação espontânea de folículos primordiais, ou seja, ativação folicular em meio de cultivo de base, tem sido demonstrada no cultivo *in situ* em diversas espécies incluindo ovinos (BEZERRA et al., 2018; SANTOS et al., 2017) murinos (NILSSON et al., 2001), bovinos (TANG et al., 2012), equinos (HAAG et al., 2013), caprinos (MATOS et al., 2007) e primatas (WANDJI et al., 2001). Essa ativação pode ocorrer pela liberação de fatores estimulantes ou remoção de fatores de inibição de origem medular (CUSHMAN et al., 2002).

Uma das vias responsáveis pela ativação de folículos primordiais é a via de sinalização fosfatidil inositol 4,5-bifosfato 3 quinase/ Proteína quinase B (PI3K/AKT) (REDDY et al., 2005). Essa via tem sido investigada devido às funções encontradas na ativação e desenvolvimento folicular (REDDY et al., 2008; LIU et al., 2006; REDDY et al., 2005; LIU et al., 2007; REDDY et al., 2009) como demonstrado em um estudo *in vitro*, onde AKT (proteína quinase) e FOXO3a, que são componentes importantes para o funcionamento da via PI3K, foram expressos em oócitos de ratas e camundongas (REDDY et al., 2005). Esta via é formada por moléculas de sinalização (enzimas), como quinases, fosfatases, e fatores de transcrição, que regulam a proliferação celular, sobrevivência e metabolismo. Essas enzimas podem ser classificadas em I, II ou III, de acordo com as distintas funções na sinalização celular, que pode levar à ativação de diferentes vias, regulando o metabolismo, a sobrevivência e a diferenciação celular (ORCY et al., 2008). A PI3K da classe I, possui a capacidade de fosforilar o fosfatidilinositol-bi-fosfato (PIP-2) e produzir fosfatidilinositol-3,4,5-trifosfato (PIP3), que por sua vez, ativa proteínas como a quinase B ou AKT (JANG et al., 2016). Dentre os fatores de transcrição presentes e atuantes na via PI3K podemos citar ainda os fatores da família FOXO, (FOXO1, FOXO3a e FOXO4), que atuam como substratos da via AKT (ARDEN et al., 2002; ACCILI et al., 2004).

Uma ferramenta que permite estudar a via PI3K é a utilização de inibidores farmacológicos dessa via, como por exemplo o LY294002 (GRANVILLE et al., 2006; KEATING et al. 2009, SOBINOFF et al., 2012; ZHAO et al., 2017). Na espécie ovina, estudos da nossa equipe demonstraram a primeira evidência direta de que a via PI3K medeia a ativação espontânea *in vitro* de folículos primordiais na espécie ovina, (SANTOS et al., 2017). Além disso, foi comprovado que o IGF-I promove ativação através da via PI3K/AKT (BEZERRA et al., 2018), ao contrário do EGF, que pode promover a ativação folicular através de outras vias de sinalização, exceto a via PI3K (SANTOS et al. 2017).

Além da ativação da via PI3K, pode ocorrer ativação espontânea no momento da fragmentação do ovário devido ao aumento da polimerização de actina e inibição da via de sinalização Hippo, levando a um aumento na expressão de fatores de crescimento do tecido conjuntivo e fatores relacionados que promovem o início do crescimento do folículo primordial *in vitro* (HSUEH et al., 2015).

Apesar do cultivo *in vitro* de tecido ovariano ser capaz de promover ativação e desenvolvimento de folículos primordiais até os estádios primário e secundário (YANG e FORTUNE, 2007; YANG e FORTUNE 2008; TANG et al., 2012), essa técnica não tem sido eficiente na promoção de maturação oocitária. Desta forma, para obtenção de folículos em estágios mais avançados de desenvolvimento (antrais) e de maturação oocitária, recomenda-se o cultivo de folículos secundários isolados.

2.5.2. Cultivo *in vitro* de folículos ovarianos isolados

Os folículos pré-antrais podem ainda ser isolados e cultivados individualmente, que apresenta como vantagens permitir o acompanhamento individual dos folículos durante o cultivo e seu desenvolvimento até estádio antral (PICTON et al., 2008), além de favorecer a maior perfusão do meio para o folículo (ABIR et al., 2001). Como desvantagens, deve-se considerar a dificuldade do isolamento, o limitado número de recuperação de folículos e a possibilidade de danificar os folículos durante seu isolamento (TELFER et al., 1996).

Os melhores resultados obtidos a partir do cultivo de folículos pré-antrais têm sido obtidos com o uso de folículos secundários grandes, tais como a manutenção da viabilidade, aumento do diâmetro folicular e aumento na produção de estrógeno e progesterona e produção de oócitos maduros e embriões (WANDJI et al., 1996; ARUNAKUMARI et al., 2010; MCLAUGHLIN e TELFER 2010; ARAÚJO et al., 2012; LUZ et al., 2012; ROSSETTO et al., 2013ab).

Diferentes meios de base comerciais podem ser utilizados para o cultivo de folículos isolados em diferentes espécies, dentre eles, podemos destacar o meio essencial mínimo (MEM) (ovinos: LUZ et al., 2012; caprinos: ALMEIDA et al., 2011; SÁ et al., 2017), Waymouth (murinos: O'BRIEN, 2003; humanos: LARONDA et al., 2014; bovinos: GIGLI; BYRD; FORTUNE, 2006); TCM 199 (ovinos: ARUNAKUMARI et al., 2010; bovinos: ROSSETTO et al., 2013b; ARAÚJO et al., 2015; caprinos: AMIN et al., 2013), além do meio McCoy (bovinos: JORSSEN et al., 2014; humanos: TELFER et al., 2008).

Além do meio básico, ainda são adicionados outros suplementos que ajudam no desenvolvimento folicular *in vitro*, por exemplo, hipoxantina, glutamina, insulina, transferrina e selênio, além do ácido ascórbico, que ajudam a manter a morfologia folicular e estimular o crescimento (SILVA et al., 2004; ROSSETTO et al., 2009; MURRAY et al., 2001; DEMEESTERE et al., 2005). Além dessas sustâncias, outros suplementos como hormônios e fatores de crescimento são comumente adicionados aos meios de cultivo. Dentre os fatores testados no cultivo *in vitro* de folículos ovarianos, podem-se destacar o IGF-I e o GDF-9. As seções seguintes abordarão a importância destas sustâncias na foliculogênese.

2.6. Fator de crescimento semelhante à insulina-I (IGF-I)

O fator de crescimento semelhante à insulina-I (IGF-I) exerce suas atividades biológicas através de sua associação com as proteínas de ligação (IGFPB) (MONGET e BONDY, 2000), que aumentam a biodisponibilidade de IGF-I. Os níveis de IGFBP no líquido folicular alteram-se drasticamente ao decorrer da foliculogênese (MONGET et al., 1996). Essas proteínas intrafoliculares têm o papel de regular o desenvolvimento folicular por modularem o IGF-I (MONGET et al., 1989). De forma geral, as IGFBP possuem funções essenciais na regulação das atividades dos IGF: 1) atuar como proteínas de transporte no plasma; 2) prolongar a meia-vida dos IGF por regular sua depuração metabólica; 3) modular diretamente a interação dos IGF com seus receptores e, assim, indiretamente, controlar a sua biorreatividade conforme ilustrado na figura 3.

A expressão do RNAm para o IGF-I já foi demonstrada em ovários de várias espécies: humanos (EL-ROEIY et al., 1993), camundongos (ADASHI et al., 1997; WANDJI et al., 1998; SHIOMI-SUGAYA et al., 2015), bovinos (YUAN et al., 1998; REBOUÇAS et al., 2013), caprinos (MARTINS et al., 2010) e bubalinos (SINGH et al., 2015). Na espécie ovina, o RNAm do IGF-I foi expresso nas células da granulosa e teca de folículos antrais precoces (LEEUWENBERG et al., 1995), em oócitos e células da granulosa de folículos preantrais (BONNET et al., 2013), e ambos os

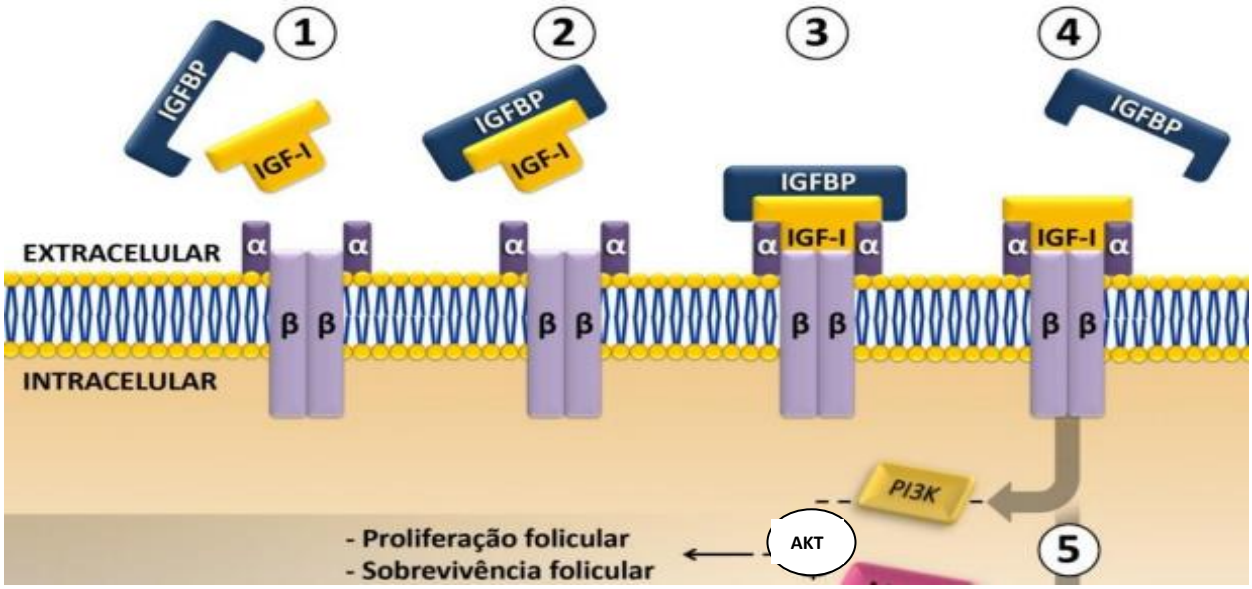
receptores de IGF estão presentes em células da granulosa de folículos primários, secundários e antrais (MONGET, 1989). Além disso, a proteína para o IGF-I foi localizada em todos os estádios foliculares em caprinos (MARTINS et al., 2010).

Estudos *in vitro* demonstraram que o IGF-I estimulou o desenvolvimento folicular em diferentes espécies (ovino: ARUNAKUMARI et al., 2010; caprinos: ZHOU e ZHANG, 2005; MAGALHÃES-PADILHA et al., 2012; ratas: ZHAO et al., 2001; bovinos: GUTIERREZ et al., 2000; humanos: LOUHIO et al., 2000)., De um modo geral, o IGF-I promoveu a ativação de folículos primordiais em caprinos e ovinos (MARTINS et al., 2010; BEZERRA et al., 2018). aumentou o diâmetro folicular em ovinos (ARUNAKUMARI et al., 2010), bovinos (WALTERS et al., 2006), suínos (GUTHRIE et al., 1998) e ratas (DEMEESTERE et al., 2005) e aumentou a taxa de recuperação de oócitos e de maturação após cultivo de folículos secundários isolados em ovinos (ARUNAKUMARI et al., 2010).

Além disso, o IGF-I estimulou a proliferação das células da granulosa e reduziu a apoptose em suínos (GUTHRIEet al., 1998), ovinos (BEZERRA et al., 2018) e bovinos (QUIRK et al., 2004), e ainda estimulou a esteroidogênese em folículos pré-antrais de camundongas (DEMEESTERE et al., 2004), ratas (DEMEESTERE et al., 2005) e bovinos (THOMAS et al., 2007). Entretanto, em bovinos, a adição de 50 ng/mL de IGF-I ao meio de cultivo de folículos pré-antrais não promoveu nenhum efeito benéfico ao desenvolvimento folicular (ARAÚJO et al., 2014). Por outro lado, quando folículos caprinos foram cultivados *in vitro* na presença de IGF-I, FSH e insulina, houve efeitos benéficos no desenvolvimento folicular, nas taxas de recuperação oocitária, além de aumento na retomada da meiose (MAGALHÃES-PADILHA et al., 2012).

Os efeitos benéficos do IGF-I no cultivo de folículos ovarianos podem estar relacionados à ativação da via de sinalização PI3K. A ativação dessa via leva à fosforilação da proteína quinase B (AKT), sobrevivência celular (CECCONI et al., 2013) e ativação de folículos primordiais (JOHN et al., 2008), além de ter efeito protetor contra apoptose em células da granulosa na espécie bovina (HU et al., 2004). A via PI3K/AKT tem ação na proliferação de células da granulosa luteinizadas na espécie humana (GOTO et al., 2009), e na maturação de oócitos de camundongos (LI et al., 2016) sob estímulo do IGF-I. Além disso o IGF-I, age sinergicamente com o FSH promovendo a proliferação das células da granulosa e esteroidogênese via ativação da cascata de sinalização PI3K/AKT (Figura 4) (MANI et al., 2010; MAGALHÃES-PADILHA et al., 2012; BEZERRA et al., 2018).

Figura 4. Cascata de ativação das vias de sinalização PI3K sob ação do IGF-I. 1) IGF-I e IGFBP disponível para conjugação; 2) Conjugação IGF-I e IGFBP; 3) Transporte do complexo IGFI/IGFBP até IGFR-I; 4) Liberação da IGFBP; 5) Ativação da via PI3K e mediação dos processos de proliferação e sobrevivência folicular pelas vias PI3K (Adaptado de ROSSETTO et al., 2013).



Embora muitos estudos já tenham sido realizados, a imunolocalização do IGF-I em ovários ovinos e os efeitos de diferentes concentrações de IGF-I sozinho ou em associação com o FSH na sobrevivência, apoptose, crescimento, estresse oxidativo e atividade mitocondrial de oócitos oriundos de folículos ovarianos cultivados *in vitro* na espécie ovina permanecem desconhecidos.

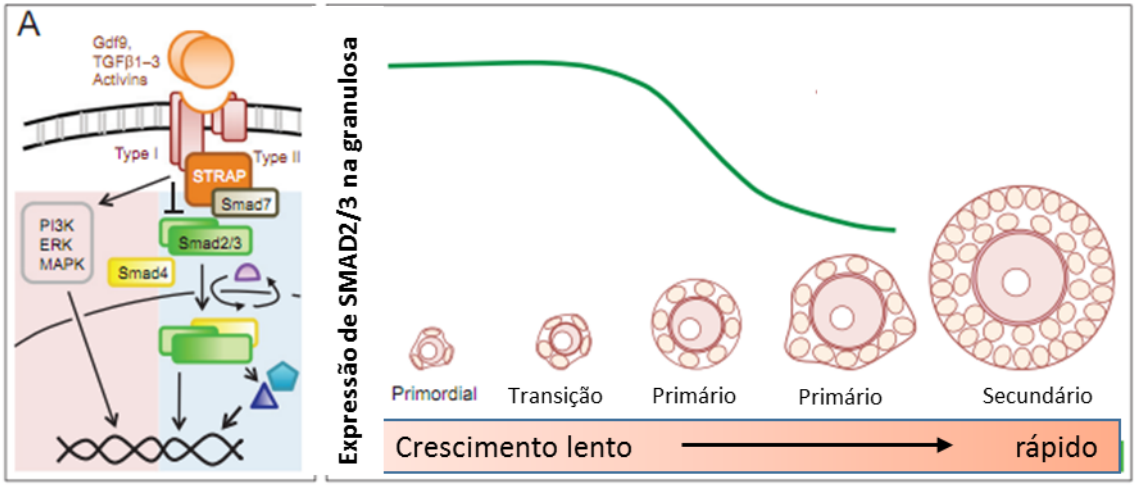
2.7. Fator de crescimento e diferenciação (GDF-9)

O GDF-9 pertence à família TGF-β, composta por diversas proteínas morfogenéticas (BMPs) e ativinas (CHANG et al., 2002; JUENGEL e MCNATTY, 2005, YOSHINO et al., 2006). Essa família desempenha um papel importante no desenvolvimento de folículos ovarianos pré-antrais (JUENGEL et al., 2005). A ação do GDF-9 é iniciada através da sua ligação aos receptores de membrana específicos dos tipos I e II com atividade serina-treonina quinase. O GDF-9 liga-se ao receptor de BMP tipo II (BMPR-II) e, em seguida, ao receptor de ativina tipo kinase-5 (ALK5) (MASSAGUÉ, 1998; SPICER et al., 2008).

Sua ativação induzida pela ligação ligante-receptor, que fosforila o SMAD 2/3 em resposta ao GDF9 (KRETZSCHMAR E MASSAGUE, 1998). Posteriormente, o Smad2/3 fosforilado e

ativados heterodimerizam com um Smad, Smad4 comum, que translocam-se para o núcleo, onde modulam a transcrição de vários genes-alvo, e ativam a via PI3K (ORISAKA et al., 2006) (Figura 4).

Figura 5. Desenho esquemático das vias ativadas pelo GDF-9 (Adaptado de SHARUM et al., 2017).



Na espécie ovina, o GDF-9 é produzido exclusivamente pelo oócito, em todas as fases do desenvolvimento folicular (SCARAMUZZI et al., 2011). Um estudo de expressão quantitativa de RNA total em ovinos demonstrou que a expressão de GDF9 foi observado em oócitos de todos os estágios de desenvolvimento dos folículos *in vivo* (KONA et al., 2015), semelhante às observações nas mulheres, porcas e cabras (SILVA et al., 2005; SUN et al., 2010; PANNETIER et al., 2004). Estes achados foram confirmados por Crawford et al (2011), que estudaram a expressão de GDF9 no CCOs isolados de folículos antrais em ovelhas e relataram que o GDF9 estava ausente nas células do cumulus, mas presente nos oócitos.

O RNAm para o GDF-9 já foi localizado em oócitos de ovários bovinos e ovinos (BODENSTEINER et al., 1999), em todas as categorias foliculares de caprinos (SILVA et al., 2005; ALMEIDA et al., 2011) e em humanos (AALTONEN et al., 1999). Estudos demonstraram que os receptores para GDF-9 estão expressos em todas as categorias foliculares em ovários da espécie caprina (SILVA et al., 2005), o que sugere que o GDF-9 desempenha um importante papel na foliculogênese inicial.

Um estudo mostrou que ovelhas imunizadas contra o GDF-9 (JUENGEL et al., 2002) ou homozigotas para mutações que ocorrem naturalmente no gene GDF-9 (HANRAHAN et al. 2004) perdem a capacidade de ovular e apresentam uma redução no número de folículos em desenvolvimento. Em camundongos, na ausência deste fator de crescimento, não ocorre formação

de folículos secundários, devido à degeneração dos oócitos inclusos em folículos primários, caracterizando um bloqueio nos estágios iniciais da foliculogênese (DONG et al.,1996; MCNATTY et al., 2007). Já a injeção intraovariana de gene codificador do GDF-9, promoveu ativação e avanço dos folículos para o estádio de antral inicial (SHIMIZU et al., 2006).

O GDF-9 promove o crescimento de folículos primários e a proliferação de células da teca de ratas (NILSON e SKINNER, 2002). A adição de GDF-9 no cultivo de folículos primordiais manteve a sobrevivência folicular, bem como promoveu o crescimento para o estádio secundário após 7 dias de cultivo em folículos humanos (HREISSON et al., 2002) e caprinos (MARTINS et al., 2008), e progressão para folículo primário *in vivo* (VITT et al., 2000) e *in vitro* em roedores (HAYASHI et al., 1999; NILSSON e SKINNER 2002).

Além disso, o GDF-9 é essencial para o desenvolvimento de folículos secundários isolados cultivados *in vitro*, tendo um efeito positivo na sobrevivência folicular, na formação de antro em caprinos (ALMEIDA et al., 2011) e ratas (ORISAKA et al., 2006). Este fator também aumentou a produção de oócitos capazes de reiniciar a meiose (ALMEIDA et al., 2011), além de manter a sobrevivência folicular e promover a transição para a fase antral pela supressão da apoptose nas células da granulosa de ratos (ORISAKA et al., 2006).

Em associação com o FSH, o GDF-9 aumentou o crescimento e a diferenciação de folículos pré-antrais murinos (HAYASHI et al., 1999), além de induzir a expansão das células do cumulus e promover a maturação oocitária em folículos de camundongos (ELVIN et al., 1999), o que não foi possível na ausência de FSH (DRAGOVIC et al., 2005). A adição de GDF-9 a CCOs de camundongos cultivados com FSH antes da fecundação *in vitro* e transferência para fêmeas receptoras aumentou o número de fetos viáveis, sugerindo que esse fator apoia o desenvolvimento embrionário e a viabilidade fetal (YEO et al., 2008).

O GDF-9 também desempenha um papel importante durante os estágios finais do crescimento folicular, como a ovulação. Antes do aumento de LH, as células do cumulus requerem suporte de GDF-9 nas cascatas metabólicas, como glicólise e biossíntese de esteróis (SUGIURA et al. 2005), na regulação da expressão gênica durante o estágio pré-ovulatório e aumentando a produção do receptor do LH (ELVIN et al., 1999).

Em um estudo utilizando folículos pré-antrais de ratas, o GDF-9 exerceu efeitos antiapoptóticos e protegeu células da granulosa contra a apoptose via ativação PI3K/AKT (ORISAKA et al., 2006). Esse efeito anti-apoptótico parece ser mais efetivo durante os estágios iniciais, mas não tardios, do desenvolvimento folicular (OTSUKA et al., 2011).

O GDF-9 também desempenha um papel fundamental no controle das funções das células da teca, tendo seus efeitos inicialmente descobertos por um estudo *in vivo*, no qual injeções

intraperitoneais de GDF-9 em ratos imaturos promoveram a progressão de folículos primordiais e primários para pequenos folículos pré-antrais e aumentaram a expressão do marcador específico de células da teca CYP17 (VITT et al., 2000). Já em folículos bovinos, o GDF-9 estimulou a proliferação e inibiu a diferenciação de células da teca derivadas de pequenos folículos (SPICER et al., 2008).

2.8. Estado atual do cultivo *in vitro* de folículos pré-antrais

Diversos avanços foram obtidos no tocante ao cultivo *in vitro* de folículos pré-antrais em diferentes espécies animais. Em primatas não humanos, foi obtido embrião na fase de clivagem com cultivo tridimensional e adição de FSH (Xu et al., 2011).

Apesar do grande avanço no cultivo *in vitro* de folículos pré-antrais com as referidas espécies, os resultados mais satisfatórios foram observados em animais de laboratório com a obtenção do nascimento e sobrevivência de 59 camundongos a partir de folículos primordiais crescidos *in vitro* (O'BRIEN et al., 2003).

No entanto, ainda não foi possível repetir esse resultado em outros mamíferos. Em humanos, foi possível produzir oócitos maduros a partir de folículos secundários (XIAO et al., 2015). Na espécie bovina, até o momento, o cultivo de folículos pré-antrais tem como melhor resultado a obtenção de folículos no estágio antral a partir de folículos primários (SUN e LI, 2013), já com cultivo de folículos antrais iniciais, foi possível a produção de blastocistos (HUANG et al., 2013) e o nascimento de crias vivas a partir de complexos granulosa-oócito (YAMAMOTO et al., 1999; HIRAO et al., 2004).

Em ovinos (ARUNAKUMARI et al., 2010; LUZ et al., 2012), suínos (WU et al., 2001), bubalinos (GUPTA et al., 2008), primatas não humanos (XU et al., 2011, 2013b) e caprinos (SARAIVA et al., 2010; MAGALHÃES et al., 2011a; SILVA et al., 2014), foi possível produzir oócitos maduros a partir de folículos secundários e após fecundação destes oócitos, houve a produção de embriões *in vitro*. No entanto, as taxas de maturação oocitária ainda são baixas quando comparadas com as obtidas a partir de oócitos provenientes de folículos crescidos in vivo.

Desta forma, estes resultados demostram a necessidade de mais estudos visando melhorar a eficiência dos meios e/ou sistemas de cultivo atuais.

3. JUSTIFICATIVA

O cultivo *in vitro* de folículos ovarianos consiste em resgatar folículos pré-antrais dos ovários e cultivá-los *in vitro* com o objetivo de completar a foliculogênese, evitando a atresia, e potencializando a produção de oócitos maduros fertilizáveis. O desenvolvimento desta biotécnica pode contribuir no entendimento da foliculogênese, aumentando exponencialmente o potencial reprodutivo de diversas espécies de valor zootécnico ou contribuir para a preservação de animais silvestres ou ainda, preservar a fertilidade em mulheres submetidas a tratamentos gonadotóxicos. No entanto, atualmente, o principal entrave dessa técnica, em mamíferos de médio e grande porte, são as baixas taxas de maturação oocitária e produção de embriões obtidas.

Diversos modelos animais vêm sendo utilizados no desenvolvimento desta biotécnica, dentre os quais destacam-se os ovinos. Esta espécie tem importância sócio-econômica principalmente para a região Nordeste do Brasil, onde encontramos cerca de 63% do rebanho (IBGE, 2018), sendo uma importante fonte de carne e pele. Nesta espécie, apesar de já terem sido produzidos oócitos maduros e embrião em estádio de mórula, as taxas de maturação e produção de embriões permanecem baixas quando comparado àquelas alcançados *in vivo*.

Diversas variáveis podem afetar o cultivo de folículos pré-antrais, considerando-se a natureza dinâmica do processo de foliculogênese, com grande variação na expressão de proteínas entre as diferentes fases de desenvolvimento folicular. Desta forma, as necessidades foliculares podem variar ao longo do seu desenvolvimento, o que pode ser a chave para o desenvolvimento de futuros sistemas de cultivo que reproduzam a foliculogênese completa, com meios de cultivo específicos para cada uma dessas fases.

Diferentes fatores de crescimento já comprovaram regular a foliculogênese e promover o desenvolvimento de folículos pré-antrais cultivados *in vitro*. Dentre esses fatores podemos destacar o IGF-I e o GDF-9. Esses dois fatores já promoveram a ativação de folículos primordiais, e desenvolvimento de folículos secundários isolados em diversas espécies. No entanto, na espécie ovina, ainda não foi demonstrado a imunolocalização do IGF-I e o efeito desse fator e do GDF-9 no desenvolvimento de folículos secundários *in vitro* em diferentes sistemas de desenvolvimento. Assim, este trabalho poderia ajudar a compreender a foliculogênese *in vitro* e descrever padrões de crescimento folicular que possam predizer a maturação oocitária, o que poderia acarretar no aumento significativo da eficiência dos sistemas de cultivo atuais.

4. HIPÓTESES

- A adição de IGF-I sozinho ou associado ao FSH no meio de cultivo *in vitro* de folículos secundários isolados ovinos promove manutenção da viabilidade e efeitos benéficos no desenvolvimento folicular e produção de oócitos maduros.
- A adição de GDF-9 ao meio de cultivo de tecido ovariano ovino promove ativação e crescimento folicular através da via PI3K.
- A adição de GDF-9 ao meio de cultivo de folículos secundários ovinos isolados promove manutenção da viabilidade e efeitos benéficos no desenvolvimento folicular e produção de oócitos maduros.

5. OBJETIVOS

5.1. Objetivo Geral

Estudar os efeitos do IGF-I e do GDF-9 sobre o desenvolvimento *in vitro* de folículos pré-antrais ovinos.

5.2. Objetivos Específicos

- Estudar a imunolocalização do IGF-I em ovários ovinos;
- Avaliar o efeito de diferentes concentrações de IGF-I, associado ou não ao FSH, sobre o cultivo *in vitro* de folículos secundários ovinos isolados, tendo como parâmetros:

  - a morfologia, viabilidade, a formação de antro bem como o crescimento folicular;
  - a taxa de recuperação oocitária;
  - a imunolocalização do receptor de LH antes e após o cultivo;
  - a fragmentação de DNA, pela marcação com TUNEL de fluorescência;
  - os níveis de ERO, glutationa e mitocôndrias ativas;
  - as taxas de maturação *in vitro* dos oócitos ovinos.

- Avaliar o efeito de diferentes concentrações de GDF-9 sobre o cultivo de tecido ovariano ovino, avaliando os seguintes parâmetros:

  - a morfologia;
  - ativação de folículos primordiais e posterior crescimento folicular e oocitário;

- a proliferação celular;
- a apoptose;
- a via de ativação/proliferação PI3K/AKT.

- Avaliar o efeito de diferentes concentrações de GDF-9 sobre o cultivo *in vitro* de folículos secundários ovinos isolados, tendo como parâmetros:
  - a morfologia, a formação de antro e o crescimento folicular;
  - a taxa de recuperação oocitária;
  - os níveis de ERO e mitocôndrias ativas após a MIV;
  - as taxas de maturação *in vitro* dos oócitos ovinos.

6. CAPÍTULO I

IMMUNOHISTOCHEMICAL LOCALIZATION OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) IN THE SHEEP OVARY AND THE SYNERGISTIC EFFECT OF IGF-I AND FSH ON OOCYTE DEVELOPMENT *IN VITRO* AND LH RECEPTOR EXPRESSION

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Running head: IGF-I and FSH on sheep oocyte growth

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Abstract

This study analyzed IGF-I protein expression in sheep ovaries, the effect of IGF-I alone or associated with FSH on the culture of secondary follicles, and the expression of LHR protein in antral follicles before and after culture. Ovaries were collected for IGF-I protein analysis. In experiment 1, secondary follicles were cultured in α-MEM$^+$ (control) or α-MEM$^+$ supplemented with IGF-I (10, 50 or 100 ng/mL). In experiment 2, follicles were cultured in the same media of experiment 1 plus 750 ng/mL FSH. Moreover, LHR expression was analyzed in fresh antral follicles and after culture in 50 ng/mL IGF-I + FSH. The IGF-I protein was expressed in oocytes from all stages of follicle development and in the granulosa cells from secondary and antral follicles. IGF-I did not influence ($P>0.05$) follicular viability and growth (experiment 1). However, in experiment 2, 50 ng/mL IGF-I + FSH stimulated oocyte growth ($P<0.05$) and LHR expression in antral follicles. Control medium, 10 or 50 ng/mL IGF-I + FSH showed similar levels of reactive oxygen species, glutathione and active mitochondria ($P>0.05$). In conclusion, the IGF-I protein is expressed in all ovarian follicle stages in sheep. Moreover, the association between 50 ng/mL IGF-I and FSH has a synergistic effect *in vitro*, increasing the percentage of fully grown oocytes and the expression of LHR protein in oocytes and granulosa cells of cultured antral follicles.

Keywords: Preantral follicles, Hormone, *In vitro* culture, *In vitro* maturation

1. Introduction

In vitro follicle culture provides a tool for new discoveries regarding the physiology and mechanisms governing follicle development and oocyte maturation [1]. These systems have proven successful in producing high-quality oocytes and live offspring from murine follicles cultured *in vitro* [2], but the number of embryos produced from *in vitro* grown preantral follicles from large animal species is very low (swine: [3]; buffalo: [4]; ovine: [5]; caprine: [6]). An important limitation is that oocyte quality is significantly impacted by the culture medium and conditions [1]. Therefore, improvements in the culture system, such as the use of optimal media components, would help to support normal follicle development.

Among the factors that regulate ovarian follicular development, a key role is played by the insulin-like growth factor-I (IGF-I), which belongs to the IGF system [7]. Expression of IGF-I mRNA was reported in the ovaries of human [8], mouse [9; 10; 11], bovine [12; 13], caprine [14] and bubaline [15]. In sheep species, the IGF-I mRNA was localized in both granulosa and theca

layers of antral follicles [16; 17], and also in oocytes and granulosa cells of preantral follicles [18]. Nevertheless, IGF-I protein was localized in preantral and antral follicles in caprine [19] and in preovulatory follicles in bubaline [15]. The biological functions of IGF-I are mediated through the association with IGF binding proteins (IGFBPs) and tyrosine kinase type I receptors (IGF-IR) [20], which are expressed in the theca and granulosa cells in cattle [21]. After binding to its receptor, two signaling pathways could be activated: a phosphatidylinositol 3-kinase (PI3K) pathway and/or a mitogen-activated protein kinase (MAPK) [22].

In vitro studies have shown that addition of IGF-I to the culture medium of ovarian tissue culture promoted primordial follicle activation (human: [23]; caprine: [19; 24; bovine: [25]. Additionally, IGF-I stimulated the antrum formation (bovine: [26]), and reduced follicular atresia (feline: [27]) after culture of isolated secondary follicles. Moreover, IGF-I acted synergistically with FSH to improve caprine preantral follicle development and the production of meiotically competent oocytes [28], and to increase follicle diameter in mice [29]. Furthermore, IGF-I enhances the expression of LH receptor (LHR) in rat granulosa cells cultured in the presence of FSH, whereas IGF-I alone did not induce LHR mRNA expression [30].

Thus, it is clear that IGF-I is important for follicle development and there are species-dependent variations of IGF-I expression and action. Therefore, additional immunohistochemistry to detect IGF-I protein and *in vitro* follicle culture could be used to evaluate the effect of IGF-I on sheep follicle growth [18]. Furthermore, we hypothesized that association between IGF-I and FSH could modulate the expression of LHR protein after culture, but LHR protein expression in sheep antral follicles before or after *in vitro* culture is not yet known.

The aims of this study were: (i) to characterize protein expression for IGF-I in sheep ovaries, (ii) to evaluate the effect of IGF-I alone or associated with FSH on the morphology, follicular and oocyte growth, intracellular levels of reactive oxygen species, glutathione and active mitochondria, and resumption of meiosis of oocytes from isolated ovine secondary follicles cultured *in vitro*, (iii) to analyze the expression of LHR protein in the antral follicles, and therefore verify whether the association between IGF-I and FSH could regulate the expression of LHR protein after culture.

2. Material e Methods

Unless indicated, media, supplements 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 adult (1–3 years old) mixed-breed sheep (*Ovis aries*). Immediately postmortem, the ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil) followed by twice in Minimum Essential Medium 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 h to the laboratory in tubes containing MEM-HEPES with antibiotics at 4°C [31].

2.2. Immunohistochemistry

Immunohistochemistry was performed according to previous studies [32]. Ovaries (n = 16) from four sheeps were collected and fixed in 10% buffered formalin (Dinâmica). After 18 h of fixation, the ovarian tissue was dehydrated with increasing concentrations of ethanol (Dinâmica), clarified in xylene (Dinâmica), and embedded in paraffin (Dinâmica). Sections (5 µm thick) from each block were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted in Starfrost glass slides (Knittel, Braunschweig, Germany). The slides were incubated in citrate buffer (Dinâmica) at 95◦ C in a deckloaking chamber (Biocare, Concord, USA) for 40 min to retrieve antigenicity, and endogenous peroxidase activity was prevented by incubation with 3% H_2O_2 (Dinâmica) and methyl ethanol (QEEL, São Paulo, Brazil) for 10 min. Non specific binding sites were blocked using 1% normal goat serum (Biocare) and diluted in phosphate-buffered saline (PBS; Sigma Aldrich Chemical Co., St. Louis, MO, USA). Subsequently, the sections were incubated in a humidified chamber for 60 min at room temperature with polyclonal rabbit anti-IGF-I (1:50; reference: (H-70) sc 9013, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Thereafter, the sections were incubated for 20 min with MACH4 Universal HRP-polymer (Biocare). Protein localization was demonstrated with diaminobenzidine (DAB; Biocare), and the sections were counterstained with hematoxylin (Vetec, São Paulo, Brazil) for 1 min. Negative controls (reaction control) underwent all steps except the primary antibody incubation.

Preantral follicles were classified as defined previously [33], in primordial (oocyte surrounded by a single layer of squamous or squamous and cuboidal granulosa cells), primary (oocyte surrounded by a single layer of cuboidal granulosa cells), secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells) or antral follicles (presence of an antral cavity). In the different follicular compartments (oocyte, granulosa and theca cells), the immunostaining was classified as absent, weak, moderate or strong. The slides were examined using a microscope (Nikon, Tokyo, Japan) under 400x magnification.

2.3. Isolation and selection of ovine secondary follicles

Isolation, selection, culture and follicular evaluation were performed according to Pessoa et al. [34]. In the laboratory, the surrounding fatty tissues and ligaments were stripped from the ovaries; large antral follicles and corpora lutea were removed. Ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in holding medium consisting of MEM-HEPES with antibiotics. Ovine secondary follicles, approximately 250-300 μm in diameter, without antral cavities, were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and mechanically isolated by microdissection using 26-gauge needles (26 G). These follicles were then transferred to 100 μL droplets containing base culture medium for the evaluation of quality. Only follicles that displayed the following characteristics were selected for culture: an intact basement membrane, two or more layers of granulosa cells and a visible and healthy oocyte that was round and centrally located within the follicle, without any dark cytoplasm. Isolated follicles were pooled and then randomly allocated to the treatments with approximately 55 follicles per group. After selection, the follicles were individually cultured (one follicle per droplet) in 100 μL droplets of culture medium under mineral oil in petri dishes (60 x 15 mm, Corning, USA). All follicles were cultured at 39 °C under 5% CO_2 for 18 days. Every two days, in all treatments, 60 μL of the culture medium was replaced with fresh medium in each droplet.

2.4. Experiment 1: *In vitro* culture of secondary follicles in the presence or absence of IGF-1

The base control medium consisted of α-MEM$^+$ (pH 7.2–7.4) supplemented with 3.0 mg/mL BSA, 10 ng/mL insulin, 2 mM glutamine, 2 mM hypoxanthine, 5.5 μg/mL transferrin, 5.0 ng/mL selenium, and 50 μg/mL ascorbic acid. In experiment 1, follicles were cultured in the control medium (α-MEM$^+$) or in α-MEM$^+$ supplemented with different concentrations of IGF-I (10, 50 or 100 ng/mL). The concentrations of IGF-I were chosen based on previous studies [20; 35]. For this culture, 5 replicates were carried out using 30 ovaries.

The morphological aspects of all follicles were assessed every 6 days using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon) at x 100 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, and (ii) extruded follicles, characterized by a follicle with ruptured basement membrane and a bright and intact oocyte (with no signs of an irregular contour, a darkened oocyte and/or granulosa cells) [34].

Follicular atresia was recognized when a darkening of the oocytes and 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, (ii) the diameter of healthy follicles, measured from the basement membrane, which included two perpendicular measurements of each follicle, and (iii) the daily growth rate, calculated as the diameter variation during the culture period (18 days).

After 18 days of culture, intact follicles were carefully and mechanically opened with 26 G needles under a stereomicroscope for oocyte recovery. The percentage of fully grown oocytes, i.e. oocyte ≥110 μm, was calculated as the number of acceptable quality oocytes (≥ 110 μm) recovered out of the total number of cultured follicles (x 100).

2.5. Assessment of follicular viability after culture

For live/dead fluorescent labeling, after 18 days of culture, approximately 20 follicles per treatment were placed in droplets of 100 µL of phosphate buffered saline (PBS) with 4 mM calcein-AM and 2 mM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), followed by incubation at 39 °C for 15 min. Thereafter, the follicles were washed in PBS and were examined using a fluorescence microscope (Nikon E200, Tokyo, Japan) at a magnification of 400x. The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. The follicles were considered live if the cytoplasm was labeled positively with calcein-AM (green) and dead if cellular chromatin was labeled with ethidium homodimer-1 (red).

2.6. Experiment 2: Effect of the association between IGF-1 and FSH on the *in vitro* culture of isolated follicles

In experiment 2, the control medium was α-MEM$^+$ added with 750 ng/mL FSH (recombinant human FSH; Gonal-F®; MerkSerono S.p.A., Bari, Italy). Approximately 55 secondary follicles per treatment were cultured in the control medium (α-MEM$^+$ + FSH) or in the control medium supplemented with 10, 50 or 100 ng/mL IGF-I. The concentration of FSH was chosen according to a recent study performed by our group with ovine secondary follicle culture (Barros *et al.* unpublished data). For this experiment 5 replicates were carried out using 30 ovaries.

As described for experiment 1, every 6 days of culture, the follicles were assessed for morphological aspects (surviving follicles, antral cavity formation, follicle diameter, growth rate

and percentage of fully grown oocytes). Furthermore, healthy oocytes retrieved from the experiment 2 were evaluated for reactive oxygen species (ROS) and reduced glutathione (GSH) intracellular levels, mitochondrial activity, *in vitro* maturation and detection of DNA fragmentation.

2.7. Histological analysis of cultured isolated follicles

To obtain a more detailed view of the follicle structures after *in vitro* culture, follicles were processed for histological examination as previously described [36], with some modifications. After 18 days of culture, isolated follicles from all treatments were fixed in 4% paraformaldehyde for 1 h. Thereafter, the follicles were washed twice in PBS. Samples were dehydrated by incubation with increasing concentrations of ethanol (70 to 100%). Follicles were then embedded in paraffin and serial 3-μm sections were cut. The sections were mounted, stained with hematoxylin-eosin and analyzed by light microscopy (x400; Nikon). For this analysis, the follicles were classified as morphologically normal or atretic. Normal follicles showed intact oocyte (without a pyknotic nucleus or cytoplasmic retraction) surrounded by organized granulosa cells. Conversely, atretic follicles were defined as those with retracted oocyte, nuclear pyknosis, and/or disorganization of granulosa cells and discontinuity of the basement membrane.

2.8. Immunohistochemical expression of LHR protein in antral follicles before and after *in vitro* culture

To verify the hypothesis that the interaction between IGF-I and FSH could increase the LHR protein, immunohistochemical analysis was carried out to address the expression of LHR protein in antral follicles from fresh ovarian tissue, and also in isolated follicles cultured for 18 days in the treatment that showed the highest percentage of fully grown oocytes (50 ng/mL IGF-I associated with FSH). The immunohistochemistry of fresh tissues was carried out with the same ovaries used for IGF-I protein analysis. Cultured isolated follicles were fixed and processed for histology. Thereafter, immunohistochemistry was performed as described above using anti-LHR antibody (1:500; reference: (H-50), sc 25828 Santa Cruz Biotechnology).

2.9. Assessment of oxidative stress markers (ROS and GSH intracellular levels) and metabolically active mitochondria

After culture, the oocytes were recovered and intracellular GSH, ROS levels and mitochondrial activity were measured as previously described [32]. Briefly, 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker® Blue; CMF2HC; Invitrogen Corporation), 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Corporation) and MitoTracker Red (MitoTracker® Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) were used to detect intracellular GSH, ROS and mitochondrial activity levels as blue, green and red fluorescence, respectively. Approximately 20 oocytes per treatment were incubated in the dark for 30 minutes in PBS supplemented with 10 mM of CellTracker® Blue, 10 mM of H2DCFDA and 100 MitoTracker® Red at 39 ºC. Thereafter, the oocytes were washed in PBS and the fluorescence was observed under an epifluorescence microscope with UV filters (370 nm for GSH, 460 nm for ROS and 579-599 nm for active mitochondria). Fluorescence intensities of the oocytes were analyzed by using the Image J software (National Institutes of Health, Bethesda, MD, USA).

2.10. Maturation of ovine oocytes from *in vitro* cultured secondary follicles

In vitro maturation (IVM) was performed to confirm that oocytes derived from *in vitro* grown preantral follicles cultured in medium containing 50 ng/mL IGF-I + FSH were able to resume meiosis. 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, all oocytes enclosed in healthy follicles were carefully collected with 26-G needles under a stereomicroscope. Only oocytes ≥110 μm of diameter with a homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for IVM as previously described [37; 38]. The cumulus–oocyte complexes (COCs) were transferred to drops of 100 μL of maturation medium composed of TCM 199 supplemented with 10% FCS, 1 μg/mL FSH and 1 μg/mL LH (ovine pituitary) under oil, and incubated for 24 h under 5% CO_2 [39]. 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 (including germinal vesicle breakdown [GVBD] or metaphase I [MI]) or nuclear maturation (metaphase II [MII]).

2.11. Assessment of DNA fragmentation by TUNEL assay

For evaluation of oocyte DNA fragmentation after IVM, oocytes were subjected to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay as previously described [40]. Briefly, healthy oocytes were fixed in 4% paraformaldehyde solution for 1 h at room temperature. Then, oocytes (15 oocytes per treatment) were washed three times in solution of PBS/polyvinylpyrrolidone (PVP) and stored at 4°C in eppendorf with PBS/PVP until the beginning of TUNEL procedure. Thereafter, oocytes were incubated in droplets of 100 µL of permeabilizing solution (0.1% [v/v] Triton X-100 in 10 Mm PBS) for 3 h 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 h 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, 7.5 µL terminal deoxynucleotidyl transferase (TDT) enzyme and 67.5 µL of marker solution of 2-deoxyuridine triphosphate 5-FITC were made to obtain 75 µL of TUNEL mixture for reaction. The experimental groups and the positive control were incubated with 15 µL of this solution for 1 h at 37°C in a moist chamber in the dark. The negative control was incubated at 15 µL with the marker solution. Oocytes were washed three times in drops of PBS/PVP and incubated in PBS containing 10 mM Hoechst 33342 for 15 min at room temperature in the dark. Then, 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 x400. Oocytes were considered TUNEL-positive (with DNA fragmentation) when they have marked chromatin with green fluorescence.

2.12. Statistical analysis

Data from follicle survival, extruded follicles, antrum formation, viability, and maturation rates after *in vitro* culture were expressed as percentages and compared by the Chi-squared test. Data from follicular diameter, growth rate, mitochondrial activity, GSH and ROS levels were submitted to the D´Agostino 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 mean (SEM), and differences were considered significant when $P < 0.05$.

3. Results

3.1.Immunohistochemical localization of IGF-I in sheep ovarian follicles

Figure 1 and Table 1 show the expression pattern of immunohistochemical staining for IGF-I in ovine ovaries. Oocytes from primordial, primary and secondary follicles showed a strong, moderate and weak reaction for IGF-I protein, respectively (Fig. 1A-C), while oocytes from antral follicles showed a moderate reaction (Fig. 1D). The intensity of the immunostaining was strong in the granulosa cells of secondary follicles (Fig. 1C), and moderate in antral follicles (Fig. 1D). No staining was observed in the theca or stromal cells (Fig. 1D) neither in negative controls (Fig. 1E).

Figure 1. Immunolocalization of IGF-1 protein (A) in sheep ovarian follicles: primordial (A), primary (B), secondary (C), antral (D) follicle, and negative control (E). O: oocyte; GC: granulosa cells; TC: theca cells; A: Antrum (400x). Scale bar: 50 µm.

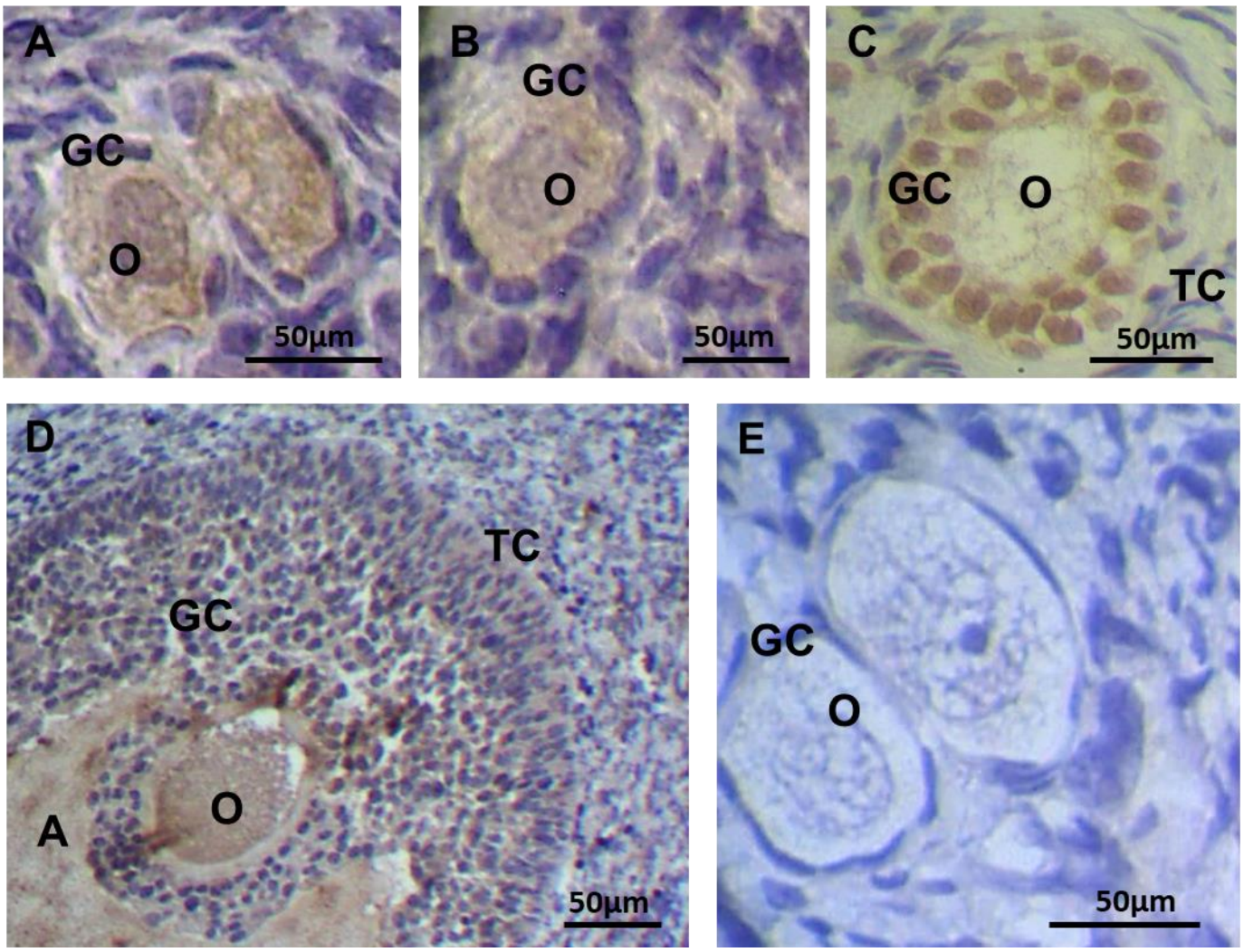


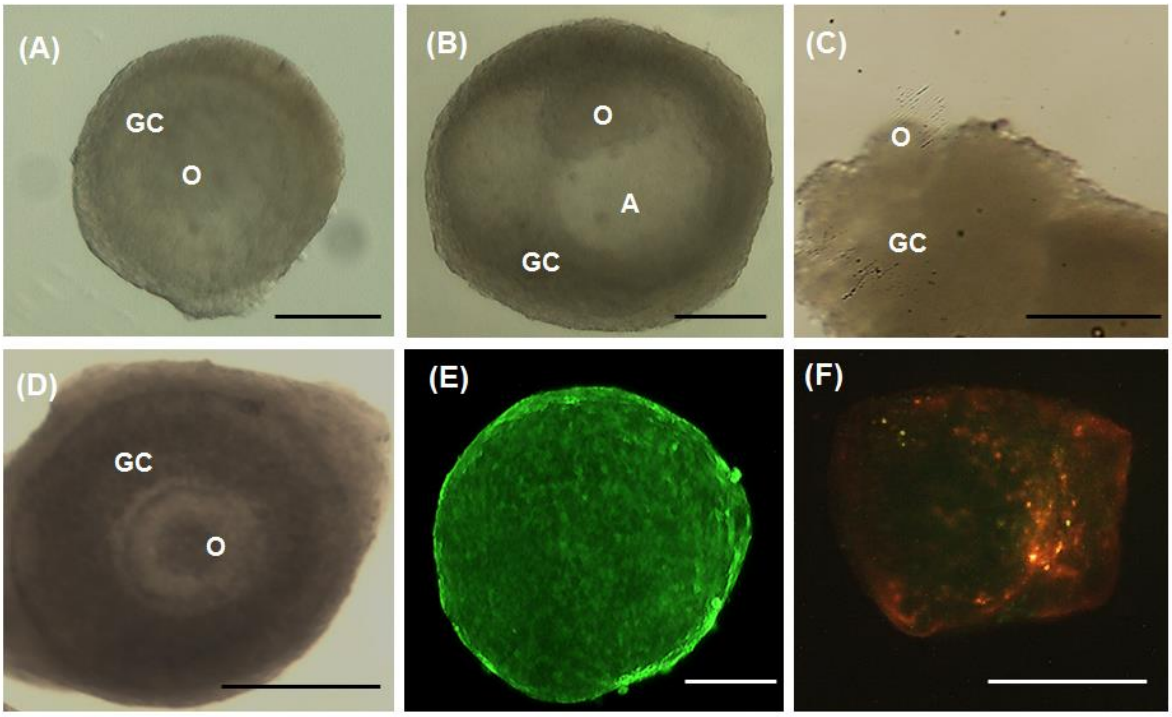
Table 1. Relative intensity of immunohistochemical staining for IGF-1 in the ovaries of sheep.

Structure	Primordial follicle	Primary follicle	Secondary follicle	Antral follicle
Oocyte	+++	++	+	++
Granulosa cell	-	-	+++	++
Theca cell			-	-

3.2. Experiment 1: *In vitro* culture of secondary follicles in the presence or absence of IGF-I

Morphologically normal secondary follicles showed centrally located oocytes and normal granulosa cells, which were enclosed by an intact basement membrane (Fig. 2A). As early as 6 days of culture, antral cavity was observed (Fig. 2B). Follicle with ruptured basement membrane and extruded intact oocytes (Fig. 2C), and atretic follicles (Fig. 2D) were also observed after 18 days of culture.

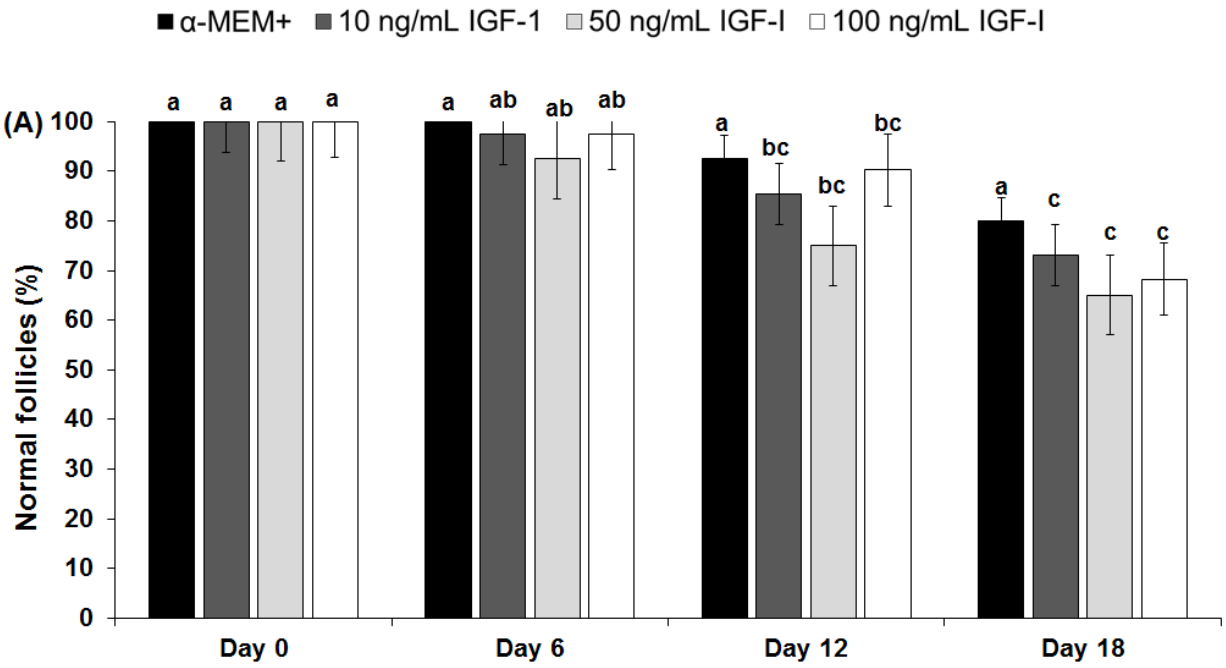
Figure 2. Morphologically normal follicle at day 0 (A), antral follicle after 6 days of culture in 50 ng/mL IGF-I (B), extruded follicle at 100 ng/mL IGF-I (C), atretic follicle at 10 ng/mL IGF-I (D), viable follicle after culture in 50 ng/mL IGF-I (E), and non-viable follicle cultured in α-MEM (F) for 18 days. GC: granulosa cell; O: oocyte. A: antrum. Scale bar: 100 μm.



The percentage of morphologically normal follicles (follicular survival) decreased significantly from day 0 to day 18 in all treatments, except in the control medium that maintained ($P>0.05$) follicular survival throughout the culture (Fig. 3). After 18 days, the percentage of normal follicles was similar ($P>0.05$) among all treatments (82.1%, 71.8%, 65% and 70% for the control medium, 10, 50 and 100 ng/mL IGF-I, respectively). These results were confirmed by fluorescence analysis (Fig. 2E-F) in which no differences ($P>0.05$) were observed in the viability rates among the treatments (87.5%; 96.4%; 96.5% and 100% for α-MEM^{+}, 10, 50 and 100 ng/mL IGF-I, respectively).

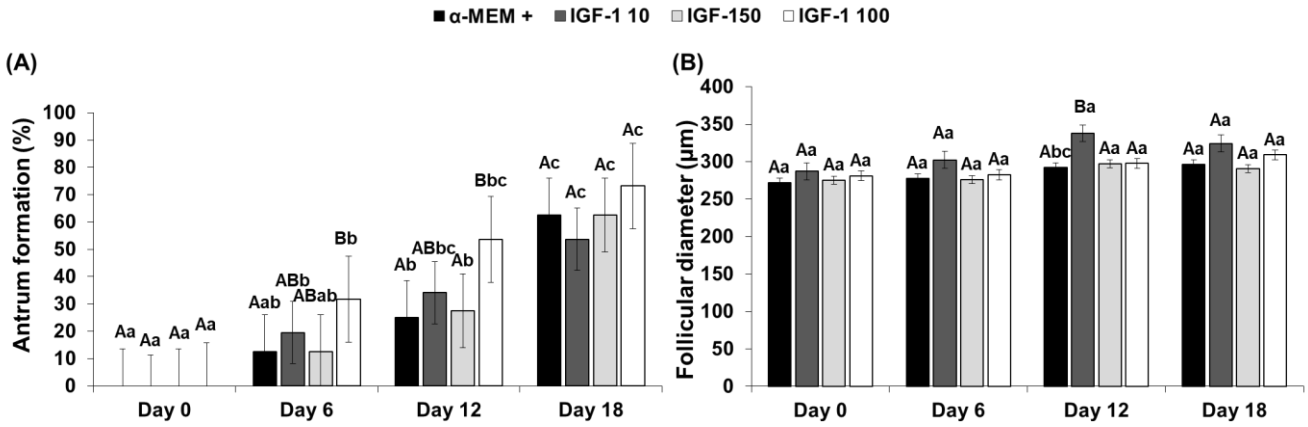
Figure 3. Percentages of morphologically normal follicles cultured in α-MEM$^+$ or in different concentrations of IGF-I (10; 50 or 100 ng/mL).

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



Compared to day 0, the rates of antral cavity formation increased significantly at day 6 only in 10 ng/mL IGF-I (20.5% of antral follicles) or 100 ng/mL IGF-I (32.5% of antral follicles) (Fig. 4A). At the end of culture, there was no significant difference in the antrum formation among treatments (61.5%, 56.4%, 62.5% and 72.5% for α-MEM$^+$, 10, 50 and 100 ng/mL IGF-I, respectively; $P>0.05$; Fig. 4A). Comparing the treatments in the same period of culture, only at day 12, a significant increase in the follicular diameter was observed after culture in the presence of 10 ng/mL IGF-I (Fig. 4B). Moreover, there was no significant difference ($P>0.05$) in the daily growth rates among treatments (1.5 µm, 2.1 µm, 2.1 µm and 2.0 µm for α-MEM$^+$, 10, 50 and 100 ng/mL IGF-I, respectively) or in the rate of oocytes ≥ 110 µm (71.8%, 66.7%, 62.5% and 65.0% for α-MEM$^+$, 10, 50 and 100 ng/mL IGF-I, respectively).

Figure 4. Experiment 1: antrum formation (A) and follicular diameter (B) after *in vitro* culture in α-MEM^{+} or in different concentrations of IGF-1 (10; 50 or 100 ng/mL).
($^{a, b, c}$) Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$); ($^{A, B}$) Different letters denote significant differences among treatments in the same period ($P < 0.05$).

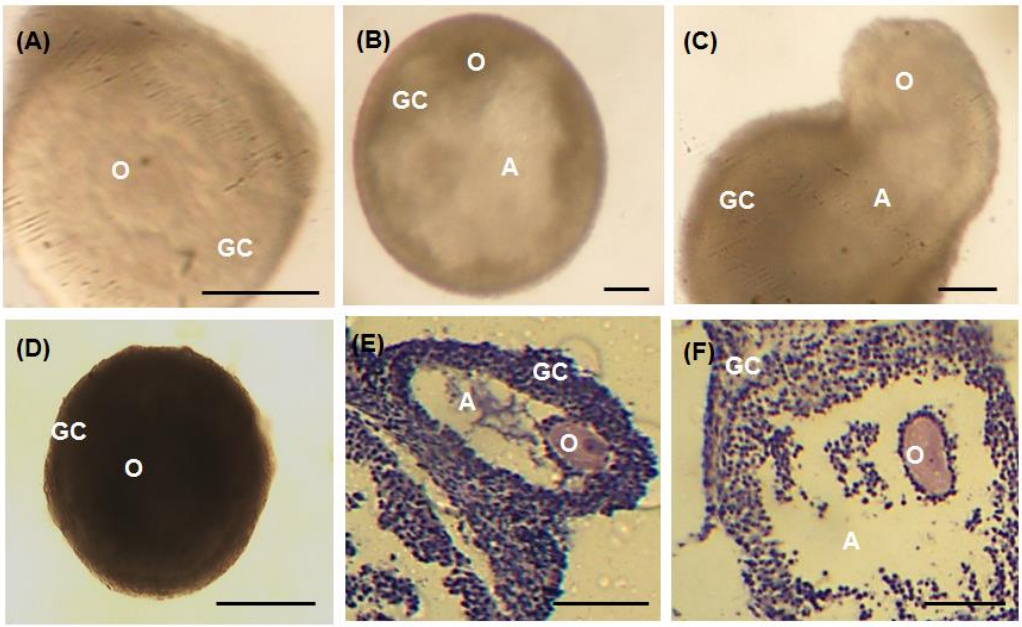


3.3. Experiment 2: Effect of the association between IGF-I and FSH on the *in vitro* culture of isolated follicles

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

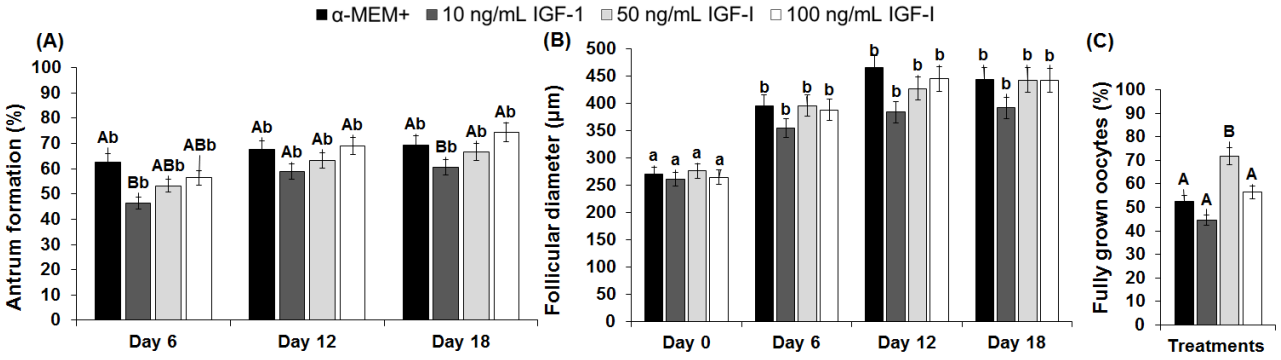
Figure 5 shows normal secondary follicle before culture (Fig. 5A), an antral follicle after 6 days of culture in 50 ng/mL IGF-I + FSH (Fig. 5B), as well a follicle with ruptured basement membrane and extruded intact oocytes (Fig. 5C) and atretic follicle (Fig. 5D) after 18 days of culture in α-MEM^{+} + FSH. The follicular survival was maintained in all treatments throughout the culture and there was no difference ($P>0.05$) among treatments after 18 days (98.3%, 96.4%, 98.3% and 96.4% of normal follicles for the control medium, 10 ng/mL IGF-I + FSH, 50 ng/mL IGF-I + FSH and 100 ng/mL IGF-I + FSH, respectively). Histological examination confirmed that normal follicles were found after *in vitro* culture (Fig. 5E-F).

Figure 5. Morphologically normal follicle at day 0 (A), antral follicle after 6 days of culture in 50 ng/mL IGF-I + FSH (B), extruded follicle at 100 ng/mL IGF-I + FSH (C), and atretic follicle cultured for 18 days in 10 ng/mL IGF-I+ FSH (D). Histological sections showing normal antral follicles after 18 days of culture in α-MEM^{+} (E) or in 50 ng/mL IGF-I+ FSH (F). O: oocyte, GC: granulosa cells, A: antrum. Scale bar: 100 µm.



Similar to experiment 1, as early as day 6 of culture, antral cavity formation was observed in all treatments ($P<0.05$; Fig. 6A). However, after 18 days, the medium containing 10 ng/mL IGF-I + FSH showed the lowest rate of antral cavity formation ($P<0.05$; Fig. 6A). The follicular diameter increased significantly in all treatments on day 6 of culture compared to day 0 (Fig. 6B). However, after 18 days, there was no difference ($P>0.05$) either in the follicular diameter (Fig. 6B) or in the daily growth rate among the treatments (9.9 µm, 6.1 µm, 8.1 µm and 9.1 µm for α-MEM^{+}, 10, 50 and 100 ng/mL IGF-I + FSH, respectively). However, it is important to highlight that the interaction between 50 ng/mL IGF-I and FSH promoted a significant increase in the percentage of fully grown oocytes compared to the other treatments (52.5%, 44.6%, 71.7% and 56.4% for α-MEM^{+}, 10, 50 and 100 ng/mL IGF-I + FSH, respectively; Fig. 6C).

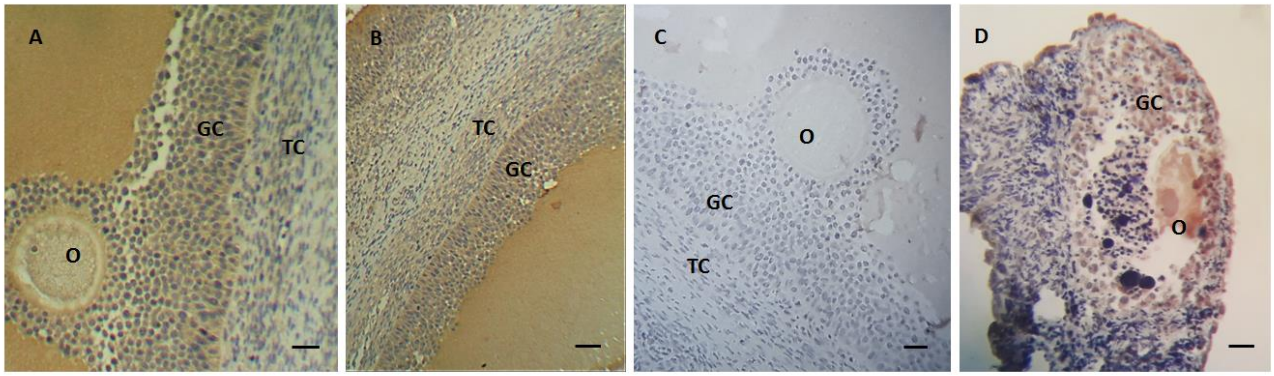
Figure 6. Experiment 2: antrum formation (A), follicular diameter (B), and percentages of fully grown oocytes (C) after *in vitro* culture in α-MEM^{+} or in different concentrations of IGF-I (10; 50 or 100 ng/mL) associated with FSH. ($^{a, b, c}$) Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$); ($^{A, B}$) Different letters denote significant differences among treatments in the same period ($P < 0.05$).



3.3.2. Expression of LHR in non-cultured antral follicles and in isolated follicles after culture in medium containing IGF-I and FSH

In the fresh ovaries, a moderate immunostaining for LHR protein was observed in oocytes, granulosa and theca cells of antral follicles (Fig. 7A-B). No staining was observed in the stromal cells neither in negative controls (Fig. 7C). After 18 days of culture in the association between 50 ng/mL IGF-I and FSH, it was possible to observe a strong reaction for LHR protein in the granulosa cells of antral follicles (Fig. 7D).

Figure 7. Immunolocalization of LHR protein in ovine non-cultured antral follicles (A-B), negative control (C), and isolated follicle cultured in the association between 50 ng/mL IGF-I and FSH (D). O: oocyte; GC: granulosa cells; TC: theca cells (400x). Scale bar: 50 µm.

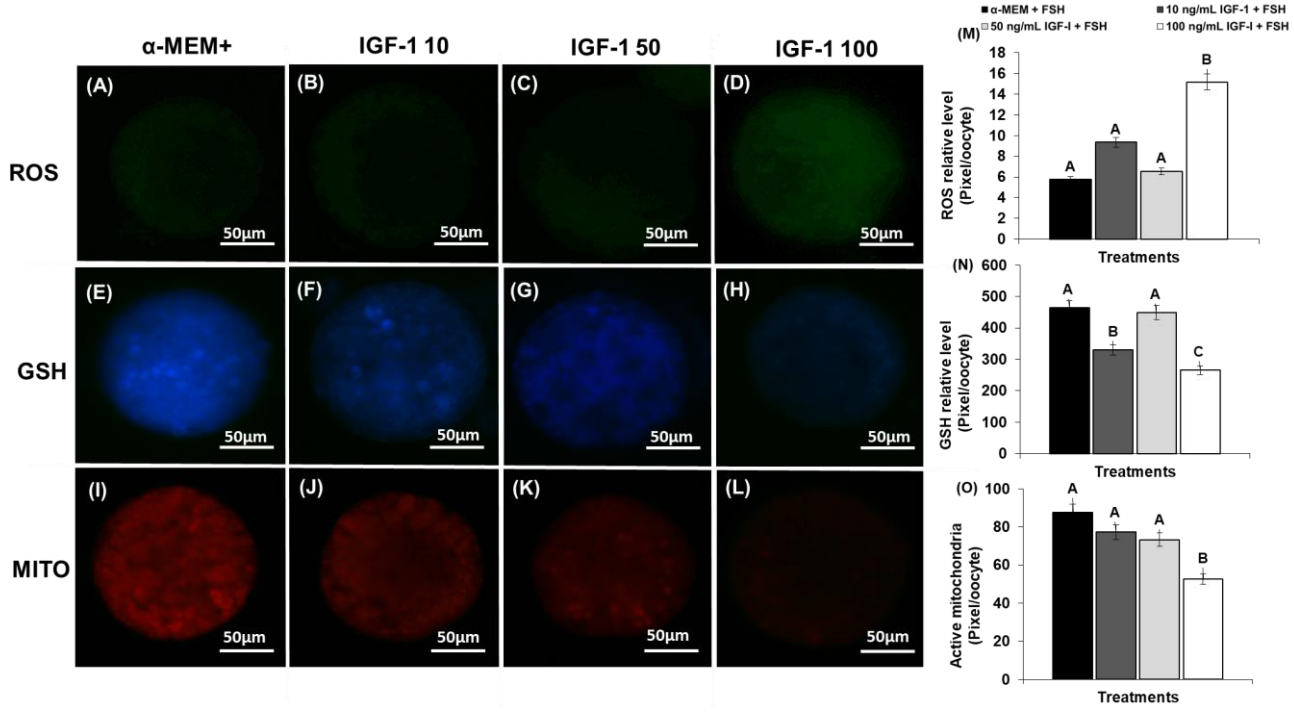


3.3.3. Intracellular levels of ROS, GSH and mitochondrial activity after culture

At day 18, the oocyte levels of ROS were higher ($P<0.05$) in the medium containing 100 ng/mL IGF-I + FSH than the other treatments (Fig 8A-D, and M). Moreover, GSH levels were similar ($P<0.05$) between the control medium alone or added with 50 ng/mL IGF-I + FSH and higher ($P<0.05$) than the medium containing 10 or 100 ng/mL IGF-I + FSH (Fig 8E-H, and N). In addition, mitochondrial activity was similar ($P<0.05$) between the control medium alone or medium supplemented with 10 or 50 ng/mL IGF-I + FSH ($P>0.05$) and higher ($P<0.05$) than 100 ng/mL IGF-I + FSH (Fig. 8I-L, and O).

Figure 8. Detection of intracellular levels of ROS, GSH, and mitochondrial activity in follicles cultured in α-MEM^{+} (A, E, I), 10 ng/mL IGF-I (B, F, J), 50 ng/mL IGF-I (C, G, K) or 100 ng/mL IGF-I (D, H, L). Intracellular levels of ROS (M), GSH (N), and active mitochondrial (O) in oocytes from different experimental groups.

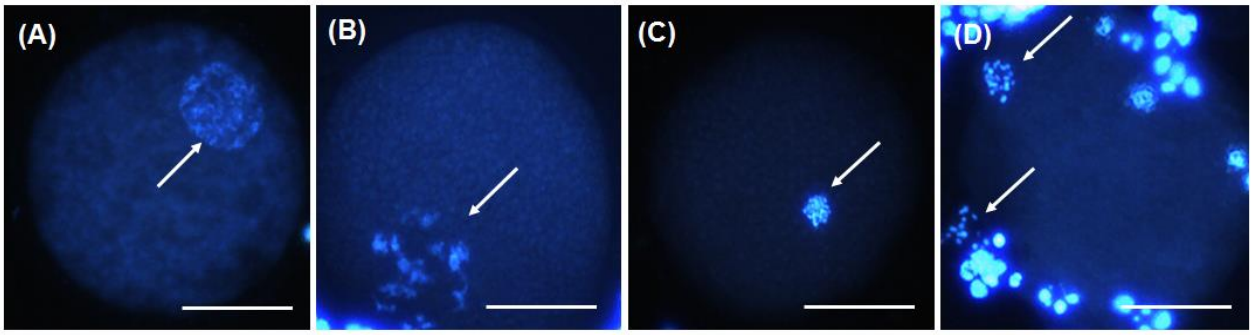
($^{A, B, C}$) Within each group (ROS, GSH, or mitochondrial function), bars with different letters are significantly different ($P < 0.05$). Scale bars: 50 µm.



3.3.4. Chromatin configuration and DNA fragmentation after IVM

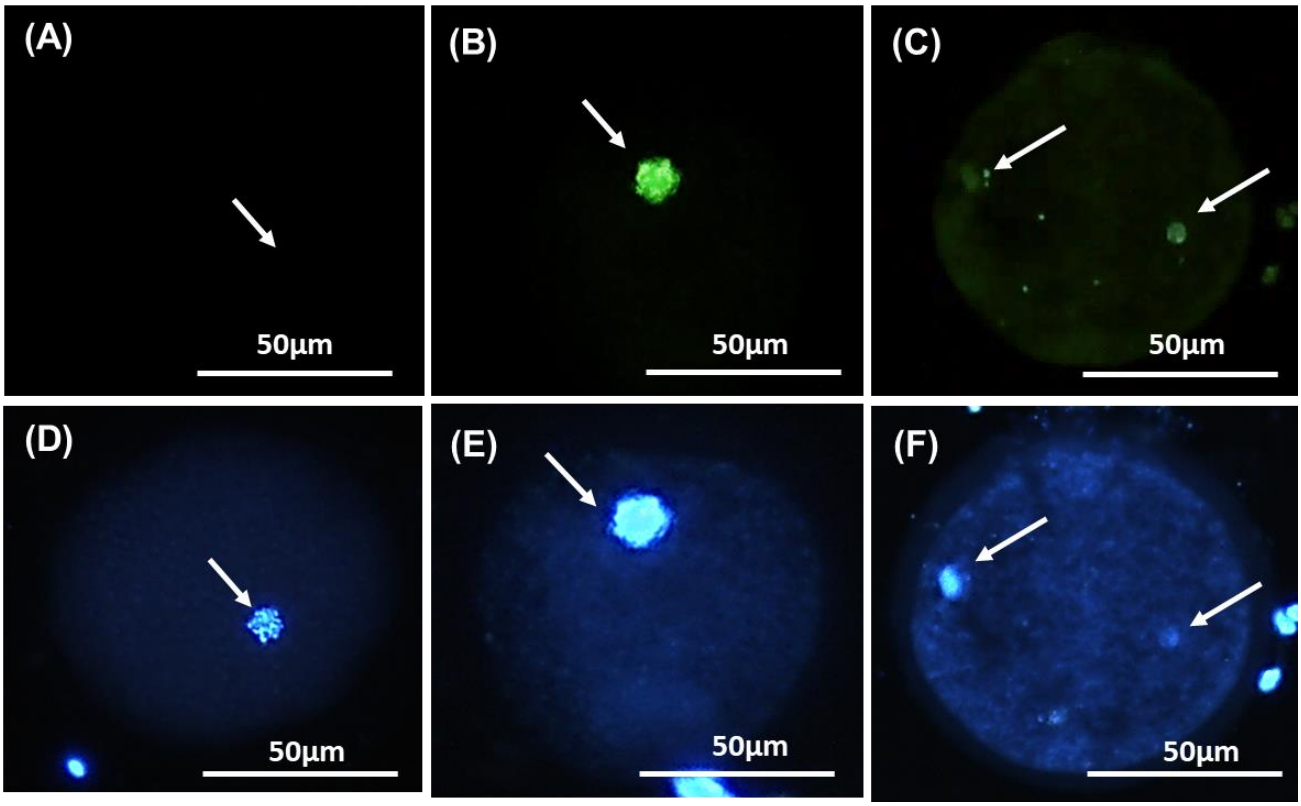
After the evaluation of the chromatin configuration, some oocytes cultured in the interaction between 50 ng/mL IGF-1 and FSH remained in GV (20.83%; Fig. 9A). However, the majority (79.16%) of the oocytes were able to resume meiosis (GVBD: 29.17% [Fig. 9B]; MI: 41.66% [Fig. 9C], and MII: 8.33% [Fig. 9D].

Figure 9. Epifluorescent photomicrographic images of ovine oocytes stained with Hoechst 33342 after IVM. Oocytes obtained from follicles cultured in 50 ng/mL IGF-1 + FSH in GV (A), GVBD (B), MI (C) or MII (D). Arrow: nuclear chromatin. Scale bars: 50 μm.



The TUNEL assay revealed the absence (Fig. 10A and D) or very few oocytes with DNA fragmentation after IVM. All oocytes were apoptotic in the positive control (Fig. 10B) and negative control did not show staining for TUNEL analysis (Fig. 10C). All oocytes showed chromatin stained with Hoechst 33342 in blue fluorescence (Fig. 10 D–G). No significant differences were found between the control medium and 50 ng/mL IGF-1 + FSH (data not shown).

Figure 10. Representative DNA fragmentation of sheep oocytes after 18 days of culture. Normal follicle (A, D), follicle with DNA fragmentation (B, E), and positive control (C and F). Ocytes stained with TUNEL (A, B, C) and Hoechst 33342 (D, E, F). Scale bars: 50 µm.



4. Discussion

This study demonstrated for the first time the immunolocalization of IGF-1 protein in sheep ovaries and the effects of IGF-1 alone or in association with FSH on the *in vitro* development of isolated secondary follicles and LHR protein expression in antral follicles. In the sheep, the IGF-1 protein was expressed in oocytes from all stages of follicle development (primordial, primary, secondary, and antral) and in the granulosa cells from secondary and antral follicles. Previously, IGF-1 mRNA have been localized in the ovaries of other species (human: [8]; mouse: [9; 10; 11]; bovine: [12; 13]; caprine: [14]; bubaline: [15], and IGF-1 protein was expressed in caprine [14]) and bubaline [15] ovarian follicles. Moreover, the IGF-1 mRNA was expressed in the granulosa and thecal cells of antral follicles [16; 17] and in oocytes and granulosa cells of preantral follicles [18] in sheep. It is noteworthy that IGF-1 expression is species specific, suggesting different mechanisms of action. Therefore, expression of IGF-1 protein in early follicular development supports the hypothesis that it could be involved in the ovarian function in sheep.

Using this knowledge, we further assess the effects of IGF-1 on the *in vitro* culture of ovine secondary follicles. However, IGF-1 did not influence follicular viability or growth as demonstrated by the results of experiment 1. Similarly, after seven days of culture of goat ovarian tissue, the presence of 50 or 100 ng/mL IGF-I had no influence on the follicular survival rate [24]. Nevertheless, addition of 10 ng/mL IGF-1 to the culture medium (TCM199) of sheep isolated secondary follicles enhanced the antrum formation and growth after short-term culture [5]. These different results may be explained by the differences in the base media used (α-MEM x TCM199). The content of some amino acids (arginine, glutamine, leucine, and tyrosine) is higher in TCM-199 than α-MEM [41]. Furthermore, TCM199 contain adenine sulphate, an exogenous precursor of adenosine, which would reduce ischemia and oxidative stress in ovarian follicles during *in vitro* culture [42].

Although we did not evaluate IGF-1 immunostaining after culture, it can be suggested that the presence of IGF-1 protein in the granulosa cells of ovine secondary and antral follicles might be sufficient to support follicular integrity and growth observed in our control medium (α-MEM^{+} without IGF-1). Moreover, a previous study with IGF-1 null mice has indicated that IGF-I is an indispensable component of the ovulatory pathway, but not an obligatory participant in the early follicular development [43], which may explain our results in experiment 1. These authors also observed an interdependence of the gonadotropin and IGF-I signaling pathways [43]. In mice, the effect of IGF1 depend on the follicle size because it promoted the growth of early secondary follicles (under 150 μm), while the growth of follicles with a diameter of over 150 μm was not promoted [11]. The latter study indicated that the growth of follicles over 150 μm mainly depended on FSH and LH. In fact, IGF-1 can act synergistically with FSH to increase preantral follicle growth and estradiol production in mice [29]. Therefore, we hypothesized that the association between IGF-1 and FSH (experiment 2) could enhance the *in vitro* development of ovine secondary follicles.

Although similar among treatments, it is important to note that the rates of normal follicles in experiment 2 (average of 97.5%) were higher than those observed in experiment 1 (average of 68.7%), in which FSH was not present in the media. In addition, more antral follicles were observed at day 6 of culture in experiment 2 (54.7%) compared to experiment 1 (18.8%). Taken together, these findings demonstrate the importance of FSH to the maintenance of follicular viability and antrum formation [39; 44]. Compared to the control medium with FSH, addition of 50 ng/mL IGF-1 + FSH to the culture medium did not result in any additive effect either in follicular morphology or antrum formation in both caprine [45] and bovine species [46], or in the viability of bovine follicles [47]. Moreover, similar rates of normal follicles were observed after seven days of culture of goat

ovarian tissue in the control medium and in the medium containing 100 ng/mL IGF-I combined with FSH [48].

However, both 50 ng/mL IGF-1 and FSH are apparently required for increasing the percentage of fully grown oocytes (70%). These data support the hypothesis that the association between IGF-1 and FSH has a synergistic effect on follicle and oocyte development in sheep, which was also reported in other *in vitro* studies. For example, in caprine species, the percentages of fully grown oocytes after 18 days of culture were also higher in follicles cultured in IGF-I (50 or 100 ng/mL) + FSH than in the control group [28]. In addition, FSH acts synergistically with IGF-1 to increase bovine granulosa cell proliferation when compared to IGF-1 alone, suggesting that FSH enhance the sensitivity of granulosa cells to the mitotic effect of IGF-1 [49]. An important finding of the present study is that after *in vitro* culture in medium containing 50 ng/mL IGF-1 + FSH, a high proportion of the oocytes resumed meiosis (79.16%) and 8.33% of them were mature (metaphase II). Therefore, these results present an advantage over those reported by Luz et al. [50] in which ovine follicles cultured in medium containing IGF-1 had 51.7% of fully grown oocytes, 60% of meiosis resumption and none of the oocytes reached metaphase II stage. However, we believe that our meiotic resumption rate would be enhanced in an extended maturation period (36 to 40 hours) as performed in other studies [37; 51].

IGF-I is capable of synergizing with FSH, increasing the expression of LHR mRNA in rat granulosa cells in a dose-and time-dependent manner [30]. Thus, we further analyzed the expression of LHR protein in non-cultured antral follicles (from fresh ovarian tissue) and in the antral follicles obtained from the culture with the association between 50 ng/mL IGF-1 and FSH. To our knowledge, this is the first study that demonstrates the expression of LHR protein in oocytes, granulosa and theca cells of antral follicles, suggesting that LH may have a physiological role in sheep follicles at this stage. Interestingly, similarly to our findings, the presence of mRNA for LHR was also observed in mouse and human oocytes from antral follicles [52; 53]. The expression of LHR in oocytes may indicate a new mechanism for oocyte maturation mediated by gonadotrophins directly [53]. The mRNA for LHR was also demonstrated in ovarian cells of rat [30], mouse [54], bovine [55; 56], canine [57], and human [58; 59] follicles. In this study, it is important to highlight that an increase of the LHR protein expression in oocytes and granulosa cells (from moderate to strong immunostaining) was observed after culture of the follicles in medium containing both 50 ng/mL IGF-1 and FSH. The expression of LHR is one of the major markers of the FSH-induced differentiation of granulosa cells, and this process is also modified by many growth factors [30]. Therefore, as suggested by Hirakawa et al., [30], we may speculate that IGF-1 has a primary effect on FSH receptor expression that secondarily potentiates FSH action and results in augmentation of

LHR protein expression in the granulosa cells. Other study has also demonstrated that IGF-1 increased the expression of LHR mRNA in the granulosa cells of small and large bovine antral follicles [60]. Moreover, in the presence of FSH, IGF-1 also increases LHR synthesis in rat theca-interstitial cells [61] and in granulosa cells [62] from antral follicles. Our data may also indicate a physiological role of the LHR in the oocyte maturation process. Further studies are required to elucidate these mechanisms completely.

Similar levels of ROS, GSH and metabolically active mitochondria were noted in the control medium with FSH or in medium containing 50 ng/mL IGF-1 and FSH. Therefore, it appears that these findings may have contributed to the same rates of DNA fragmentation observed between these treatments. Nevertheless, 100 ng/mL IGF-1 and FSH was not adequate for oocyte development because this combination increased ROS levels and decreased GSH and mitochondrial activity. These data illustrate the dose-dependent effects of IGF-1 in ovarian function.

In conclusion, the IGF-1 protein is expressed in all ovarian follicle stages in sheep. Moreover, the association between 50 ng/mL IGF-1 and FSH has a synergistic effect *in vitro*, increasing the percentage of fully grown oocytes and the expression of LHR protein in oocytes and granulosa cells of antral follicles. Therefore, the synergistic action of IGF-I with FSH is likely to be of key importance for maintaining a critical level of LHR expression for oocyte maturation in the sheep ovary.

5. Acknowledgements

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Figure captions

Figure 1. Immunolocalization of IGF-1 protein (A) in sheep ovarian follicles: primordial (A), primary (B), secondary (C), antral (D) follicle, and negative control (E). O: oocyte; GC: granulosa cells; TC: theca cells. (400x). Scale bar: 50 μm.

Figure 2. Morphologically normal follicle at day 0 (A), antral follicle after 6 days of culture in 50 ng/mL IGF-1 (B), extruded follicle at 100 ng/mL IGF-I (C), atretic follicle at 10 ng/mL IGF-1 (D), viable follicle after culture in 50 ng/mL IGF-1 (E), and non-viable follicle cultured in α-MEM (F) for 18 days. GC: granulosa cell; O: oocyte. A: antrum. Scale bar: 100 μm.

Figure 3. Percentages of morphologically normal follicles cultured in α-MEM^{+} or in different concentrations of IGF-1 (10; 50 or 100 ng/mL).
($^{a, b, c}$) Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$)

Figure 4. Experiment 1: antrum formation (A) and follicular diameter (B) after *in vitro* culture in α-MEM^{+} or in different concentrations of IGF-1 (10; 50 or 100 ng/mL).
($^{a, b, c}$) Different letters denote significant differences among culture periods in the same treatment ($P < 0.05$); ($^{A, B, C}$) Different letters denote significant differences among treatments in the same period ($P < 0.05$).

Figure 5. Morphologically normal follicle at day 0 (A), antral follicle after 6 days of culture in 50 ng/mL IGF-1 + FSH (B), extruded follicle at 100 ng/mL IGF-I + FSH (C), and atretic follicle cultured for 18 days in 10 ng/mL IGF-1+ FSH (D). Histological sections showing normal antral follicles after 18 days of culture in α-MEM^{+} (E) or in 50 ng/mL IGF-1+ FSH (F). O: oocyte, GC: granulosa cells, A: antrum. Scale bar: 100 μm.

Figure 6. Experiment 2: antrum formation (A), follicular diameter (B), and percentages of fully grown oocytes (C) after *in vitro* culture in α-MEM^{+} or in different concentrations of IGF-1 (10; 50 or 100 ng/mL) associated with FSH.

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

Figure 7. Immunolocalization of LHR protein in ovine non-cultured antral follicles (A-B), negative control (C), and isolated follicle cultured in the association between 50 ng/mL IGF-1 and FSH (D). O: oocyte; GC: granulosa cells; TC: theca cells (400x). Scale bar: 50 µm.

Figure 8. Detection of intracellular levels of ROS, GSH, and mitochondrial activity in follicles cultured in α-MEM^{+} (A, E, I), 10 ng/mL IGF-1 (B, F, J), 50 ng/mL IGF-1 (C, G, K) or 100 ng/mL IGF-1 (D, H, L). Intracellular levels of ROS (M), GSH (N), and active mitochondrial (O) in oocytes from different experimental groups.
($^{A, B, C}$) Within each group (ROS, GSH, or mitochondrial function), bars with different letters are significantly different ($P < 0.05$). Scale bars: 50 µm.

Figure 9. Epifluorescent photomicrographic images of ovine oocytes stained with Hoechst 33342 after IVM. Oocytes obtained from follicles cultured in 50 ng/mL IGF-1 + FSH in GV (A), GVBD (B), MI (C) or MII (D). Arrow: nuclear chromatin. Scale bars: 50 µm.

Figure 10. Representative DNA fragmentation of sheep oocytes after 18 days of culture. Normal follicle (A, D), follicle with DNA fragmentation (B, E), and positive control (C and F). Ocytes stained with TUNEL (A, B, C) and Hoechst 33342 (D, E, F). Scale bars: 50 µm.

7. CAPÍTULO 2

Growth and differentiation factor-9 (GDF-9) reduces apoptosis and promotes *in vitro* activation of sheep primordial follicles through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway

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Running head: GDF-9 and the PI3K/AKT pathway in ovine ovary

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Abstract

This study aimed to evaluate the effects of GDF-9 on the morphology, activation, apoptosis and granulosa cell proliferation of ovine preantral follicles cultured *in situ* and to verify whether GDF-9 could improve follicular activation through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway. Ovine ovarian fragments were fixed for histological analysis (fresh control) or cultured in α-MEM$^+$ (control) or α-MEM$^+$ with GDF-9 (1, 50, 100, 200 or 400 ng/mL) for 7 days. Cleaved caspase-3 and PCNA analyses were performed in the fresh control, α-MEM$^+$ and 1 or 50 ng/mL GDF-9. Inhibition of PI3K activity was performed through pretreatment with LY294002 and phosphorylated AKT (pAKT) expression was analyzed after culture in the absence or presence of LY294002. The results showed that 50 ng/mL GDF-9 had ($P<0.05$) more morphologically normal follicles when compared to all treatments, except 1 ng/mL GDF-9. Moreover, 50 ng/mL GDF-9 increased primordial follicle activation compared to all treatments, except α-MEM$^+$ and 1 ng/mL GDF-9. At 50 ng/mL GDF-9, the percentage of PCNA-positive cells was significantly higher than other treatments, and the rate of apoptotic follicles was lower than all treatments (apoptosis was similar to the fresh control). The pretreatment of the ovarian tissue with LY294002 significantly inhibited the activation of primordial follicles stimulated by both α-MEM$^+$ and 50 ng/mL GDF-9 and reduced pAKT expression in the follicles. In conclusion, GDF-9 at 50 ng/mL maintains follicular survival and promotes primordial follicle activation by reducing apoptosis and stimulating granulosa cell proliferation through activation of the PI3K/AKT pathway.

Keywords: Ovary. Organ culture. Preantral follicle. Intracellular signaling. Ovine

Introduction

In vitro culture of ovarian tissue is an important tool to investigate early follicle biology and the factors that promote and/or restrict the initial steps of folliculogenesis (Shea et al. 2014), especially the activation of dormant primordial follicles. Considering that primordial follicles constitute the entire ovarian reserve of the female (Monniaux et al., 2014), an *in vitro* activation protocol could benefit infertile patients with a reduced pool of primordial follicles by providing a large supply of mature oocytes from human and other mammals, including endangered species and economically important animals (Li et al., 2010). Using *in vitro* culture, several ovarian paracrine factors have been found to be important for the activation of primordial follicles, including growth and differentiation factor-9 (GDF-9).

GDF-9 is a member of the superfamily of transforming growth factor-β (TGF-β), known to be important regulators of cell proliferation, growth and follicular survival (Vitt et al., 2000; Orisaka et al., 2006; Dipaz-Berrocal et al., 2017). Supplementation of the culture medium with GDF-9 maintained follicular survival and/or promoted *in vitro* activation of primordial follicles, which develop into primary and secondary follicle stage (human: Hreinsson et al., 2002; caprine: Martins et al., 2008; bovine: Tang et al., 2012). Moreover, after *in vitro* culture of isolated preantral follicles, GDF-9 reduced atresia, stimulated antrum formation (rat: Orisaka et al., 2006; caprine: Almeida et al., 2011), oocyte growth (mouse: Cook-Andersen et al., 2016) and the production of oocytes able to resume meiosis (caprine: Almeida et al., 2011). In sheep ovaries, GDF-9 has been localized in oocytes of all stages of follicle development (Bodensteiner et al., 1999; Mery et al., 2007; Scaramuzzi et al., 2011; Kona et al., 2015), which indicate that this factor has a key role in the folliculogenesis in this species (Dong et al., 1996). However, the effects of GDF-9 on the survival and activation of ovine primordial follicles using ovarian tissue culture is not yet known.

The biological effect of GDF-9 occurs after binding to bone morphogenetic protein (BMP) receptor type II (BMPR-II) (Mazerbourg et al., 2004), which is able to activate the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway (Orisaka et al., 2006), one of the major signaling pathways involved in the control of follicular survival and activation (Zhao et al., 2014). More specifically, GDF-9 improves rat secondary follicle growth and inhibits granulosa cell apoptosis through activation of the PI3K/AKT pathway (Orisaka et al., 2006). However, it is not known whether the PI3K/AKT pathway is involved in GDF-9 action in the sheep ovary. Therefore, pharmacological inhibition of this pathway, using PI3K specific inhibitors, could potentially give a better understanding of the regulatory mechanisms of GDF-9 (Granville et al. 2006; John et al. 2008, 2009).

Therefore, the aims of this study were (i) to analyze the effects of different concentrations of GDF-9 on the morphology, apoptosis, primordial follicle activation and growth after *in vitro* culture of sheep ovarian tissue, (ii) to evaluate whether GDF-9 could improve activation of primordial follicles through the activation of PI3K/AKT pathway.

Materials and methods

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

Source of ovarian tissue

Ovine ovaries (n=10) from adult crossbred sheep (n=5) were collected at a local slaughterhouse. Immediately postmortem, the ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil), and twice in 0.9% saline solution supplemented with antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). Next, ovaries were transported within 1 h to the laboratory

in tubes containing 0.9% saline solution and antibiotics at 4°C (Soares et al., 2018). The approval of the ethics committee was not required since the research involved tissues of slaughtered animals.

In vitro culture of ovarian tissue

The *in vitro* culture was performed according to Bezerra et al. (2018). In the laboratory, each ovarian pair was fragmented into slices of approximately 3 mm x 3 mm (1 mm thick) in size using a scalpel under sterile conditions. Subsequently, one ovary fragment of each animal was immediately fixed in 10% buffered formaldehyde (Dinâmica) for classical histology and served as fresh control. The remaining slices of the ovarian cortex were randomly distributed to different treatments and cultured individually for 7 days in 1 mL of culture medium in 24-well culture dishes at 39°C in an atmosphere of 5% CO_2 in the air. The base culture medium (control) consisted of α-MEM (pH 7.2–7.4) supplemented with 10 ng/mL insulin, 5.5 µg/mL transferrin, 5.0 ng/mL selenium, 2 mM glutamine, 2 mM hypoxantine, 50 µg/mL ascorbic acid and 1.25 mg/mL bovine serum albumin (BSA), which was referred to α-MEM$^+$. For the experimental conditions, the fragments were distributed in α-MEM$^+$ (control medium) or in medium supplemented with GDF-9 at different concentrations (1, 50, 100, 200 or 400 ng/mL). The concentrations of GDF-9 were chosen based on a previous study (Martins *et al.* 2008) in which this factor has been used for *in vitro* culture of goat ovarian cortex. The culture medium was replenished every two days. Each treatment was repeated five times.

Morphological analysis of preantral follicles

Tissues from all treatments (fresh control, control medium and GDF-9 treatments) were fixed in 10% buffered formaldehyde (Dinâmica) for 18 h and then dehydrated in increasing

concentrations of ethanol (Dinâmica). After paraffin embedding (Dinâmica), ovarian cortex were cut into 5 µm sections, and every section was mounted on glass slides and stained with hematoxylin and eosin (Vetec, São Paulo, Brazil). The preantral follicles were evaluated by light microscopy (Nikon, Tokyo, Japan; 400x magnification) in the section where the oocyte nucleus was visible. The follicles were classified as histologically normal when an intact oocyte was present and surrounded by granulosa cells that were well organized and had no pyknotic nuclei. The atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. Overall, 150 follicles were evaluated for each treatment (30 follicles per treatment x 5 replicates = 150 follicles), totaling 1,050 preantral follicles.

Assessment of the in vitro follicular activation and growth

The evaluation of the follicular activation (transition from primordial to growing follicles, when surrounding squamous pregranulosa cells become cuboidal and begin to proliferate) was performed by quantifying the follicles at different stages of follicular development (Silva et al. 2004), as primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened to cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells, and secondary: two or more layers of cuboidal granulosa cells around the oocyte). The proportion of primordial and growing follicles was calculated at day 0 (fresh control) and after 7 days of culture and only histologically normal follicles were recorded. In addition, from the basement membrane, the major and minor axes of each oocyte and follicle were measured by using the Image-Pro Plus® software (Media Cybernetics, Warrendale, PA, USA). The average of these two measurements was used to determine the diameters of both the oocyte and the follicle.

Immunohistochemistry

After histological analysis, for a more in-depth evaluation of follicular quality, immunohistochemical analyses were performed in the fresh control and in treatments that showed the best results of follicular morphology and activation *in vitro*. For this, additional pairs of sheep ovaries (n=10 ovaries) were collected, transported to the laboratory, fragmented and cultured in α-MEM$^+$ or in α-MEM$^+$ supplemented with 1 or 50 ng/mL GDF-9 as described above. Immunohistochemistry was performed as described previously (Bezerra et al., 2018) with some modifications. Briefly, sections (4 μm thick) were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted in Starfrost glass slides (Knittel, Braunschweig, Germany). The slides were incubated in citrate buffer (Dinâmica) at 95ºC in a deckloaking chamber (Biocare, Concord, USA) for 40 min to retrieve antigenicity, and endogenous peroxidase activity was prevented by incubation with 3% H_2O_2 (Easypath) for 10 min. Nonspecific binding sites were blocked using 1% normal goat serum (Easypath) and diluted in phosphate-buffered saline (PBS). Subsequently, the sections were incubated in a dark humidified chamber for 50 min at room temperature with (i) rabbit polyclonal anti-activated caspase-3 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and (ii) rabbit polyclonal anti-PCNA (proliferating cell nuclear antigen; 1:300; Santa Cruz Biotechnology). Thereafter, the sections were incubated for 20 min polymer EasyLink One (Easypath). Protein localization was demonstrated with diaminobenzidine (DAB; Easypath), and the sections were counterstained with hematoxylin (Vetec) for 1 min. The negative control underwent all steps except the primary antibody incubation.

For apoptotic marker and proliferating cell assay, only follicles that contained an oocyte nucleus were analyzed using light microscope (Nikon) connected to a computer equipped with Image-Pro Plus® software (Media Cybernetics). For activated caspase-3 expression, follicles containing at least one positively stained cell (oocyte/granulosa cell) were counted manually, and

the follicle was considered apoptotic (Desmeules and Devine, 2006). The percentage of apoptotic follicles was calculated as the number of apoptotic follicles out of the total number of follicles (× 100). The number of PCNA-positive granulosa cells (brown staining) was counted in 10 random fields per treatment and the percentage of PCNA-positive cells was calculated as the number of proliferating cells out of the total number of cells (× 100).

Pharmacologic inhibition of PI3K pathway

To evaluate the effect of the pharmacological inhibition on the PI3K pathway on primordial follicle survival and activation *in vitro*, additional pairs of sheep ovaries (n=8 ovaries) were collected, transported to the laboratory and fragmented as described above. The ovarian fragments were cultured in the control medium (α-MEM$^+$) or in α-MEM$^+$ supplemented with 50 ng/mL GDF-9 without the PI3K inhibitor, or in these treatments in the presence of the PI3K inhibitor (α-MEM$^+$ + PI3K inhibitor or 50 ng/mL GDF-9 + PI3K inhibitor). For PI3K inhibition, 10 µM of the PI3K-specific inhibitor LY294002 (Cell Signaling Technologies, Danvers, USA) was added to the control medium (α-MEM$^+$) and incubated for 30 min (before GDF-9 supplementation) at 39 °C under 5% CO_2 in the air. Thereafter, the ovarian tissues were further cultured for 7 days. Media were changed and treatments replenished every two days (including the inhibitor). The concentration (10 µM) and incubation period (30 min) of LY294002 were chosen according to Orisaka et al. (2006). After this culture, the ovarian cortex was fixed and processed for histological evaluation as described above. Preantral follicles were examined for morphological aspects (morphologically normal or atretic) and developmental stages (primordial or growing follicles).

Immunohistochemistry for the evaluation of the expression of phosphorylated AKT (pAKT)

To check the effectiveness of LY294002 in the PI3K pathway suppression, we evaluated AKT phosphorylation (pAKT) in the ovarian cortex, an indirect measurement of PI3K activity and a marker of primordial follicle activation (Orisaka et al., 2006). Following *in vitro* culture for 7 days, the ovarian fragments from the cultured control (α-MEM^{+}) or those treated with 50 ng/mL GDF-9 in the absence or presence of the PI3K inhibitor were destined to immunohistochemistry that was carried out by the same steps that were previously mentioned, except for the antibody used, which was rabbit polyclonal anti-p-AKT (1:40; Santa Cruz Biotechnology). Using a light microscope (Nikon) under 400x magnification, only follicles that contained an oocyte nucleus were analyzed and the immunostaining (brown staining) was classified as absent, weak, moderate or strong.

Statistical analysis

The percentages of morphologically normal (survival), primordial and growing follicles (activation) from both cultures (without and with the PI3K inhibitor), PCNA-positive cells, and apoptotic follicles were compared by Chi-squared test. Data of follicular and oocyte diameters were submitted to Shapiro Wilk test. Therefore, ANOVA and the Tukey´s tests were applied for comparison among treatments. The results were expressed as the mean ± SEM. The differences were considered to be statistically significant when $P<0.05$.

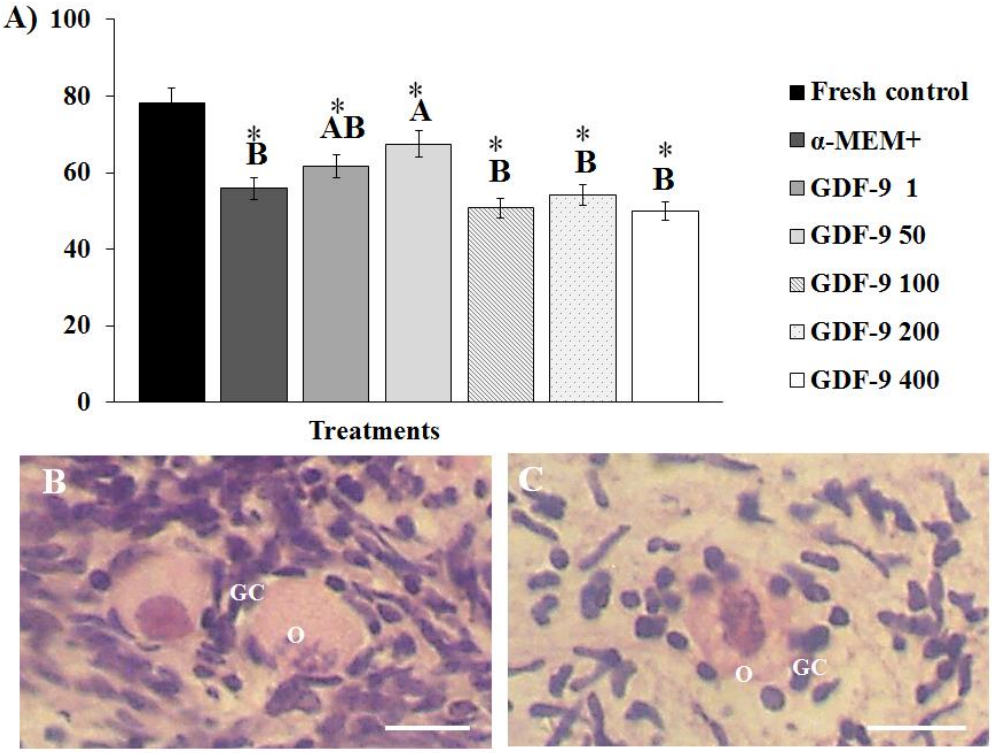
Results

Follicular morphology and in vitro activation

The percentage of morphologically normal follicles (survival) decreased significantly after 7 days of culture in all treatments compared to the fresh control (78.33%) (Fig. 1A). In addition, the concentration of 50 ng/mL GDF-9 (67.5%) presented similar ($P>0.05$) percentages of morphologically normal follicles as observed in 1 ng/mL GDF-9 (61.7%), but significantly more normal follicles than α-MEM$^+$ (55.83%) or 100 (50.8%), 200 (54.2%) and 400 (50.0%) ng/mL GDF-9. The preantral follicles cultured in the presence of 50 ng/mL GDF-9 had centrally located oocytes rounded by well-organized granulosa cells (Fig. 1B), while atretic follicles with retracted oocyte, pyknotic nucleus and disorganized granulosa cells detached from the basement membrane could be observed after culture in α-MEM$^+$ (Fig. 1C).

Fig. 1 Percentages of morphologically normal follicles in the fresh control and after *in vitro* culture in α-MEM^{+} or in different concentrations of GDF-9 (A). Histological sections of ovine ovarian fragments showing normal follicles after *in vitro* culture in medium containing 50 ng/mL GDF-9 (B) and atretic follicle in the control medium (C). O: oocyte; GC: granulosa cells. Scale bars: 25 µm (400x).

*Differs significantly from fresh control (P<0.05). ($^{A, B}$) Different letters denote significant differences among treatments (P<0.05)



After culture, there was a significant reduction in the percentage of primordial follicles (Fig. 2A) and an increase in the percentage of growing follicles (Fig. 2B) in all treatments compared to fresh control. Furthermore, the treatment containing 50 ng/mL GDF-9 showed higher (P<0.05)

follicular activation (81.48% growing follicles) than 100 (72.13%), 200 (76.92%) and 400 (76.67%) ng/mL GDF-9, and similar to α-MEM^{+} (80.60% growing follicles) and 1 ng/mL GDF-9 (78.38%) (P>0.05). However, the concentration of 1 ng/mL of GDF-9 significantly decreased follicular and oocyte diameters compared to the fresh control and 100 ng/mL GDF-9 (Table 1).

Fig. 2 Percentages of primordial (A) and growing (B) follicles in the fresh control and after *in vitro* culture in α-MEM^{+} or in different concentrations of GDF-9.

*Differs significantly from fresh control (P < 0.05). ($^{A, B, C}$) Different letters denote significant differences among treatments (P<0.05)

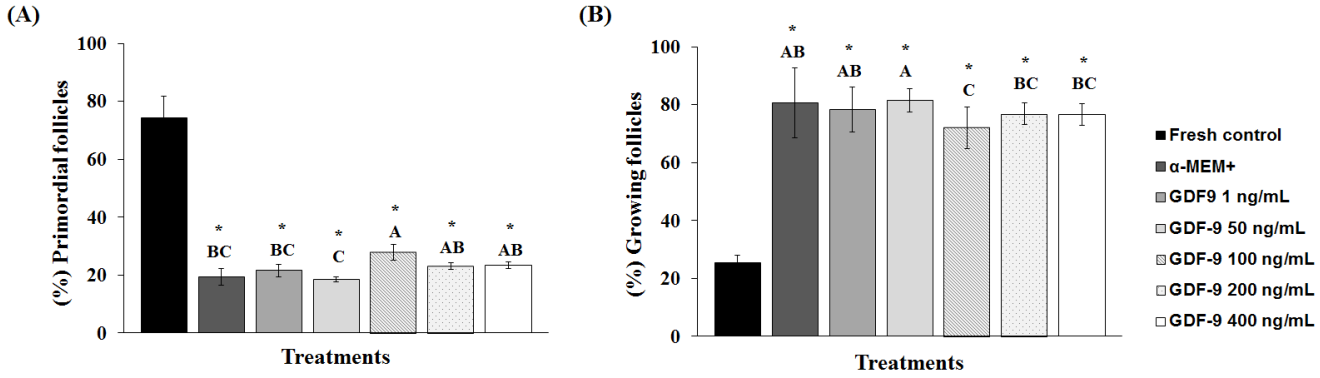


Table 1. Mean (mean ± SEM) follicular and oocyte diameters in the fresh control and after *in vitro* culture of sheep ovarian tissue in control medium (α-MEM^{+}) or in different concentrations of GDF-9.

Treatments	Follicular diameter (µm)	Oocyte diameter (µm)
Fresh control	49.93 ± 2.15	34.38 ± 2.03
α-MEM^{+}	44.8 ± 2.88 ^{AB}	29.80 ± 2.85 ^{AB}
1 ng/mL GDF-9	43.30 ± 2.72 *^{B}	28.86 ± 2.64 * ^{B}
50 ng/mL GDF-9	44.96 ± 3.24 ^{AB}	29.78 ± 2.99 ^{AB}
100 ng/mL GDF-9	47.83 ± 2.99 ^{A}	34.16 ± 2.84 ^{A}
200 ng/mL GDF-9	44.29 ± 4.12 ^{AB}	30.96 ± 3.87 ^{AB}
400 ng/mL GDF-9	46.69 ± 2.84 ^{AB}	32.93 ± 2.80 ^{AB}

* Differs significantly from fresh control (P<0.05).

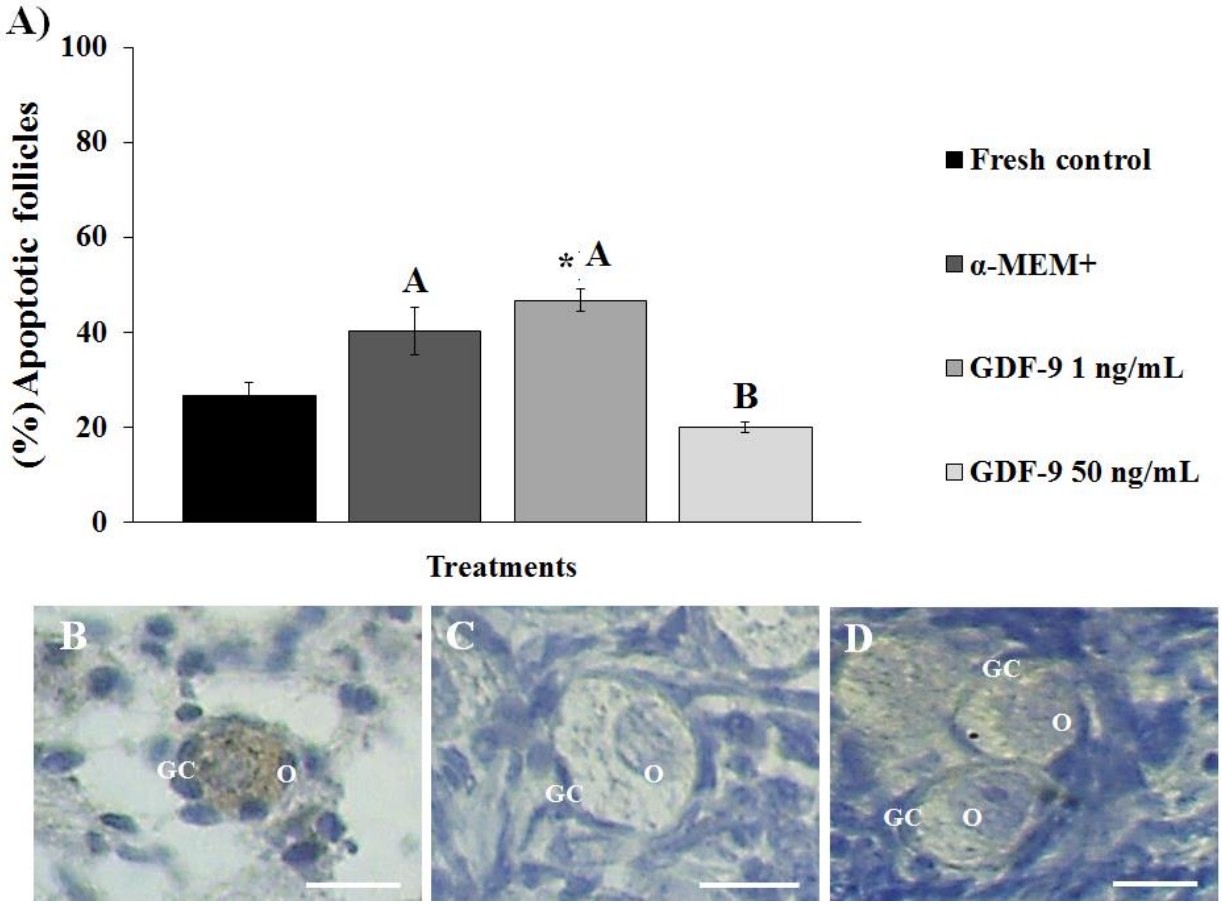
(^{A,B}) Different letters denote significant differences among treatments (P<0.05).

Evaluation of apoptosis and granulosa cell proliferation

Immunohistochemical analyses for apoptosis (activated caspase-3) and proliferating cell (PCNA) markers were performed in the fresh control and in treatments that showed the best results for follicular morphology and activation *in vitro* (control medium and media containing 1 or 50 ng/mL GDF-9). Culture of ovarian tissue in 50 ng/mL GDF-9 maintained the percentage of follicular apoptosis similar (P>0.05) to that observed in the fresh control and significantly lower than that observed in the α-MEM^{+} and 1 ng/mL GDF-9 (Fig. 3A-C).

Fig. 3 Percentages of apoptotic follicles in the fresh control and after *in vitro* culture in α-MEM^{+} or in 1 or 50 ng/mL of GDF-9 (A). Immunohistochemical positive detection of activated caspase-3 (follicular apoptosis) after culture in α-MEM^{+} (B) and negative detection after culture in 50 ng/mL GDF-9 (C); negative control for immunohistochemical analysis (D). O: oocyte; GC: granulosa cells. Scale bars: 25 µm (400x).

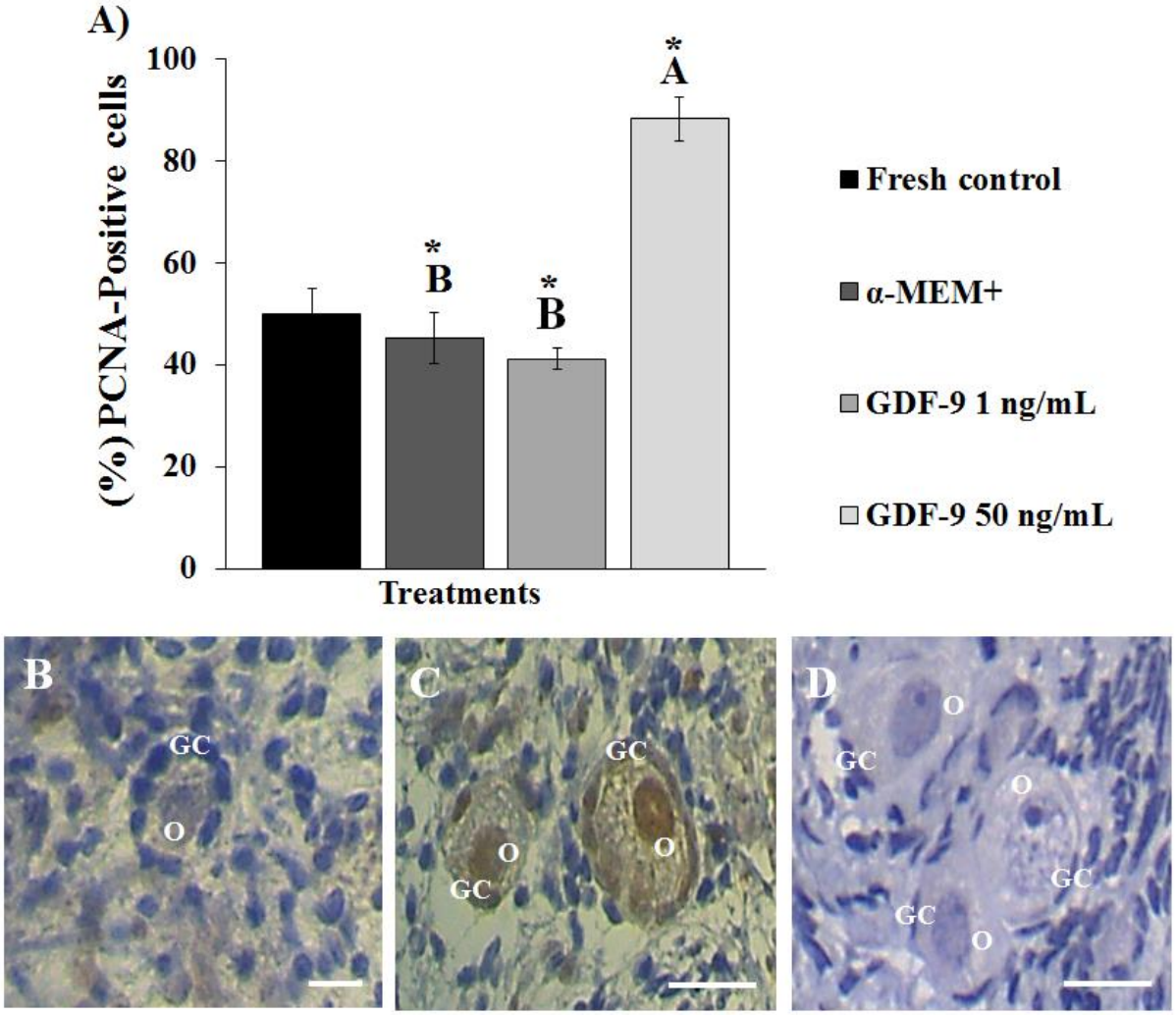
*Differs significantly from fresh control ($P<0.05$). ($^{A,\ B}$) Different letters denote significant differences among treatments ($P<0.05$)



The percentage of PCNA-positive cells in 50 ng/mL GDF-9 was significantly higher than in the fresh control, α-MEM^{+} and 1 ng/mL GDF-9 (Fig. 4A-C). Negative control did not show staining for immunohistochemical analyses (Fig. 3D and 4D).

Fig. 4 Percentages PCNA-positive cells in the fresh control and after *in vitro* culture in α-MEM^{+} or in 1 or 50 ng/mL of GDF-9 (A). Immunohistochemical negative detection of proliferating cell after culture in α-MEM^{+} (B) and positive detection after culture in 50 ng/mL GDF-9 (C); negative control (D). O: oocyte; GC: granulosa cells. Scale bars: 25 μm (400x).

*Differs significantly from fresh control (P < 0.05). ($^{A, B, C}$) Different letters denote significant differences among treatments (P<0.05)



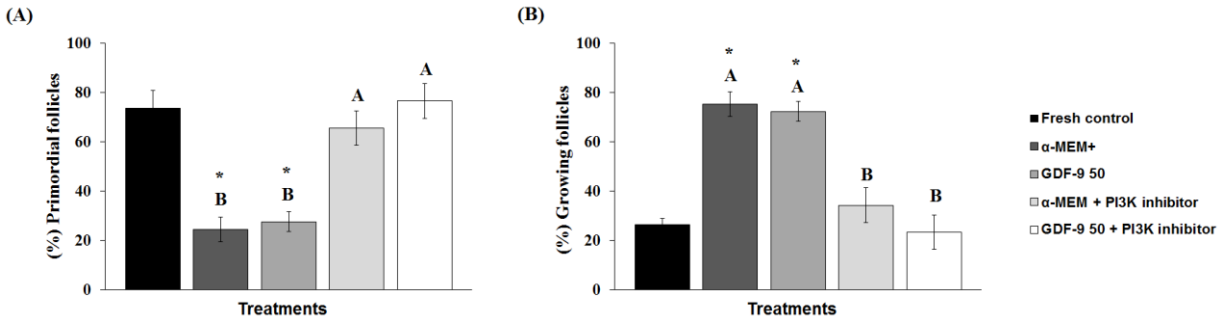
Pharmacologic inhibition of PI3K pathway

In vitro culture of ovarian cortex with PI3K inhibitor did not influence (P>0.05) the percentage of normal follicles (data not show). As observed previously, in the absence of the

inhibitor LY294002, follicular activation was significantly higher after culture in both α-MEM^{+} (75.34%) and 50 ng/mL GDF-9 (72.31%) than fresh control (26.37%). Moreover, there was no difference (P>0.05) between the treatments (Fig. 5). Nevertheless, the pretreatment of the ovarian tissue with LY294002 significantly inhibited the primordial follicle activation stimulated by both α-MEM^{+} and 50 ng/mL GDF-9. In these treatments, the percentage of primordial and growing follicles was similar (P>0.05) to the fresh control after culture (Fig. 5).

Fig. 5 Percentages of normal primordial (A) and growing (B) follicles in the fresh control, after *in vitro* culture in α-MEM^{+} or in 50 ng/mL GFD-9 in the absence or presence of LY294002 (PI3K inhibitor).

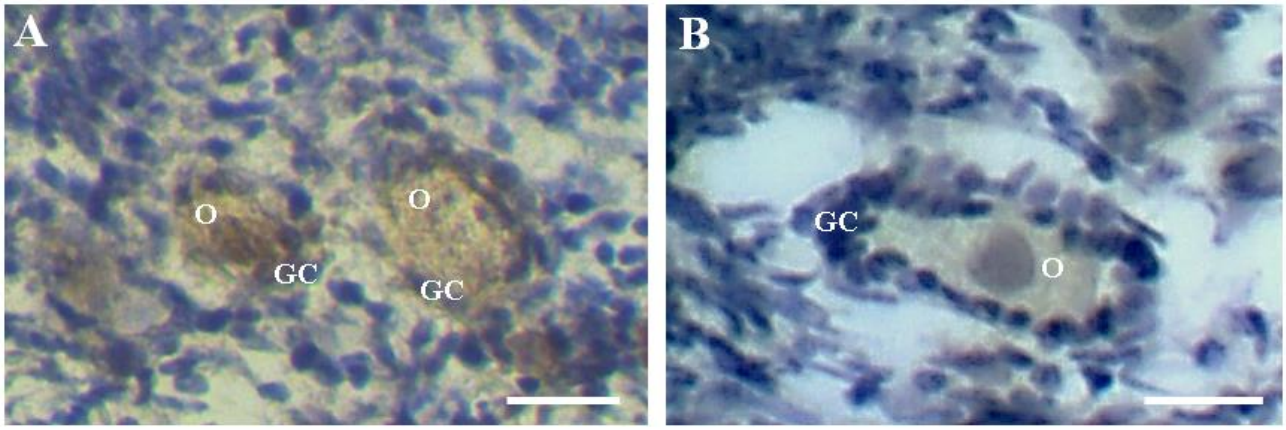
*Differs significantly from fresh control (P<0.05). ($^{A,\ B}$) Different letters denote significant differences among cultured treatments (P<0.05)



Effect of PI3K inhibition on pAKT protein staining

After *in vitro* culture in the absence of the PI3K inhibitor (α-MEM^{+} and 50 ng/mL GDF-9), immunohistochemistry staining demonstrated that oocytes from primordial or intermediate follicles showed a moderate staining for pAKT (Fig. 6A). Nevertheless, after PI3K inhibition, pAKT immunostaining in oocytes was markedly reduced (weak staining) in these treatments (Fig. 6B). Negative control did not show staining for pAKT.

Fig. 6 Immunohistochemical expression of pAKT in ovine ovary: follicles after 7 days of *in vitro* culture in 50 ng/mL GDF-9 in the absence (A) or presence (B) of LY294002 (PI3K inhibitor). GC: granulosa cells; O: oocyte. Scale bars: 20 µm (400x).



Discussion

The present study showed that 50 ng/mL GDF-9 preserved follicle morphology and decreases apoptosis, as well as enhances *in vitro* activation of primordial follicles and granulosa cell proliferation after culture of ovine ovarian tissue. In addition, we demonstrated that inactivation of the PI3K pathway inhibited primordial follicle growth and that AKT phosphorylation is involved in GDF-9 action in the sheep ovary.

After *in vitro* culture, the concentration of 50 ng/mL GDF-9 showed higher percentage of histologically normal follicles than control medium (α-MEM^{+}) and other GDF-9 concentrations (except to 1 ng/mL GDF-9). Furthermore, it is clear that culture in 50 ng/mL GDF-9 may have beneficial effects on the ovarian tissue because the follicle apoptosis rate in this treatment was only 20% (similar to fresh control) versus 40.18% and 46.67% in α-MEM^{+} and 1 ng/mL GDF-9, respectively. Previous studies have also demonstrated that GDF-9 maintains survival of preantral follicles after ovarian tissue culture (human: Hreinsson et al., 2002; caprine: Martins et al., 2008;

bovine: Tang et al., 2015) and suppresses apoptosis of granulosa cells after culture of isolated secondary follicles (rat: Orisaka et al., 2006).

In the current study, all treatments promoted the activation of dormant ovarian follicles compared to the fresh control. However, in comparison to the control medium (α-MEM$^+$), no influence of GDF-9 on the percentage of growing follicles was observed. In fact, the spontaneous activation of primordial follicles was reported previously when cortical pieces were placed in culture (Wandji et al., 1996; Braw-Tal and Yossefi, 1997; Santos et al., 2017; Bezerra et al., 2018). Moreover, recent studies suggest that it may be possible to activate primordial follicles using a combination of mechanical signaling and biochemical factors (Kawamura et al., 2013; Cheng et al., 2015). In this case, the mechanical disruption of ovarian structure and polymerization of actin led to inhibition of the Hippo pathway, followed by oocyte growth (reviewed by Hsueh et al., 2015). Nevertheless, we observed that addition of 50 ng/mL GDF-9 to the culture medium increased granulosa cell proliferation. Similar results were reported after *in vitro* culture of antral follicles in the presence of GDF-9 (rat: Vitt et al., 2000; bovine: Spicer et al., 2006). Thus, GDF-9 is likely acting on ovine primordial follicle activation by stimulating the proliferation of granulosa cells.

In order to test if PI3K/AKT pathway would be involved in primordial follicle growth, ovine ovaries were treated with LY294002, a PI3K inhibitor that has been used to inhibit this pathway in different ovarian cells (Zhang *et al.* 2014; Fujihara *et al.* 2014; Lan *et al.* 2017; Bezerra et al., 2018). In the present study, the presence of LY294002 in the culture medium did not affect the follicle survival. However, the transition from primordial to growing follicles has been inhibited by LY294002 in both α-MEM$^+$ and 50 ng/mL GDF-9 treatments. These data indicate that the PI3K pathway is potentially involved in the *in vitro* activation of ovine primordial follicles, which was recently confirmed by our team (Santos et al., 2017; Bezerra et al., 2018) and is in agreement with the findings reported for other species (rat: Keating *et al.* 2009; mice: Zhao *et al.* 2014; cat: Thuwanut *et al.* 2017).

In order to further investigate the mechanisms behind *in vitro* activation of dormant follicles, our next approach was to evaluate the phosphorylation of AKT (pAKT), which has been widely used as an indirect measure of PI3K activity (Reddy et al., 2009). The immunostaining of pAKT was increased in follicles from α-MEM$^+$ and 50 ng/mL GDF-9. However, inhibition of the PI3K pathway with LY294002 clearly reduced pAKT expression in the follicles of both treatments. The possible explanation for the involvement of the PI3K/AKT pathway in the primordial follicle growth observed in α-MEM$^+$ is the presence of insulin in the medium, which acts primarily by activating this pathway (Khorami et al., 2015). In regard to GDF-9, it has also activated the PI3K/AKT pathway in granulosa cells of early antral follicles by increasing pAKT content (Orisaka et al., 2006). Moreover, the PI3K inhibition with LY294002 prevented the protective effect of GDF-9 against apoptosis induced by ceramide, an intracellular intermediate that directs cell cycle arrest and induces apoptosis in granulosa cells and ovarian follicles *in vitro* (Kaipia et al., 1996; Tilly et al., 2004), and directly antagonizes the PI3K/AKT pathway (Summers et al., 1998; Ruvolo, 2001). These data support the hypothesis that GDF-9 is one upstream factor regulating *in vitro* activation and follicle viability through the PI3K/AKT pathway.

In conclusion, after culture of ovine ovarian cortical slices, the addition of 50 ng/ml GDF-9 maintains follicular survival and promotes primordial follicle activation by reducing apoptosis and stimulating granulosa cell proliferation through activation of the PI3K/AKT pathway. Our findings highlighted that pharmacologic manipulation of the PI3K/AKT signaling pathway may be useful in fertility preservation by increasing the pool of growing follicles, providing a large supply of mature female germ cells.

Declaration of interest

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

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Figure captions

Fig. 1 Percentages of morphologically normal follicles in the fresh control and after *in vitro* culture in α-MEM^{+} or in different concentrations of GDF-9 (A). Histological sections of ovine ovarian fragments showing normal follicles after *in vitro* culture in medium containing 50 ng/mL GDF-9 (B) and atretic follicle in the control medium (C). O: oocyte; GC: granulosa cells. Scale bars: 25 μm (400x).

*Differs significantly from fresh control (P<0.05). ($^{A, B}$) Different letters denote significant differences among treatments (P<0.05)

Fig. 2 Percentages of primordial (A) and growing (B) follicles in the fresh control and after *in vitro* culture in α-MEM^{+} or in different concentrations of GDF-9.

*Differs significantly from fresh control (P < 0.05). ($^{A, B, C}$) Different letters denote significant differences among treatments (P<0.05)

Fig. 3 Percentages of apoptotic follicles in the fresh control and after *in vitro* culture in α-MEM^{+} or in 1 or 50 ng/mL of GDF-9 (A). Immunohistochemical positive detection of activated caspase-3 (follicular apoptosis) after culture in α-MEM^{+} (B) and negative detection after culture in 50 ng/mL GDF-9 (C); negative control for immunohistochemical analysis (D). O: oocyte; GC: granulosa cells. Scale bars: 25 μm (400x).

*Differs significantly from fresh control (P < 0.05). ($^{A, B, C}$) Different letters denote significant differences among treatments (P<0.05)

Fig. 4 Percentages PCNA-positive cells in the fresh control and after *in vitro* culture in α-MEM^{+} or in 1 or 50 ng/mL of GDF-9 (A). Immunohistochemical negative detection of proliferating cell after culture in α-MEM^{+} (B) and positive detection after culture in 50 ng/mL GDF-9 (C); negative control (D). O: oocyte; GC: granulosa cells. Scale bars: 25 μm (400x).

*Differs significantly from fresh control ($P < 0.05$). ($^{A, B, C}$) Different letters denote significant differences among treatments ($P<0.05$)

Fig. 5 Percentages of normal primordial (A) and growing (B) follicles in the fresh control, after *in vitro* culture in α-MEM^{+} or in 50 ng/mL GFD-9 in the absence or presence of LY294002 (PI3K inhibitor).

*Differs significantly from fresh control ($P<0.05$). ($^{A, B}$) Different letters denote significant differences among cultured treatments ($P<0.05$)

Fig. 6 Immunohistochemical expression of pAKT in ovine ovary: follicles after 7 days of *in vitro* culture in 50 ng/mL GDF-9 in the absence (A) or presence (B) of LY294002 (PI3K inhibitor). GC: granulosa cells; O: oocyte. Scale bars: 20 μm (400x).

8. CAPÍTULO 3

Growth differentiation factor-9 improves development, mitochondrial activity and meiotic resumption of sheep oocytes after *in vitro* culture of secondary follicles

Running head: GDF-9 on sheep oocyte growth

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Contents

This study analyzed the effect of GDF-9 on the *in vitro* culture of isolated ovine secondary follicles. The follicles were cultured in α-MEM supplemented with BSA, insulin, glutamine, hypoxanthine, transferrin, selenium, ascorbic acid and FSH (α-MEM$^+$ - control medium) or α-MEM$^+$ supplemented with 1, 10, 50 or 100 ng/mL GDF-9. Next, the oocytes were destined to *in vitro* maturation (IVM). After 12 days of culture, there were no significant differences regarding to the percentage of normal follicles, antrum formation and follicle diameter between the treatments ($P>0.05$). The rates of fully grown oocytes (≥110 μm) were higher ($P<0.05$) in 100 ng/mL GDF-9 than other treatments, except for 10 ng/mL of GDF-9 ($P>0.05$). Treatment containing 100 ng/mL GDF-9 showed higher ($P<0.05$) mitochondrial activity than the control group. Moreover, 100 ng/mL GDF-9 showed more oocytes in MI than α-MEM$^+$, 1 or 50 ng/mL GDF-9 ($P<0.05$). In conclusion, 100 ng/mL GDF-9 promoted follicular growth, mitochondrial function and meiotic resumption of oocytes from *in vitro* grown sheep secondary follicles.

Keywords: Ovarian follicles, Growth, Maturation, Ovine

1. INTRODUCTION

The *in vitro* culture of isolated secondary follicles from small ruminants is an important strategy to evaluate the quality of gametes after cryopreservation (Lunardi et al., 2016; 2017) or after the exposure to plant extracts (Gouveia et al., 2016; Cavalcante et al., 2018) or to metabolic stressors (Nandi et al., 2017), as well as to increase the production of meiotically competent oocytes (Silva et al., 2017) or embryos (Arunakumari et al., 2010; Luz et al., 2012). However, in sheep, the current culture system still has low efficiency with a small number of mature oocytes and embryos

produced. In an attempt to improve these results, the addition of growth factors in the culture medium has been used to enhance the growth of secondary follicles (Santos et al., 2014; Cunha et al., 2018). Among these factors, the importance of the growth differentiation factor-9 (GDF-9) in the follicular development is remarkable.

GDF-9 is a member of Transforming Growth Factor-beta (TGF-β) superfamily and plays an important role in the early folliculogenesis and steroids synthesis in mammals (Sun et al., 2010; Cook-Andersen et al., 2016). The biological functions of GDF-9 are mediated by interacting with its type I (ALK-5) and II (BMPRII) receptors (Vitt et al., 2002; Mazerbourg et al., 2004). Expression of mRNA and/or protein for GDF-9 was demonstrated in the oocyte in several species (ovine: Mery et al., 2007; Kona et al., 2015; bovine: Pennetier et al., 2004; caprine: Silva et al., 2004; Almeida et al., 2011; bubaline: Abdel-Ghani et al., 2016; human: Aaltonen et al., 1999). The expression of the BMPRII and ALK-5 receptors has been shown in all follicular categories (caprine: Silva et al., 2004), and increasing with follicular growth (ovine: Qin Chen et al., 2009).

It has been demonstrated that GDF-9-deficient female mice exhibit primary infertility due to failed ovarian follicular development (Dong et al., 1996). *In vitro* studies have demonstrated that GDF-9 maintains viability and induces primordial follicle activation (human: Hreinsson et al., 2002; caprine: Martins et al., 2008; Dipaz-Berrocal et al., 2017), as well as stimulates proliferation of granulosa and/or theca cells from secondary (human: Hreinsson et al., 2002) and antral (rat: Vitt et al., 2000; bovine: Spicer et al., 2008) follicles. Moreover, GDF-9 decreased follicular atresia and promoted secondary follicle development (mouse: Hayashi et al., 1999; Cook-Andersen et al., 2016; rat: Orisaka et al., 2006; caprine: Almeida et al., 2011). In addition, GDF-9 acts synergistically with FSH to promote *in vitro* growth of rat isolated preantral follicles (Hayashi et al., 1999), to maintain the survival and stimulate activation and growth of primordial follicles after culture of bovine ovarian cortex (Tang et al., 2012). However, the effects of GDF-9 on *in vitro* culture of sheep isolated secondary follicles remains unknown.

Therefore, the aim of this study was to evaluate the effects of different concentrations of GDF-9 on the morphology, development, mitochondrial activity, reactive oxygen species (ROS) levels and meiotic resumption of sheep secondary follicles cultured *in vitro*.

2. MATERIAL E METHODS

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

2.1. Source of ovaries

Ovaries (n = 80) were collected at an abattoir from 40 adult (1–3 years old) mixed-breed sheep. The pairs of ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil) and twice in 0.9% saline solution supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. Next, the ovaries were transported within 1 h to the laboratory in tubes containing 0.9% saline solution and antibiotics at 4°C (Soares et al., 2018). The approval of the ethics committee was not required since the research involved tissues of slaughtered animals.

2.2. Isolation and selection of secondary follicles

Isolation, culture and follicular evaluation were performed according to Cavalcante et al. (2018). After removal of the large antral follicles and corpora lutea, ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade and subsequently placed in holding medium consisting of minimum essential medium buffered with HEPES (MEM-HEPES) and antibiotics.

Secondary follicles (approximately 250-300 μm), without antral cavities, were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and mechanically isolated by microdissection using 26-gauge needles. These follicles were transferred to 100 μL droplets containing base culture medium for quality evaluation. The follicles selected for culture showed an intact basement membrane, two or more layers of granulosa cells and a visible and healthy oocyte that was round and centrally located within the follicle, without any dark cytoplasm.

2.3. *In vitro* culture of secondary follicles

The follicles were individually cultured (one follicle per droplet) in 100 μL droplets of culture medium under mineral oil in petri dishes (60 x 15 mm, Corning, Sarstedt, Newton, NC, USA) at 39 °C under 5% CO_2 for 12 days. Every two days, 60 μL of the culture medium was replaced with fresh medium in each droplet. The base culture medium, which is referred to as α-MEM$^+$ (control medium), consisted of α-MEM (pH 7.2–7.4) supplemented with 3.0 mg/mL BSA, 10 ng/mL insulin, 2 mM glutamine, 2 mM hypoxanthine, 5.5 μg/mL transferrin, 5.0 ng/mL selenium, 50 μg/mL ascorbic acid, and 750 ng/mL follicle stimulating hormone (human recombinant FSHr; Gonal-F®; Serono Laboratórios, São Paulo, Brazil). For the experimental conditions, the follicles were randomly distributed (approximately 55 follicles per group) in the control medium (α-MEM$^+$) or in this medium supplemented with different concentrations of GDF-9 (1, 10, 50 or 100 ng/mL). The concentration of FSH was chosen according to a recent study performed by our group with ovine secondary follicle culture (Barros et al., unpublished data). The concentrations of GDF-9 were chosen based on a previous study (Martins et al., 2008) in which this growth factor has been used for *in vitro* culture of goat ovarian cortex.

2.4. Morphological evaluation of follicle development

The morphological aspects of all follicles were assessed every 6 days using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon) at 100x magnification. Only those follicles showing an intact basement membrane, with bright and homogeneous granulosa cells and an absence of morphological signs of atresia were classified as morphologically normal follicles. Follicular atresia was recognized when a darkening of the oocytes and surrounding cumulus cells or misshapen oocytes was noted. In the normal follicles, the following characteristics were analyzed: (i) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers, (ii) follicle diameter, measured from the basement membrane, which included two perpendicular measurements of each follicle, and (iii) the daily growth rate, calculated as the diameter variation during the culture period.

After 12 days of culture, intact follicles were mechanically opened with 26 G needles under a stereomicroscope for oocyte recovery. The percentage of fully grown oocytes, i.e. oocyte with diameter ≥110 μm, was calculated as the number of acceptable quality oocytes recovered out of the total number of cultured follicles (x 100).

2.5. *In vitro* maturation of ovine oocytes from *in vitro* cultured secondary follicles

At the end of culture period, oocytes from all treatments enclosed in healthy follicles were collected with 26-G needles under a stereomicroscope. Only oocytes ≥110 μm of diameter with a homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for IVM (Lunardi et al., 2017). The cumulus–oocyte complexes (COCs; n=12-16 per treatment) were transferred to drops of 100 μL of maturation medium composed of tissue culture medium 199 (TCM 199) supplemented with 10% fetal calf serum (FCS), 1 μg/mL FSHr (Gonal-F;

Serono Laboratórios) and 1 μg/mL luteinizing hormone (LH; ovine pituitary) under oil (Cecconi et al., 1999), and incubated for 36-40 h under 5% CO_2 (Lunardi et al., 2017).

2.6. Assessment of active mitochondria, reactive oxygen species and chromatin configuration after IVM

After IVM, the oocytes were washed in phosphate buffered saline (PBS) and mitochondrial activity and ROS intracellular levels were measured as previously described (Lins et al., 2017). Briefly, oocytes (n = 12-16 per treatment) were incubated in the dark at 39ºC for 30 minutes in PBS supplemented with 100 nM MitoTracker® Red (CMXRos, Molecular Probes, Melbourne, Victoria, Australia) and 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen Corporation, Carlsbad, CA, USA) to detect mitochondrial activity and ROS levels as red and green fluorescence, respectively. Then, the oocytes were washed in PBS and observed under an epifluorescence microscope (Nikon) with UV filters (579 nm for active mitochondria and 460 nm for ROS). Fluorescence intensities of the oocytes were analyzed by using the Image J software (National Institutes of Health, Bethesda, MD, USA).

Thereafter, the oocytes were washed and incubated in drops of PBS containing 10 mM Hoechst 33342 for 15 min at room temperature in the dark and visualized under fluorescence microscopy (Nikon) with UV filter (483 nm). The chromatin configuration (blue fluorescence) was analyzed through observation of the intact germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) or nuclear maturation (metaphase II [MII]).

2.7. Statistical analysis

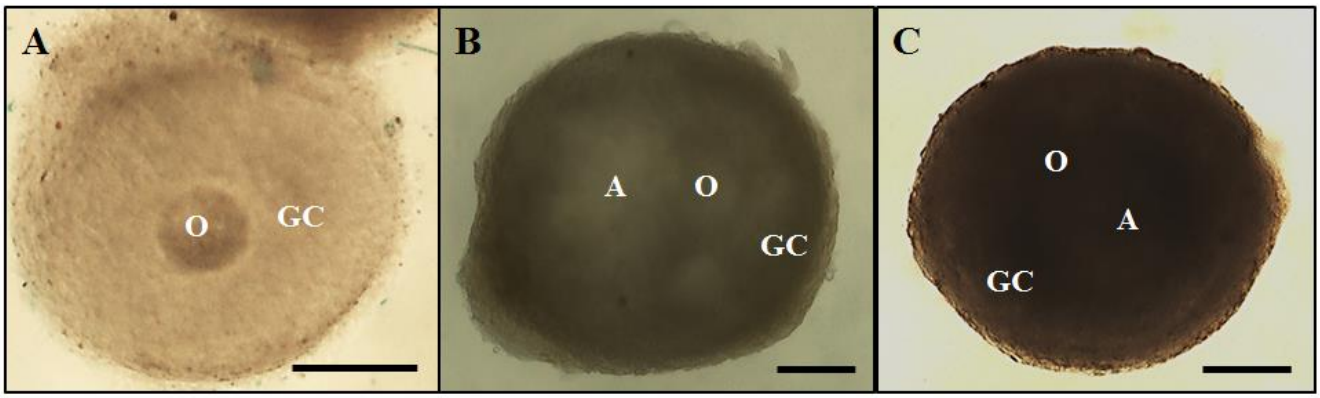
Data from follicle survival, antrum formation, fully grown oocytes and maturation rates after culture were expressed as percentages and compared by the Chi-squared test. Data from follicular diameter, growth rate, mitochondrial activity and ROS levels were submitted to the D´Agostino test to verify normal 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 mean (SEM), and differences were considered significant when $P<0.05$.

3. RESULTS

3.1. Follicular morphology and development after culture

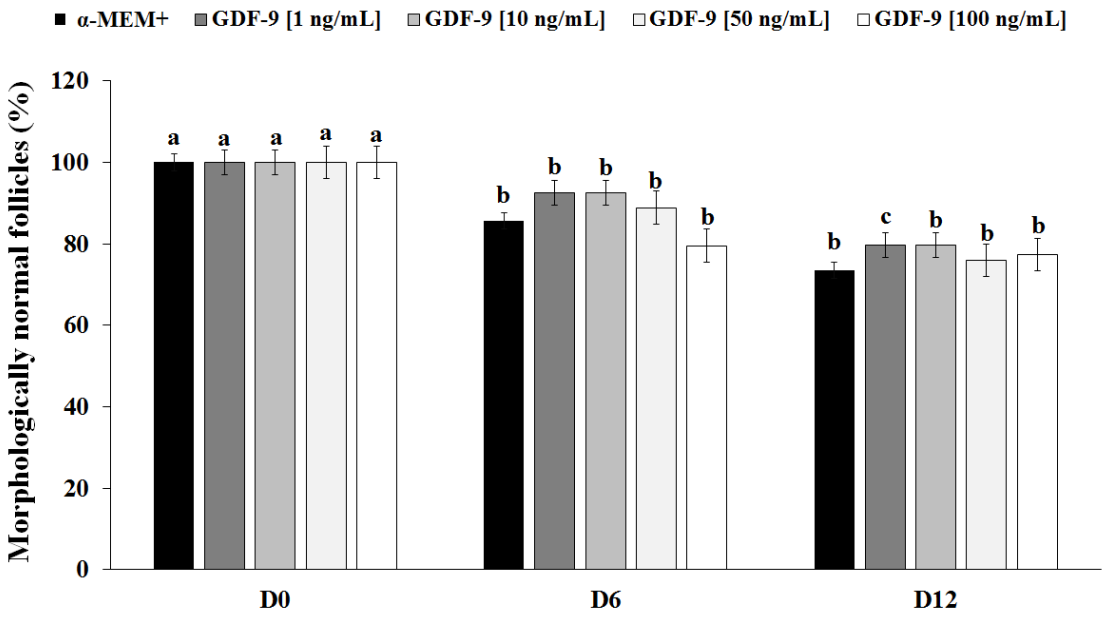
Morphologically normal secondary follicles showed visible and centrally located oocytes, normal granulosa cells and intact basement membrane (Fig. 1A). At day 6 of culture, antral (Fig. 1B) and atretic (Fig. 1C) follicles could be observed.

Figure 1. Morphologically normal secondary follicle at day 0 (A), antral follicle after 6 days of culture in 100 ng/mL GDF-9 (B), and atretic follicle after 6 days of culture in α-MEM^{+} (C). GC: granulosa cell; O: oocyte. A: antrum. Scale bar: 100 μm.



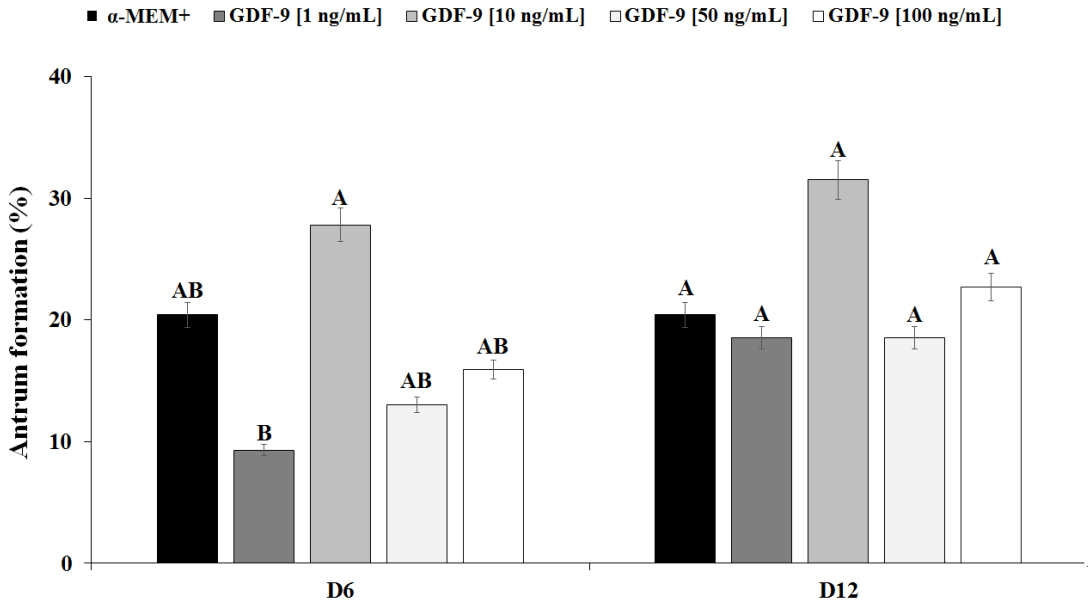
The percentage of morphologically normal follicles decrease significantly from day 0 to day 6 in all treatments and remained until the end of culture period, except for 1 ng/mL GDF-9, which decreased during all the culture period (Fig. 2). Moreover, all treatments showed similar (P>0.05) percentages of normal follicles throughout the culture period (73.5%, 79.6%, 79.6%, 75.9% and 77.3% for α-MEM^{+}, 1, 10, 50 and 100 ng/mL GDF-9 at day 12, respectively).

Figure 2. Percentages of morphologically normal follicles cultured in α-MEM^{+} or in different concentrations of GDF-9. ($^{a, b, c}$) Different letters denote significant differences among culture periods in the same treatment (P<0.05).



The rates of antral cavity formation remained unchanged from day 6 until the end of culture in all treatments (P>0.05). Moreover, after 12 days, antrum formation was similar (P>0.05) among treatments (24.4%, 18.5%, 31.5%, 18.5% and 22.7% for α-MEM^{+}, 1, 10, 50 and 100 ng/mL GDF-9, respectively; Fig. 3).

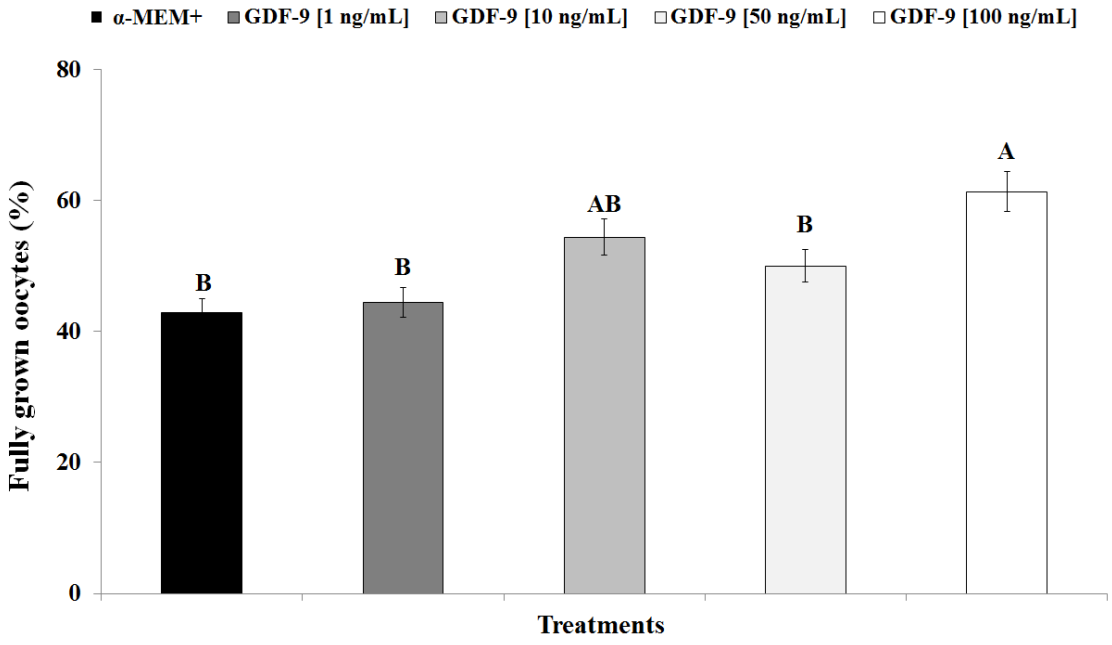
Figure 3. Percentages of antrum formation after follicle culture in α-MEM^{+} or in different concentrations of GDF-9. ($^{A, B}$) Different letters denote significant differences among treatments in the same period (P<0.05).



Regarding follicular diameter and daily growth rate, there was no difference (P>0.05) among treatments (data not show). At day 12, the concentration of 100 ng/mL of GDF-9 showed similar rates (P>0.05) of fully grown oocytes (≥110 µm; 61.4%) to 10 ng/mL GDF-9 (54.4%) and greater rates (P<0.05) than other treatments (42.9%, 44.4% and 50.0% for α-MEM^{+}, 1, and 50 ng/mL GDF-9, respectively; Fig. 4).

Figure 4. Percentages of fully grown oocytes after *in vitro* culture of secondary follicles in α-MEM^{+} or in different concentrations of GDF-9.

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

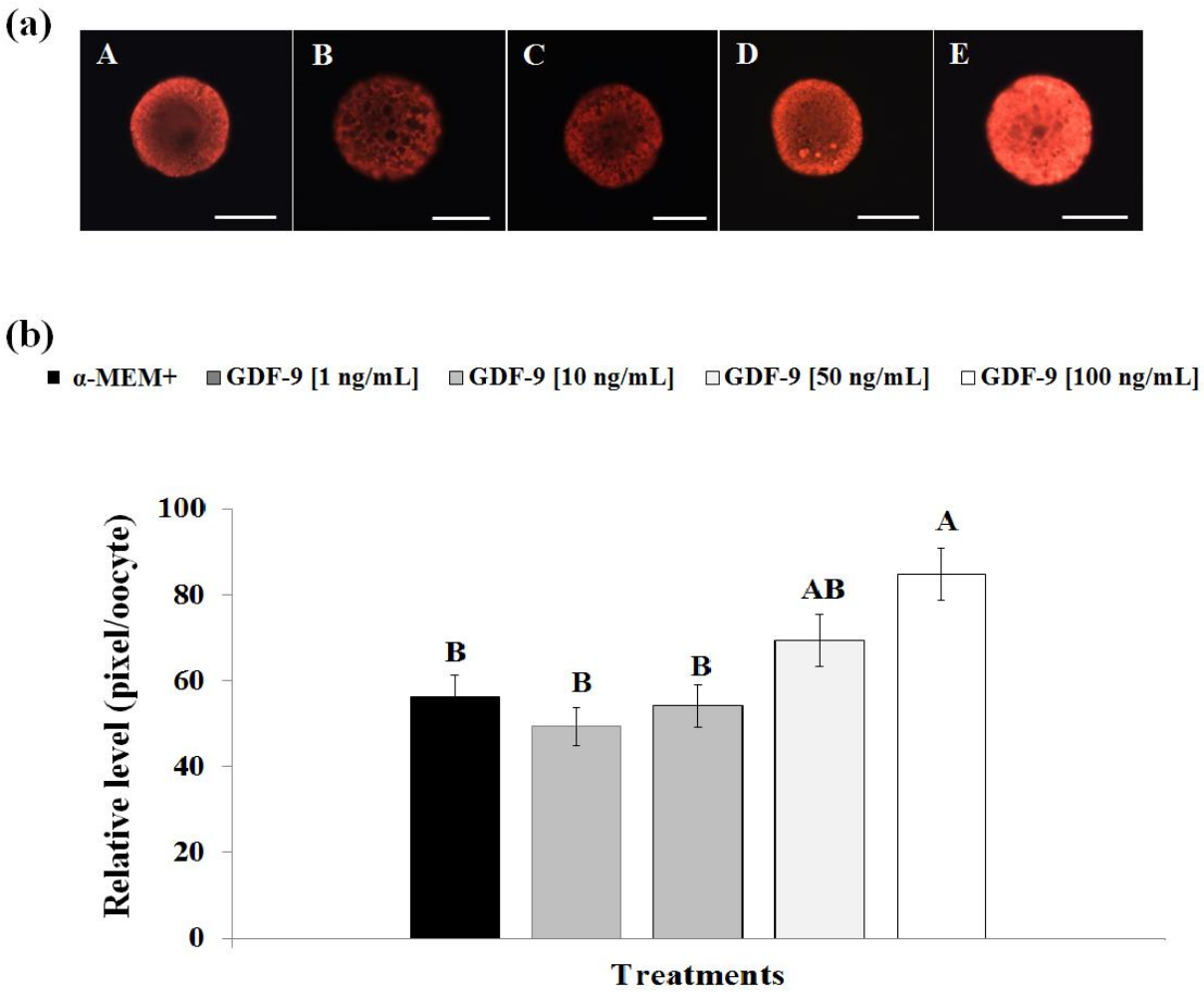


3.2. Mitochondrial activity and ROS levels after IMV

After IVM, oocytes cultured in medium containing 100 ng/mL GDF-9 showed higher (P<0.05) mitochondrial activity than the other groups, except for 50 ng/mL GDF-9 (P>0.05; Fig. 5). Moreover, ROS levels were similar (P>0.05) among treatments (data not show).

Figure 5. (a) Epifluorescent photomicrographic images of ovine oocytes from *in vitro* grown secondary follicles showing activity mitochondrial after IVM: oocytes cultured in α-MEM^{+} (A) or in α-MEM^{+} containing 1 (B), 10 (C), 50 (D) or 100 (E) ng/mL GDF-9. Scale bars: 75 µm (100x). (b) Active mitochondria levels in oocytes after IVM.

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



3.3. Chromatin configuration after IVM

There were oocytes in GV (Fig. 6A), GVBD (Fig. 6B) and MI (Fig. 6C) in all treatments. No oocytes reached the MII stage. More GV stage oocytes were observed at 1 ng/mL GDF-9 compared to 10 ng/mL and 100 ng/mL GDF-9 ($P<0.05$).

Figure 6. Epifluorescent photomicrographic images of ovine oocytes from *in vitro* grown secondary follicles stained with Hoechst 33342 after IVM. Oocytes in GV (A), GVBD (B), and MI (C) cultured in 100 ng/mL GDF-9. Arrow: nuclear chromatin. Scale bars: 50 μm (100x).

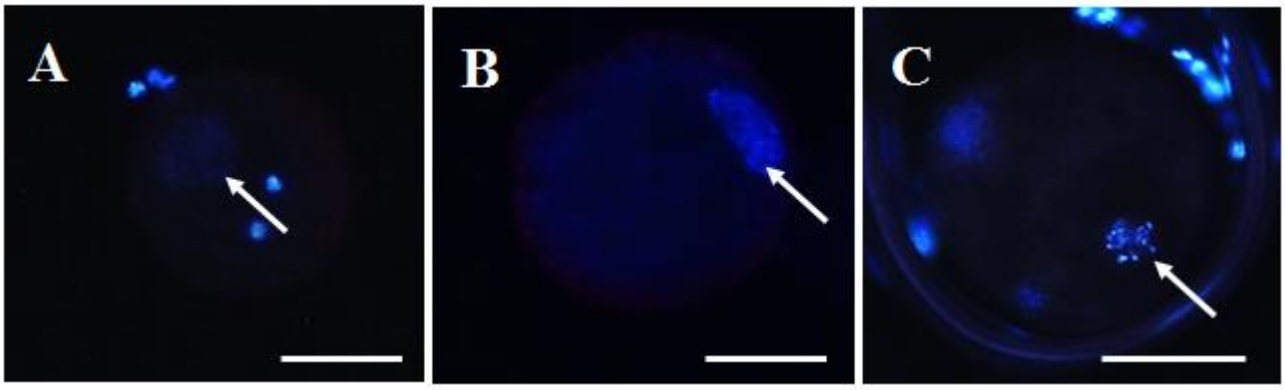


Table 1. Meiotic stages (%) after IVM of sheep oocytes from secondary follicles cultured in α-MEM^{+} or in different concentrations of GDF-9.

Treatments	% GV (n)	% GVDB (n)	% MI (n)
α-MEM^{+}	41.66 (5/12)^{AB}	33.33 (4/12)^{A}	25.00 (3/12)^{B}
1 ng/mL GDF-9	64.28 (9/14)^{A}	21.42 (3/14)^{A}	14.28 (2/14)^{B}
10 ng/mL GDF-9	25.00 (4/16)^{B}	37.5 (6/16)^{A}	37.5 (6/16)^{AB}
50 ng/mL GDF-9	33.33 (5/15)^{AB}	46.67 (7/15)^{A}	33.33 (3/15)^{B}
100 ng/mL GDF-9	15.38 (2/15)^{B}	15.38 (2/15)^{A}	69.24 (9/15)^{A}

($^{A, B}$) Different letter denote significant differences between treatments ($P<0.05$).

No differences were observed between treatments in the percentage of GVBD stage oocytes. However, 100 ng/mL GDF-9 showed higher ($P<0.05$) percentage of oocytes in MI than α-MEM^{+}, 1 or 50 ng/mL GDF-9 (Table 1).

4. DISCUSSION

To our knowledge, the present study is the first which demonstrated that 100 ng/mL GDF-9 stimulates the growth, mitochondrial activity and meiotic resumption of sheep oocytes from secondary follicles cultured *in vitro*.

In this study, GDF-9 did not influence survival, antrum formation and follicular diameter compared to the control medium. Similar results were reported after culture of goat secondary follicles in medium containing 100 or 200 ng/mL GDF-9 associated with FSH (Almeida et al., 2011). Although FSH is important for follicle survival and growth (Barros et al., 2013; Gouveia et al., 2016), when both GDF-9 and FSH were added to the culture medium, the positive effects of GDF-9 alone were not recorded (Almeida et al., 2011). Therefore, our results could be justified by the fact that FSH may induce the expression of intra-ovarian factors inhibiting any additional effect of GDF-9. Moreover, FSH may also inhibit GDF-9 by regulating the expression of specific receptors (BMPRII and ALK-5) as observed in ovine isolated granulosa cell culture (Qin Chen et al., 2009).

Despite the fact that it did not have an additional effect on antrum formation, the treatment containing 100 ng/mL GDF-9 showed the highest rates of fully grown oocytes (61.4%) at the end of culture. Conversely, other authors did not find any influence of GDF-9 on oocyte growth after culture of preantral follicles (rat: Orisaka et al., 2006; goat: Martins et al., 2008; Almeida et al., 2011). This difference in the results could be related to species specificity and the form that FSH

was added to the medium (fixed concentration in our study and sequential and increasing concentrations in the study performed by Almeida et al., 2011). Moreover, the presence of GDF-9 in the oocyte of all follicular categories in the sheep ovary and the increase of its expression in the oocyte and cumulus cells after follicular culture may indicate its direct role in oocyte growth (Mery et al., 2007; Kona et al., 2015).

The results of the current study also support the findings that there is a relationship between oocyte diameter (≥110 μm) and acquisition of the ability to resume meiosis (Crozet et al., 2000; Cadenas et al., 2018) because the concentration of 100 ng/mL GDF-9 also showed higher rates of oocytes in MI stage (69.24%). GDF-9 is able to regulate oocyte meiotic resumption in preovulatory follicles, activating the mitogen-activated protein kinase pathway by modification of cumulus cell function after the preovulatory LH surge (Norris et al., 2008). Moreover, GDF-9 plays a functional role in the maintenance of a cohesive interaction between cumulus cells and oocytes that influences subsequent fertility (Yan et al., 2001) and in the expression of essential genes for cumulus cell expansion during oocyte maturation, such as hyaluronic synthase 2 and cyclooxygenase 2 (Pangas & Matzuk, 2005; Lin et al., 2014). Additionally, GDF-9 knockdown by RNAi (RNA interference) injection into the murine oocyte eliminates the expansion of cumulus cells (Gui & Joyce, 2005). These findings support the essential role of GDF-9 in oocyte maturation.

The rates of meiotic resumption of the oocytes cultured in 100 ng/mL GDF-9 may also be explained by the increased levels of active mitochondria, which influences the quality of oocytes and is important for oocyte maturation, fertilization and subsequent embryo development (Dumollard et al., 2007; Castaneda et al., 2013). Moreover, low mitochondrial function would seem to be a feature of oocytes that have not undergone adequate maturation (Tarazona et al., 2006). The fact that no MII oocytes were obtained is a clear indication that an optimization of the IVM conditions (for example, use of antioxidants and/or additional energy substrates in the IVM medium) may be required to achieve high developmental competence.

In conclusion, the concentration of 100 ng/mL GDF-9 increased the growth, mitochondrial function and meiotic resumption of oocytes from *in vitro* grown sheep secondary follicles. This provides an encouraging step toward increasing the number of *in vitro* mature oocytes and further production of embryos.

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CONFLICT OF INTEREST

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

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Figure captions

Figure 1. Morphologically normal secondary follicle at day 0 (A), antral follicle after 6 days of culture in 100 ng/mL GDF-9 (B), and atretic follicle after 6 days of culture in α-MEM$^+$ (C). GC: granulosa cell; O: oocyte. A: antrum. Scale bar: 100 μm.

Figure 2. Percentages of morphologically normal follicles cultured in α-MEM$^+$ or in different concentrations of GDF-9.

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

Figure 3. Percentages of antrum formation after follicle culture in α-MEM$^+$ or in different concentrations of GDF-9.

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

Figure 4. Percentages of fully grown oocytes after *in vitro* culture of secondary follicles in α-MEM$^+$ or in different concentrations of GDF-9.

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

Figure 5. (a) Epifluorescent photomicrographic images of ovine oocytes from *in vitro* grown secondary follicles showing active mitochondria after IVM: oocytes cultured in α-MEM$^+$ (A) or in α-MEM$^+$ containing 1 (B), 10 (C), 50 (D) or 100 (E) ng/mL GDF-9. Scale bars: 75 μm (100x). (b) Active mitochondria levels in oocytes after IVM.

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

Figure 6. Epifluorescent photomicrographic images of ovine oocytes from *in vitro* grown secondary follicles stained with Hoechst 33342 after IVM. Oocytes in GV (A), GVBD (B), and MI (C) cultured in 100 ng/mL GDF-9. Arrow: nuclear chromatin. Scale bars: 50 μm (100x).

9. CONCLUSÂO

Em conclusão, este estudo demonstrou a presença de IGF-I no ovário de ovelha. Além disso, 50 ng/mL de IGF-1 associado à FSH (750 ng/mL) mantém níveis de EROS e GSH e atividade mitocondrial, aumentando o crescimento e o desenvolvimento de oócitos competentes à retomada da meiose.

Após o cultivo de fragmentos do córtex ovariano de ovinos, a adição de 50 ng/ml de GDF-9 mantém a sobrevivência folicular e promove a ativação do folículo primordial, reduzindo a apoptose e estimulando a proliferação de células da granulosa através da ativação da via PI3K/AKTA concentração de 100 ng/mL de GDF-9 aumentou o crescimento, a atividade mitocondrial e a retomada meiótica de oócitos de folículos secundários ovinos cultivados *in vitro*.

10. PERSPECTIVAS

Mais estudos são necessários com a utilização da fertilização *in vitro* de oócitos oriundos de folículos ovarianos cultivados *in vitro* em meio contendo 50 ng/mL de IGF-I e 750 ng/mL de FSH para verificar a capacidade de desenvolvimento embrionário.

Estudos com a manipulação farmacológica da via PI3K adicionados ao meio de cultivo de tecido ovariano associado ao GDF-9 demonstram que a manipulação farmacológica dessa via de sinalização pode ser útil na preservação da fertilidade, aumentando o pool de folículos em crescimento, fornecendo um grande suprimento de células germinativas femininas maduras

A utilização da concentração de 100 ng/mL de GDF-9 no cultivo in vitro de folículos secundários isolados ovinos proporciona um passo encorajador para o aumento do número de oócitos maduros *in vitro* e posterior produção de embriões.

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12.ANEXOS

Anexo 1: Comprovante de submissão do artigo 1

Gmail

Alane Pains <alanepainsvet@gmail.com>

Fw: Fwd: Thank you for your submission to Theriogenology

1 mensagem

----- Mensagem encaminhada -----

De: "Theriogenology" <eesserver@eesmail.elsevier.com>

Para: "helena.matos" <helena.matos@univasf.edu.br>, "htmatos" <htmatos@yahoo.com>

Enviadas: Sexta-feira, 27 de abril de 2018 11:14:31

Assunto: Thank you for your submission to Theriogenology

Dear Dr. Matos,

Thank you for sending your manuscript IMMUNOHISTOCHEMICAL LOCALIZATION OF INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) IN THE SHEEP OVARY AND THE SYNERGISTIC EFFECT OF IGF-1 AND FSH ON OOCYTE DEVELOPMENT IN VITRO AND LH RECEPTOR EXPRESSION for consideration to Theriogenology. Please accept this message as confirmation of your submission.

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Anexo 2: Comprovante de submissão do artigo 2

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Cell and Tissue Research - Notification of co-authorship

1 mensagem

Cell and Tissue Research <onbehalfof@manuscriptcentral.com> 29 de junho de 2018 19:09

Responder a: ctr@springer.com

Para: ctr@springer.com

29-Jun-2018

Dear Authors!

A manuscript titled Growth and differentiation factor-9 (GDF-9) reduces apoptosis and promotes in vitro activation of sheep primordial follicles through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway (CTR-18-0313) has been submitted by Dr. Maria Helena de Matos to Cell and Tissue Research.

You are receiving this email because you have been listed as an author for this manuscript.

The full author list for the submission is: Oliveira do Monte, Alane Pains; Bezerra, Maria Éllida; Menezes, Vanúzia; Gouveia, Bruna Bortoloni; Barberino, Ricássio; Lins, Thae; Barros, Vanessa; Santos, Jamile

Maiara da Silva; Donfack, Nathalie; de Matos, Maria Helena Tavares

If you are not aware of this submission, or if you should not be listed as a co-author, then please contact the journal office at ctr@springer.com

Thank you for your participation.

Sincerely,

Ms Jutta Jäger
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Anexo 3: Comprovante de submissão do artigo 3

Gmail **Alane Pains <alanepainsvet@gmail.com>**

Reproduction in Domestic Animals - Manuscript ID RDA-OA-Jun-2018-0308 - Submission received

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Lorraine Quiambao <onbehalfof@manuscriptcentral.com> 27 de junho de 2018 16:15

Responder a: rdaoffice@wiley.com

Para: helena.matos@univasf.edu.br

Cc: alanepainsvet@gmail.com, jamile_maiara@hotmail.com, vanuziagm@yahoo.com.br, bruna.bortoloni@gmail.com, thae.lanne@hotmail.com, cassiobarberino@hotmail.com, oliveiraliandro@gmail.com, njiatsa@gmail.com, helena.matos@univasf.edu.br

27-Jun-2018

Dear Dr Matos

Thank you for your submitting your manuscript to Reproduction in Domestic Animals. Your manuscript entitled "Growth differentiation factor-9 improves development, mitochondrial activity and meiotic resumption of sheep oocytes after in vitro culture of secondary follicles" (by Monte, Alane; Santos, Jamile; Menezes, Vanúzia; Gouveia, Bruna; Lins, Thae; Barberino, Ricássio; Oliveira Jr., Joãozito; Donfack, Nathalie; Matos, Maria Helena), has been successfully submitted online and is presently processed for review by members of our Editorial team.

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