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CULTIVO *IN SITU* DE FOLÍCULOS OVARIANOS PRÉ-ANTRAIS DE CABRAS COM ESFINGOSINA 1-FOSFATO (S1P) E FATOR INIBIDOR DA LEUCEMIA (LIF).

TESE DE DOUTORADO

Janduí Escarião da Nóbrega Jr.

Santa Maria, RS, Brasil

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Por

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(UFSM, RS), como requisito parcial para obtenção do grau de
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Orientador: Prof. Dr. João Francisco Coelho de Oliveira.

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Como requisito parcial para obtenção do grau de
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

CULTIVO *IN SITU* DE FOLÍCULOS OVARIANOS PRÉ-ANTRAIS DE CABRAS COM ESFINGOSINA (S1P) E FATOR INIBIDOR DA LEUCEMIA (LIF).

Autor: Janduí Escarião da Nóbrega Jr.
Orientador: João Francisco Coelho de Oliveira.
Data e Local da Defesa: Santa Maria, 25 de fevereiro de 2011.

Palavras-chaves: Caprino, folículo pré-antral, fator inibidor da leucemia, Esfingosina 1fosfato

A Manipulação de Oócito Incluso em Folículos Ovariano Pré-Antral (MOIFOPA) possibilita o aumento do potencial reprodutivo das fêmeas a partir do isolamento e cultivo de FOPA. A viabilidade folicular pode sofrer influências negativas com a idade, desnutrição, exposição a rádio ou quimioterápicos e condições sanitárias adversas. As alternativas para aproveitamento desses FOPA são pesquisas voltadas para elaboração de meios de cultivos que proporcione o manutenção da viabilidade a possibilite a ativação e o desenvolvimento folicular *in vitro*, para posterior fertilização. Fisiologicamente vários fatores de crescimento estão envolvidos durante o processo da foliculogênese, muitos dos quais até o momento não foram testados em cabras. Dentre eles, a Esfingosina 1-fosfato (S1P) e o Fator Inibidor da Leucemia (LIF), uma vez que, o desenvolvimento folicular foram obtido com esses fatores em outras espécies, possibilitando crescimento folicular e efeito anti-apoptótico no tecido ovariano. O objetivo desse trabalho foi avaliar a adição de diferentes concentrações de S1P e o do LIF separadamente aos meios de cultivo, avaliando a manutenção da viabilidade, ativação e desenvolvimento folicular. Dessa forma, os FOPA cultivados *in situ* com S1P e LIF por 7 dias possibilitaram desenvolvimento folicular conforme a manutenção da viabilidade, ativação e crescimento dos FOPA *in vitro*. Concluindo que a S1P 1ng/ml e o LIF 10ng/ml, foram as concentração que condicionaram a manutenção da viabilidade e a ativação dos FOPA *in situ* cultivados *in vitro* por 7 em dias melhor mantiveram a viabilidade, ativação e crescimento folicular.

ABSTRACT

Tese de doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

CULTURE *IN SITU* OF OVARIAN PREANTRAL FOLLICLE WITH SPHINGOSINE 1-PHOSPHATE (S1P) AND LEUKEMIA INHIBITORY FACTOR (LIF)

Autor: Janduí Escarião da Nóbrega Jr.
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Data e Local da Defesa: Santa Maria, 25 de fevereiro de 2010.

Key words: preantral follicle, leukemia inhibitory facto, Sphyngosine 1-phospate

The manipulation of oocytes enclosed in follicles Ovarian Pre-Antral, allows increasing the reproductive potential of females from the isolation and cultivation. The follicular viability can be affected negatively correlated with age, malnutrition, exposure to radio or chemotherapy and health conditions. The alternatives are to use these research for development of culture media which provides the maintenance of viability allows the activation and follicular development *in vitro*, for subsequent fertilization. Physiologically various growth factors are involved in the process of folliculogenesis, many of which have so far not been tested in goats. Among them the Sphingosine 1-phosphate (S1P) and Leukemia Inhibitory Factor (LIF), since, follicular development were obtained with these factors in other species, allowing follicular growth and ant apoptotic effect on ovarian tissue. The aim of this study was to evaluate the addition of different concentrations of S1P and the LIF to culture media separately, evaluating the maintenance of viability, activation and follicular development. Thus, the preantral follicle cultured with S1P in situ LIF for 7 days and follicular development as possible to maintain viability, activation and growth of preantral follicle *in vitro*. Concluding that the S1P 1 ng/ml LIF and 10ng/ml, were the concentration that the maintenance of viability and follicular activation of *in situ* and grown *in vitro* for 7 days improved the viability, activation and preantral follicular growth.

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LISTA DE ABREVIAÇÕES.

µl/ml – Microlitro por mililitro

BMP – Proteína morfogenética óssea

CC – Controle cultivado

cDNA – Acido desoxirribonucléico complementar

CNTF- Fator neutrofílico ciliar

EDG – Gene de diferenciação endotelial

EGF – Fator de crescimento epidermal

ERK – Sinal-regulado extracelular

FC – Fresco controle

FOPA – Folículo Ovariano Pré Antral

FSH – Hormônio folículo estimulante

GDF – Fator de crescimento e diferenciação

GP130- Glicoproteína 130

IGF – Fator de crescimento semelhante a insulina

IL - Interleucina

JAK – Janusquinase

kDA – Quilodalton

KGF – Fator de crescimento do queratinócito

KL – Kit Ligante

LIF – Fator inibidor da leucemia

MEM – Meio essencial mínimo

MOIFOPA – Manipulação de oócitos inclusos em folículo ovariano pré antral

MPK- Mitógeno ativador de proteinaquinase

ng/ml – Nanograma por mililitro

NGF – Fator de crescimento neuronal

OSM – Oncostantina -M

r-LIF – Receptor do Fator inibidor da leucemia

RNA-m – Acido ribonucléico mensageiro

r-S1P - Receptor da esfingosina 1-fosfato

S1P - Esfingosina 1-fosfato

SHP – Sinal ativador de transcrição

STAT – Sinal da tradução e ativador da transcrição.

TGF – Fator de crescimento e transformação

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1. INTRODUÇÃO

A utilização de métodos naturais de reprodução empregados em animais de produção, muitas vezes, não são suficientes para aumentar a eficiência reprodutiva nos rebanhos. A forma mais coerente para corrigir e suprir essa constante demanda é o emprego de biotécnicas aplicadas à reprodução animal. Com isso, novas biotecnologias vêm sendo desenvolvidas e aplicadas para a multiplicação de animais de alto valor comercial ou conservacionismo. Recentemente, a Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais (MOIFOPA) vem se destacando. Essa técnica consiste em resgatar os folículos ovarianos pré-antrais (FOPA) e, posteriormente cultivo *in vitro*, aumentando dessa forma o potencial reprodutivo das fêmeas com fatores genéticos desejáveis, empregado com fins preservacionistas e a criopreservação bancos de germoplasma animal (Figueiredo *et al.*, 2006).

Em tese, a possibilidade do desenvolvimento dos FOPA é possível desde que seja mantida a regulação entre os fatores estimulatórios e inibitórios presentes no ovário. *In vitro*, esses fatores estimulatórios com efeitos confirmados são: KI (PARROTT & SKINNER, 1999), GDF-9, FGF-2 (NILSSON et al., 2001), KGF (NILSSON & SKINNER, 2003), a BMP-4 e o NGF importantes para a ativação dos folículos primordiais (DISSEN et al., 2001; TANWAR et al., 2008). Cultivos com fragmentos ovarianos de cabras em meios suplementados com TGF, IGF-I, IGF-II, EGF e FSH foram capazes de promover o desenvolvimento dos FOPA, no qual EGF apresentou atividade mitogênica e o FSH induziu o crescimento folicular (RAJARAJAN et al., 2006; ROSSETTO et al., 2009).

Pesquisas com cultivos de FOPA *in vitro* de cabras têm sido realizadas utilizando a adição de vários fatores de crescimento. Alguns deles com realizado por Bruno *et al.* (2006) comprovaram que adicionando 10% de soro fetal bovino ao meio de cultivo, ocorre manutenção da morfologia folicular e, quando os meios são acrescido de 20% de soro de

cabra em estro ocorre a ativação e crescimento folicular. Lima-Verde *et al.* (2009) confirmaram que tanto o α-tocoferol como a ternatina, dois fármacos anti-oxidantes, possuem a capacidade de promover ativação dos FOPA de cabras.

Embora não haja relatos sobre cultivo *in situ* de FOPA em meios suplementados com esfingosina 1-fosfato (S1P) e com fator inibidor da leucemia (LIF), tendo em vista que em cultivos com ovários de ratas a S1P foi capaz de manter a viabilidade folicular e estimular o crescimento de FOPA em ovários de camundongas (SPIEGEL & KOLESNICK, 2002); (KAYA et al., 2008). Dessa forma, não foram apresentados resultados do papel do LIF em FOPA de cabras, entretanto, esse fator sabidamente promove o crescimento e o desenvolvimento de folículos primordiais bem como a maturação oocitária conforme os resultados obtidos em mulheres (ABIR et al., 2004). Pesquisas em com esses fatores de crescimento, sugerem que para os FOPA de ratas e camundongas, tanto a S1P quanto o LIF são importantes para a transição de folículos primordiais até folículos primários (NILSSON et al., 2002); (HAIDARI et al., 2006).

Esta pesquisa teve como objetivo testar em cultivo *in vitro* diferentes concentrações da S1P e do LIF separadamente, analisando a ativação, manutenção da sobrevivência e crescimento do FOPA *in situ*, usando como modelo experimental a espécie caprina.

2. REVISÃO BIBLIOGRÁFICA

2.1. Ovário

O ovário é composto por vários tipos celulares que atuam em conjunto para manter e exercer suas funções (MCGEE & HSUEH, 2000); (BRISTOL-GOULD & WOODRUFF, 2006). As duas funções básicas são: a exócrina, que consiste na liberação dos oócitos aptos à fecundação e a função endócrina, que produz hormônios esteróides que são os responsáveis pelo desenvolvimento das características sexuais femininas secundárias e manutenção da gestação.

O ovário apresenta duas regiões histológicas distintas, sendo uma delas a região cortical, rica em fibras colágenas, pouco vascularizada, onde se encontra todo efetivo de folículos pré-antrais, além dos folículos ovarianos em diferentes estádios de desenvolvimento. São encontrados ainda corpos hemorrágicos, corpo lúteo e corpo *albicans* (LIU et al., 2006). A segunda região é denominada de medular, localizada na zona subortical. É composta por tecido conjuntivo, fibras de tecido nervoso com vasos sanguíneos e ductos linfáticos que penetram no ovário através do hilo (SILVA et al., 2004). É importante destacar que nos equídeos essa conformação histológica se apresenta de forma inversa, em que a região medular está localizada na periferia (SQUIRES, 2006).

Durante a oogênese, em estágio fetal, ocorre a formação das células germinativas, e uma combinação entre transferência passiva e movimentos amebóides com a migração e proliferação das células germinativas primordiais do mesoderma extraembrionário,na porção posterior do intestino rudimentar que migram para sua colonização na crista gonadal (MOLYNEAUX et al., 2003). A regulação do desenvolvimento dos FOPA em parte, ocorre por fatores parácrinos secretados pelo oócito e por células somáticas do ovário. Após a ativação dos folículos primordiais até a seleção de folículos secundários, vários fatores

exercem diferentes efeitos influenciando o crescimento e a diferenciação dos oócitos e das células da granulosa (ALBERTINI et al., 2001).

É fundamental que haja uma interação entre a sinalização parácrina do oócito e as células somáticas do ovário para que ocorra o processo de oogênese e foliculogênese nos mamíferos (HUTT et al., 2006). Em ovários de fetos ovinos com 38 dias de gestação são identificados cinco tipos celulares bem diferenciados; células mesoteliais que formam a superfície epitelial do ovário, células endoteliais que formam os vasos sanguíneos, células mesenquimais que formam o estroma, células da pré-granulosa formando os folículos e as células germinais ou oogônias (SAWYER et al., 2002).

2.2. Folículo ovariano

O folículo é constituído por um oócito circundado por células foliculares e demarcado por uma membrana basal que o separa do estroma ovariano. Esta organização histológica e sua atividade fisiológica conferem ao folículo a condição de unidade morfológica e funcional do ovário, tendo como função primordial proporcionar um ambiente ideal para o crescimento e maturação do oócito bem como produzir hormônios e peptídeos que regularão o crescimento folicular (CORTVRINDT & SMITZ, 2001; GILCHRIST et al., 2004).

Os folículos ovarianos podem ser classificados em duas categorias conforme sua estrutura histológica, com presença ou ausência da cavidade do antro folicular. Os critérios para a classificação dos FOPA foram adotados por (YANG & FORTUNE, 2008); através da identificação dos diferentes estágios foliculares em fetos bovinos, não sendo observados folículos entre 80 e 90 dias de vida fetal, porém com folículos primordiais entre 91 e 140 dias, folículos primários entre 141 e 210 dias e folículos secundários após 210 dias. Os FOPA são classificados como: 1) Folículo Primordial (oócito circundado apenas por uma única camada

de células da pré-granulosa de aspecto pavimento); 2) Folículo Primário (oócito circundado por uma única camada de células da granulosa de aspecto cubóide) e 3) Folículo Secundário (oócito circundado por duas ou mais camadas de células da granulosa de aspecto cubóide).

2.3. Isolamento folicular

A qualidade dos FOPA depende do tempo, da temperatura de transporte e dos meios empregados no cultivo, que são condições essenciais para a manutenção da viabilidade folicular. (CHAVES et al., 2008) conseguiram resultados satisfatórios para manutenção da viabilidade, transportando ovários de cabras a 4^0C por até 4h em meio mínimo essencial. Várias técnicas têm sido empregadas para o isolamento de FOPA, algumas delas permitindo o resgate de grande quantidade de folículos por métodos enzimáticos utilizando pronase, colagenase e deoxiribonuclease. No entanto, seu emprego em animais domésticos apresenta efeito contestável decorrente da natureza fibrosa dos ovários. Diante dessa impossibilidade o mecanismo viável para o resgate dos folículos é o isolamento mecânico que é empregado com sucesso no isolamento de FOPA em bovinos (ITOH & HOSHI, 2000).

O isolamento mecânico de FOPA através da técnica de "tissue chopper" em córtex de ovários de vacas foi empregado por (SCHOTANUS et al., 1997), utilizando secções de 250µm espessura permitindo o isolamento de até 50 folículos por ovário. Outra forma de isolamento mecânico utilizado é a microdissecação com agulhas de 26G que permite isolar folículos entre 150 e 400µm de diâmetro (TAMILMANI et al., 2005). (WU et al., 2007) também utilizaram de micro fórceps para o isolamento de FOPA com 296µm de diâmetro de porcas.

Existem diferentes técnicas para isolar os FOPA do córtex ovariano além do cultivo *in situ*, uma delas é microdissecação que permite isolar entre 50-60 FOPA/ ovário resultados

obtidos em búfalas. Nessa mesma espécie, quando se utiliza o "tissue chooper" recuperaram de 20-35 FOPA/ovário entre 4-5h de duração. Outras técnicas que podem ser empregadas, como a técnica de esmagamento do tecido ovariano e lavagem com colagenase e tripisina e, a sonificação, que emprega ultrassom para isolar os FOPA do estroma ovariano (SHARMA & BHARDWAJ, 2009).

2.4. Esfingosina 1- fosfato (S1P)

A proliferação, diferenciação e organização celular em tecidos animais, são controlados por diversas formas de comunicação celular. Uma das quais envolve os fatores de crescimento que ativam os receptores de membrana para a sinalização de transdução, proliferação e diferenciação. Outra forma, envolve moléculas de adesão ligadas às membranas celulares ou na matriz extracelular que interagem com os receptores localizados na membrana (MASSAGUE, 1998).

A esfingomielina é um esfingolipídio presente na membrana plasmática celular, e a sua hidrólise depende da ação da enzima esfingomielinase endotelial que é induzida por citoquinas. A ceramida é uma importante molécula lipídica transdutora de sinal que atua como segundo mensageiro numa variedade de processos biológicos, tais como a proliferação e diferenciação celular, apoptose e diversas respostas inflamatórias e imunológicas do organismo (MOURA, 2003). A S1P é um derivado da esfingomielina envolvida em diversos mecanismos biológicos, incluindo crescimento, anti-apoptose, angiogênese e proliferação celular. A ceramida, a ceramida 1-fosfato, a esfingosina e a S1P atuam como segundo mensageiro, ativando a proteína quinase e os demais componentes de sinalização intracelular. Esses lipídios podem agir intracelular como segundo mensageiro e extracelular, ativando os

receptores-S1P (S1P-r) que estão dispostos na superfície da membrana celular em outros tecidos (EYSTER, 2007); (ZANIN et al., 2008).

Durante muito tempo, os lipídios foram considerados apenas como componentes da estrutura da membrana ou como fonte de energia celular. Outras pesquisas, evidenciaram que os lipídios estão envolvidos na sinalização celular e em vários outros processos biológicos como crescimento, morte e diferenciação celular. Participando ativamente nesses processos estão os esfingolipídios, a ceramida e a S1P (FERNANDIS & WENK, 2007). Estudos estão sendo direcionados para detecção e localização do S1P-r, que também é conhecida como “endothelial differentiation gene” (EDG), objetivando assim uma aplicação clínica da S1P no tratamento anti-câncer (PYNE & PYNE, 2000).

A formação da S1P ocorre na dependência da degradação da esfingomielina, considerada a principal via de formação dos esfingolipídios. A esfingomielina presente na membrana plasmática é clivada pela enzima esfingomielinase e convertida em ceramida, a qual é deacilada pela ceramidase formando a esfingosina. A esfingosina sofre ação da enzima esfingosinaquinase, presente no retículo endoplasmático e no citosol, que por consequência realiza uma reação de fosforilação para a conversão da esfingosina em S1P (TANI et al., 2007).

Em roedores a expressão de receptores S1P1/EDG1 ocorre em vários tecidos incluindo: cérebro, pulmão, coração, baço, rim, fígado, músculo esquelético, tecido adiposo, pele, testículo e útero (KLUK & HLA, 2002). A proteína esfingosinaquinase que é a ativadora da S1P foi purificada em rins de roedores com peso molecular de 49 kDa. Posteriormente, dois clones foram obtidos com 381 e 388 aminoácidos e designados como esfingosinaquinase-1 de 42.3kDa e esfingosinaquinase-2 de 43.2kDa. Essas duas isoformas diferem apenas em alguns aminoácidos na porção *N*-terminal, sugerindo que possam ser

derivados de algum "splicing" alternativo para a transcrição do RNAm (PYNE & PYNE, 2000).

Os r-S1P foram identificados nas células da veia umbilical de humanos e dentre suas funções se destacam a manutenção da sobrevivência, migração e proliferação celular favorecendo a angiogênese. As ações a S1P ocorrem através da ligação aos diferentes membros da família S1P/EDG, dentre eles o EDG1, EDG3, EDG5 e EDG8 (KLUK & HLA, 2002). Uma vez a S1P ligada ao seu receptor, o EDG1 promove uma série de sinalizações por múltiplas vias através da ligação da proteína-G, incluindo a ativação da fosfolipase C, a mobilização de Ca^{2+}, a ativação do mitógeno ativador da proteína-quinase (MAPK), a regulação da ERK e a inibição da adenilatociclase. Estudos demonstraram que o EDG3 faz parte dos receptores para lisoefingolipídeos, mais especificamente para S1P e esfingosilfosforilcolina, sendo considerado distinto dos demais EDG1 e EDG5. O EDG3 é considerado semelhante ao EDG1 na ação sobre o AMP cíclico celular, contrariamente a ação do receptor EDG8**.** É importante analisar as funções dos diferentes receptores e subtipos, bem como suas funções biológicas, uma vez que os receptores para lisoesfingolipídeo são amplamente expressos na maioria dos tecidos (OKAMOTO et al., 1999).

Pesquisas com cultivo de córtex ovariano de fetos humanos com 24 semanas em meios no qual foi adicionado S1P por 48h, demonstraram diminuição do número de folículos primordiais, com capacidade da S1P promover ativação e crescimento dos FOPA *in vitro* (OKTEM & OKTAY, 2007).

2.5. Fator inibidor da leucemia (LIF)

O LIF é uma citocina pleiotrópica altamente glicosilada, com peso molecular entre 40 e 50 kDa, pertence à família da interleucina 6 (IL-6), da qual fazem parte a oncostatina-M

(OSM), o fator neutrófico ciliar (CNTF) e a cardiotropina-1 (CT-1) (KIMBER, 2005). Dentre suas principais ações estão a manutenção de células pluripotente, a indução de hipertrofia cardíaca *in vitro*, a indução de proteínas de fase aguda hepática, a formação osteoclástica *in vitro*, a manutenção da sobrevivência e diferenciação dos neurônios (TAGA & KISHIMOTO, 1997).

Além destas funções, o LIF está relacionado com a proliferação, diferenciação e sobrevivência celular nos sistemas imune, nervoso, cardíaco e reprodutivo (OZAKI & LEONARD, 2002). (BORNSTEIN et al., 2004) relataram que as citocinas interagem com a esteroidogênese de maneira sistêmica e complexa. Essas evidências sugerem que as citocinas produzidas pelo endométrio e pelo blastocisto participam do mecanismo de interação entre o embrião e do útero, juntamente com outros fatores de crescimento (DOMINGUEZ et al., 2003); (HERRLER et al., 2003).

Nas camundongas, o LIF é considerado fundamental para a reprodução, uma vez que as fêmeas "knockout" para esse gene apresentam falhas de implantação embrionária (DIMITRIADIS et al., 2005), embora os embriões "knockout" transferidos para fêmeas com fenótipo selvagem são capazes de realizar todas as etapas da implantação (STEWART et al., 1992). (BHATT et al., 1991) também observaram efeitos similares em camundongas, no qual os receptores do LIF (r-LIF) foram detectados no endométrio, apresentando um papel essencial na implantação e no crescimento do blastocisto, bem como na manutenção da gestação. Em coelhas os r-LIF podem ser evidenciados tanto no óvulo quanto no endométrio. Esses mesmos receptores, nos estágios precoces do desenvolvimento na fase de mórula e de blastocisto, estão presentes, indicando que o LIF pode agir de maneira parácrina durante o desenvolvimento embrionário (LEI et al., 2004).

Em mulheres, o LIF desempenha um importante papel para a manutenção da fertilidade (CULLINAN et al., 1996), visto que a expressão do RNAm do LIF-r ocorre

durante o período de implantação do embrião (CHARNOCK-JONES et al., 1994); (ARICI et al., 1997). Estudos demonstraram que a concentração de LIF em lavados uterinos aumenta durante a fase de implantação embrionária, caracterizando sua importância fisiológica para a reprodução em humanos (LAIRD et al., 1997); (LEDEE-BATAILLE et al., 2002). Dessa forma, é possível associar algumas causas de infertilidade a genótipos com menor produção ou baixa atividade de LIF (HAMBARTSOUMIAN, 1998); (GIESS et al., 1999; YUE et al., 2000).

Em ovários de roedores com expressão de r-LIF promoveram aumento da sobrevivência e da migração das células germinativas primordiais, contrastando com os ovários de ratas deficientes em r-LIF, os quais apresentavam redução do número de folículos primários e falha na ovulação. Estes estudos sugerem que a presença do r-LIF é necessária para o processo de oogênese e folículogênese nesses animais (MOLYNEAUX et al., 2003).

O r-LIF apresenta estrutura intimamente relacionada com gp-130, de acordo com os resultados de (GEARING et al., 1991) que confirmaram em murinos, a presença de cDNA para o r-LIF, apresentando 70% dos aminoácidos homólogos com humanos. Possuindo atividade muitas vezes similar à IL-6, embora o LIF e a IL-6 pareçam estruturalmente independentes. Essa relação entre as duas citocinas indica uma relação comum na sinalização dos receptores e pode ajudar a explicar os efeitos biológicos semelhantes.

Estudos sobre o LIF demonstram sua atividade pleiotrópica relacionada à proliferação, diferenciação e sobrevivência celular. Isso ocorre após a ligação a um heterodímero protéico transmembrânico formado pela gp-130, que compreende uma unidade receptora específica do LIF-r (OZAKI & LEONARD, 2002). As ações do LIF são mimetizadas por membros da família IL-6, pois compartilham do mesmo receptor gp-130 e utilizam as mesmas vias de sinalização intracelular, através das JAK/STAT, SHP-2/Ras/ERK e/ou a IP3K/Akt (KIMBER,

2005). A ativação desse complexo inicia a cascata de sinalização das JAK-STAT3 e das MAPK (CHENG et al., 2001).

Diferentes fatores de crescimento possuem atividade intrínseca de tirosina quinase (TK). Conseqüentemente, para que ocorra a sinalização celular essa citocinas necessitam do acoplamento de outras proteínas da família das TK aos seus receptores. A ligação da citocina com seus respectivos receptores de superfície celular resulta na oligomerização do receptor e na ativação da JAK. Essas quinases citoplasmáticas participam da sinalização de receptores celulares de superfície com perda intrínseca da atividade da TK. Quando as JAKs são ativadas ocorre a fosforilação do domínio citoplasmático do receptor iniciando a cascata de sinalização. Após, as proteínas transdutoras de sinal e ativadoras de transcrição (STAT) são então fosforiladas, dimerizadas e translocadas para o núcleo, local em que ocorre a regulação e a transcrição gênica (VALENTINO & PIERRE, 2006).

3. CAPÍTULO 1

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

3.1. Sphingosine 1-phosphate promoter activation of caprine preantral follicle *in vitro*

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Sphingosine 1-phosphate promoter activation of caprine preantral follicle *in vitro*.

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Summary

This study aimed to evaluate the effect of sphingosine 1-phosphate (S1P) for development of preantral follicle, therefore activation and viability follicular of caprine preantral follicles cultured *in vitro*. Ovarian fragments were cultured for 1 or 7 days in Minimum Essential Medium with different concentrations of S1P (0, 1, 10, 50, 100 or 200 ng/ml). All fragments ovarian fragments were processed for histological, ultra-structural for microscopy electronic and fluorescence analysis. Only 1 ng/ml of S1P maintained the percentage of normal follicles with the progression of the culture from day 1 to 7. With the progression of the culture period from to 7 days, there was significant reduction in the percentage of primordial follicles in all groups treated S1P, compared fresh control (FC) and Control Culture (CC), which followed by an increase of activated follicle (intermediary, primary and secondary). In comparison with FC, CC other concentrations, 7 days after culture, the S1P concentrations 1 ng/ml preserve significant maintenance ultra structural follicular of organelles, in the same period, the S1P concentrations 1 ng/ml for fluorescence microscopy analysis, kept viability of preantral follicular in comparison FC and CC. In conclusion, after 7 days of culture, the 1 ng/ml of S1P, active the development of preantral follicle of caprine, cultured *in stiu* and maintains the viability oocitary and follicular.

1. Introduction

Follicular activation, i.e. transition from primordial to primary stage follicles, as well as the subsequent steps of follicular development are critical processes for reproductive biology (SKINNER, 2005). However, mechanisms regulating the activation of primordial follicles have not been elucidated so far. Because primordial follicles potentially represent a large source of oocytes in humans and animals, with several possible applications, such as infertility treatment in clinical medicine or improvement of animal reproductive potential, efforts have been focused on developing culture systems for follicles at that stage. In addition, the *in vitro* culture of preantral follicles allows the evaluation of the effects of different substances (hormones, growth factors, antibiotics, etc) on the ovarian physiology before their use *in vivo* in humans or animals.

Although sphingolipids were originally thought to play a predominantly structural role as components of the lipid bilayer (KOLESNICK, 1987), sphingolipid metabolites – including ceramide, sphingosine, and sphingosine 1-phosphate (S1P) – are active mediators that play essential roles in cell growth, survival and death (HANNUN, 1996; MERRILL et al., 1997). Among these metabolites, S1P which is produced by phosphorylation of sphingosine, has been found to have a wide range of biological actions, including Ca^{2+} mobilization, reorganization of the cytoskeleton, as well as cell growth, differentiation, survival and motility (PYNE & PYNE, 2000; SPIEGEL & MILSTIEN, 2000). S1P acts intracellularly as a second messenger and extracellularly as a ligand for G protein-coupled receptors. In human, S1P was identified in follicular fluid with an importance in follicle development (VON OTTE et al., 2006).

Some studies have demonstrated that S1P maintain the viability of human embryonic and rat germ cells after *in vitro* culture (CHAE et al., 2004; HAIT et al., 2006).

In males, S1P was shown to inhibit germ cells against radiation-induced apoptosis (OTALA et al., 2004; SUOMALAINEN et al., 2005). An in vivo study demonstrated that S1P given into the ovarian bursa before an exposure to ionizing radiation resulted in a dose-dependent preservation of the ovaries, with complete protection of preantral follicles (MORITA et al., 2000). Recently, *in vitro* studies have shown that S1P decrease follicle apoptosis after irradiation rat (KAYA et al., 2008), before chemotherapy mouse (PEREZ et al., 1997; HANCKE et al., 2007), as well as in xenografted ovarian tissue monkey (LEE et al., 2005). In addition, besides its antiapoptotic effect, S1P stimulates mouse preantral follicles growth (SPIEGEL & KOLESNICK, 2002) and also protects oocytes from heat shock (ROTH & HANSEN, 2004).

Although the roles of S1P in apoptosis inhibition and in development promotion, studies using S1P in the culture of caprine preantral follicles were not yet performed. Therefore, the aims of the present study were to: 1) investigate a possible influence of different concentrations of S1P on the viability, activation of primordial follicles and further follicular growth after *in vitro* culture of caprine ovarian tissue, and 2) evaluate the ultra-structure of caprine preantral follicles cultured *in vitro* in the absence or presence of S1P.

2. Materials and Methods

Ovaries collection

Ovarian cortical tissues (n= 10) were collected from five adult (1–3 years-old) cyclic, non-pregnant mixed-breed goats at an local abattoir. After recovery, the ovaries were washed in 70% alcohol, followed by two times in Minimum Essential Medium

(MEM) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin, being transported to the laboratory within 30 min in MEM at 4°C.The culture media, S1P and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Experimental protocol

Our organ culture system was described in detail earlier (MATOS et al., 2007). Ovarian tissue samples from each ovarian pair were cut into 13 slices (3 x 3 x 1 mm) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultra-structural analysis fresh control (FC) or placed in culture for 1 or 7 days. Caprine tissues were transferred to 24-well culture dishes containing 1 ml of culture media. Culture was performed at 39°C in 5% CO_2 in a humidified incubator and all the media were incubated for 1 h prior to use. The basic culture medium , cultured control (CC) and consisted of α-MEM (pH 7.2 – 7.4) supplemented with ITS (insulin 6.25 µg/mL, transferrin 6.25 µg/mL and selenium 6.25 ng/mL), pyruvate 0.23 mM, glutamine 2 mM, hypoxantine 2 mM, 1.25 mg/mL of bovine serum albumin (BSA). For the experimental conditions, the medium was supplemented with S1P at different concentrations (1, 10, 50, 100 or 200 ng/mL). Each treatment was repeated five times and the culture media was replenished every other day.

Histological analysis and assessment of *in vitro* follicle growth

To evaluate caprine follicular morphology, before culture FC and after 1 or 7 days in culture, all the pieces were fixed in Carnoy's solution for 12 h and then dehydrated in

increasing concentrations of ethanol. After paraffin embedding (Vetec, Rio de Janeiro, Brasil), the caprine tissues pieces were cut into 7 μm sections, and every section was mounted on glass slides and stained by Periodic Acid Schiff - hematoxylin. Follicle stage and survival were assessed microscopically on serial sections. Coded anonymized slides were examined using a microscope under 400X magnification.

The developmental stages of follicles have been defined previously (Silva et al., 2004) as primordial (one layer of flattened granulosa cells around the oocyte) or activated 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). These follicles were still classified individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells which are well organized in one or more layers and that have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte or a pyknotic nucleus, and/or are surrounded by disorganized granulosa cells, which were detached from the basement membrane.

To evaluate follicular activation, the percentages of healthy primordial and activated follicles were calculated before FC and after culture in each medium. In addition, follicular and oocyte diameters were measured only in healthy follicles. Follicle diameter was recorded as the length from edge to edge of the basement membrane or from the outside edge of the theca cell layer when present. Oocyte diameter was recorded as the length from edge to edge of the oocyte membrane. Two perpendicular diameters were recorded for each parameter, and the average of these two values was reported as follicle and oocyte diameter, respectively. Care was taken to count each follicle only once as we have also done in our earlier studies. Each follicle was examined in every section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once, regardless of its size.

Ultra-structural analysis of follicular morphology

For better evaluation of follicular morphology, ultra-structural studies were carried out on fragments of FC and those with the best results during the histological analysis after 7days of culture. Briefly, small pieces (1 mm^3) of caprine ovarian tissues were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 h at room temperature. After fixation, fragments were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h. The samples were dehydrated through an acetone gradient, and the tissues were embedded in Spurr’s resin. Semi-thin sections (3 μm) were cut on an ultramicrotome (Reichert Supernova, Heidelberg, German) for light microscopy studies and stained with toluidine blue. The ultra-thin sections (60–70 ηm) were contrasted with uranyl acetate and lead citrate and examined under a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope. Parameters such as density and integrity of ooplasmic and granulosa cell organelles, vacuolization, and basement membrane integrity were evaluated.

Viability assessment of follicles cultured *in vitro*

Based on the results of morphological and histological analysis, the viability or survival follicles cultured with the concentration of S1P, that provided the outcome height, was further analyzed using a more accurate method of assessment based on fluorescent probes. Caprine preantral follicles from ovarian fragments FC and cultured for 7 days with S1P (1 ng/ml) were isolated by the mechanical method described by (LUCCI et al., 1999a). Briefly, using a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to a sectioning interval of 75 μm, samples were cut into

small pieces, which were placed in MEM and suspended 40 times using a large Pasteur pipette (diameter of about 1600 µm) and subsequently 40 times with a smaller Pasteur pipette (diameter of approximately 600 µm) to dissociate preantral follicles from stroma. The obtained material was passed through 500- and 100-µm nylon mesh filters, resulting in a suspension containing preantral follicles smaller than 100 µm in diameter. This procedure was carried out within 10 min at room temperature.

Thereafter, the viability of preantral follicles were analyzed using a two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells by calcein-AM and ethidium homodimer-1, respectively. While the first probe detected the intracellular esterase activity of viable cells, the second probe labeled nucleic acids of non-viable cells with plasma membrane disruption. The test was performed by adding 4 µM calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) to the suspension of isolated follicles and incubating at 37°C for 10 min. After being labeled, follicles were washed three times in MEM and mounted on a glass microscope slide in 5 µl antifading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching and finally examined using an a DMLB fluorescence microscope (Leica, Germany). The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. Oocytes and granulosa cells were considered live if the cytoplasm was stained positively with calcein-AM (green) and the chromatin was not labeled with ethidium homodimer (red).

Statistical analysis

Percentages of surviving follicles at all stages, primordial and growing, obtained after 1 or 7 days in the various treatments, as well as data from follicle and oocyte

diameters, were initially submitted to Kolmogorov–Smirnov and Bartlett's tests to confirm the normal distribution and homogeneity of variance, respectively. Analysis of variance was then carried out using GLM procedure of Statistical Analysis System, 1999 and Dunnett's test applied to compare S1P-treated groups against FC and CC. In order to avoid type II error because of the high coefficient of variation, Duncan's test was applied to compare different S1P concentrations and Student's t-test was used to compare means between 1 and 7 days of culture. Differences were considered to be significant when $P<0.05$ and results were expressed as mean ± standard error of means (SEM).

3. Results

Effect of S1P on the follicular survival

In the present study, a total of 1,950 caprine preantral follicles were analyzed. As shown in Figure 1, in all treatments, after one or seven days of culture, there was a significant reduction ($P<0.05$) in the percentage of histological normal follicles when compared to the FC (86.6 %).

In addition, after 1 day, it was observed a significant lower percentage of normal follicles ($P<0.05$) in tissues cultured with 100 ng/ml of S1P (60.2 %) than CC (71.2 %). There was not any significant difference among S1P concentrations in relation to the percentage of normal follicles after 1 or 7 days of culture ($P>0.05$). However, with the progression of the culture from day 1 to 7, only the smallest concentration of S1P (1 ng/ml) maintained the percentage of normal follicles ($P>0.05$), while the other treatments significantly reduced this parameter ($P<0.05$). Figure 2 shows morphological normal

preantral follicles after seven days of culture in medium supplemented with S1P at 1 ng/ml.

Follicular activation and development after culture with S1P

The percentage of primordial and activated (intermediate, primary and secondary) follicles in fresh tissue were 39% and 61% respectively. After 1 or 7 days of culture, there was a significant reduction in the percentage of primordial follicles in all treatments compared with FC (Figure 3; P<0.05). In addition, after 7days, a significant reduction in the percentage of primordial follicles compared with CC was observed in all treatments (P<0.05). With the progression of the culture period from 1 to 7 days, it was observed a significant reduction in the percentage of primordial follicles with 50 and 200 ng/ml of S1P (P<0.05). With respect to the percentage activated follicle. After 7 days of culture, all concentrations S1P were significantly height in relationship to FC or CC and supplemented with S1P (P<0.05). However, after 7 days of culture, in all S1P concentrations, was significantly higher in comparison were low the percentage of primary follicles observed in FC and CC (P<0.05).

Regarding the follicular growth, in all treatments, after 1 day of culture, there was no significant effect of S1P on follicular diameter compared with FC, CC or any concentration of S1P. However, with the progression of the culture period from 7, it was observed a significant reduction in follicular diameter with 10 ng/ml of S1P (P<0.05). In relation to the oocyte growth, there was a significant decrease (P<0.05) oocyte diameter, except in 50 ng/ml of S1P compared with FC (P>0.05).

Ultra-structural analysis of caprine preantral follicles cultured with S1P

To better evaluation of follicular quality, ultra-structural analysis was performed using morphologically normal preantral follicles from FC, as well as from treatments which showed the best results of follicular survival (tissues cultured in S1P at 1 ng/ml) and growth (tissues cultured in S1P at 50 ng/ml) after 7 days of culture, according to previous histological analysis. Ultra-structural normal follicles from FC had intact basal and nuclear membranes and a large oocyte nucleus. In addition to the sparse vesicles, there were several organelles uniformly distributed in the ooplasm, especially mitochondrias. Both the smooth and rough endoplasmic reticula were present, either as isolated aggregations or as complex associations with mitochondria and vesicles. The oocyte nucleus had uncondensed chromatin. Granulosa cells were ultrastructurally normal and well organized around the oocyte, showing an eloganted and large nucleus with irregular membrane. Well-developed rough endoplasmic reticulum and mitochondria with well-developed lamellar cristae were the most evident organelles observed in granulosa cells (Figure 4A). After culture for 7 days, showed an increase in cytoplasmic vacuolization, although the basal and nuclear membranes remained intact. Furthermore, the lowest S1P concentration (1 ng/ml) maintained the ultra-structural integrity of granulosa cells (Figure 4B).

When cultured in 50 ng/ml of S1P for seven days, transmission electron microscope studies, revealed some changes in follicular ultra-structure, which are indicative of degeneration. Such follicles presented a substantial irregularity of the follicular, oocyte and nuclear outlines. As degeneration progresses, the ooplasm in these follicles was extremely vacuolated, with the vacuoles often being fused, thus producing a larger vacuolated area, and in some oocytes the nuclear membrane was broken. Cytoplasmic organelles were more randomly observed. In addition, signs of endoplasmic reticulum proliferation and damage to mitochondrial membranes and cristae were

observed. The oocyte nucleus appeared retracted and granulosa cells looked swollen (Figure 4C).

Viability assessment of follicles cultured with S1P

In the present study, the preantral follicles in ten ovary were isolated and analyzed before in groups FC and after seven days of culture with 1 ng/mL of S1P. This qualitative analysis showed viable follicles with both oocyte and granulosa cells stained in green with calcein-AM before and red non-viable follicle after culture (Figure 5A and B).

4. Discussion and Conclusions

The present study described morphological, changes preantral follicle caprine of primary for preantral follicle activated, after culture in medium containing S1P *in vitro*. As there are only few papers dealing with S1P effects in animals, the concentrations used in this experiment were chosen based on the plasmatic concentrations of S1P in humans, between 76-300 ng/ml. (MURATA et al., 2000).

In the current study, only the smallest concentration of S1P (1 ng/ml) maintained the percentage of normal follicles with the progression of the culture from day 1 to 7. S1P has been found to have several biological actions, including Ca^{2+} mobilization, reorganization of the cytoskeleton, as well as cell growth, differentiation and survival (PYNE & PYNE, 2000; SPIEGEL & MILSTIEN, 2000). The mechanisms of S1P in apoptosis are not fully understood. It has been shown that ceramide concentrations in the cell increase on exposure to environmental stresses such as radiation (VERHEIJ et al., 1998), heat shock (KONDO et al., 2000) or oxidative stress (CHAN & GOLDKORN,

2000). Therefore, whereas stressful stimuli increase the concentrations of ceramide and sphingosine, leading to apoptosis, survival factors activate sphingosine kinase, resulting in accumulation of S1P and consequent suppression of apoptosis (SPIEGEL & KOLESNICK, 2002). This fact may explain our satisfactory results regarding to follicular viability after culturing with 1 ng/ml of S1P. Similar to our results, previous studies have demonstrated that S1P has an ant-apoptotic effect before an exposure to radiation (MORITA et al., 2000; KAYA et al., 2008) or chemotherapy in mouse (PEREZ et al., 1997; HANCKE et al., 2007), as well as in xenografted ovarian tissue monkey (LEE et al., 2005) and also protects oocytes from heat shock (ROTH & HANSEN, 2004). (ONIONS et al., 2008) have demonstrated that S1P can act as a cryoprotectant agent, maintaining follicular viability after cryopreservation of ovarian tissue. On the other hand, (KAYA et al., 2008) observed that S1P promoted a small protection in follicular viability when associated with radiation and in rat ovaries. Stress before slaughter and process of ovaries collections, therefore slice cut for colleting of fragment ovarian cortex and culture, were aggressing cellular, but the S1P, was able to reverse process caused by ceramida, survival keeping for 7days.

From day 1to 7 day of culture, the number of primordial follicles was markedly reduced after culturing in 50 and 200 ng/ml S1P, which was not followed by a concomitant increase in the percentage of primary follicles. These results could be due to primordial follicle atresia and to the maintenance of primary follicle survival. On the other hand, after 7 days of culture, addition of any S1P concentration to the medium resulted in a reduction in the percentage of primordial follicles with a concomitant increase in the percentage stage transition, primary and secondary follicle, compared with FC and CC. These data suggest S1P treatment stimulates follicular development, when compared FC and CC. *In vivo*, follicular activation is characterized by changing granulosa cell

morphology from flattened to cuboidal, and their further proliferation, which lasts approximately 25-30 days in cow and sheep (MCNATTY et al., 1995; BRAW-TAL & YOSSEFI, 1997). In comparison, 7 days is short period, the caprine follicles after 7 days of culture, showed results similar a study of (SPIEGEL & KOLESNICK, 2002), has showed that S1P stimulates mouse preantral follicle growth. However, the molecular mechanisms of S1P action in promoting follicular activation and growth are not clear. It is important to note that a great percentage activated follicles was observed after a small period of culture (seven days) in all medium supplemented with S1P, suggesting that this substance may play an important role in follicular activation and further development.

The present ultra-structural studies revealed differences in the health of follicles cultured in 1 ng/ml of S1P, when compared to those in medium supplemented with 50 ng/ml of S1P for seven days. Using transmission electronic microscopy, although some vacuoles have been observed, important structures such as the basal and nuclear membranes, as well as granulosa cells were preserved even after seven days of culture in the presence of 1 ng/ml of S1P. Nevertheless, degenerative features were seen in follicles cultured in 50 ng/ml of S1P, such as extreme ooplasm vacuolization. In addition to ultra structural analysis, preantral follicles cultured for seven days with 1 ng/ml of S1P were further analyzed using a viability assessment, which suggests that this treatment is efficient to maintain preantral follicle viability. The fluorescent probes calcein-AM and ethidium homodimer-1 have been used successfully to assess the viability of bovine and caprine early-staged follicles (VAN DEN HURK et al., 1998; LOPES et al., 2009). This method can be used for analyze viability aspects of follicles, thereby offering a new approach for investigating metabolic and developmental aspects of folliculogenesis *in vitro*.

In conclusion, the preserving histological and ultra structural oocitary and foliculary structures is mediated by S1P (1 ng/ml), able of promotion grow factor active preantral follicle and maintain the viability of caprine preantral follicles after 7 days of *in vitro* culture.

5. Aknowledgments

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Legends of Figures

FIGURE 1. Percentage of preantral follicle Survival after 7 days evaluated for optical microscopy. Tissue fresh control (FC) strip bars, cultured control (CC) and different concentrations of S1P (black bars). * differs significantly from control follicles (P<0.05); A, B differs significantly between each treatment (P<0.05).

FIGURE 2. Histological section of tissue cultured for 7 days in 1 ng/ml of S1P, (A) showing normal and activated follicles in different stages of development. Primary follicles, with one line of cuboidal granulosa cells and Secondary follicles, with two lines of cuboidal granulosa cells (200x). (B) Secondary follicle after 7day of culture, with multilayer of granulosa cells and pellucid zone evidence (400x).

FIGURE 3. Percentages of primordial and preantral follicles activated (intermediate primary and secondary, fresh control (FC), cultured control (CC) and different concentrations of S1P. * differs significantly from FC (P<0.05); ♦ differs significantly from CC alone in each day of culture (P<0.05).

FIGURE 4. Electron micrographs of caprine ovarian follicles before (A) and after culture in 1 ng/ml of S1P for 7 days (B), in 50 ng/ml of S1P for 7 days (C). In non-cultured follicles, note the homogeneous cytoplasm with numerous rounded mitochondria (4A). In figure 4B, note the presence of some vacuoles in the oocyte and the well-preserved granulosa cells (4B). Note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration, as well as the empty space in degenerated granulosa cells after *in vitro* culture with 50 ng/ml of S1P (4C). gc: granulosa cells, m:

mitochondria, n: nucleus, nc: nucleulus, v: vacuole, arrow: oocyte membrane; arrow head: nuclear membrane.

FIGURE 5. Viability assessment of caprine preantral follicles using fluorescent probes. An isolated preantral follicle after 7day of culture, that was classified as non-viable (A) because cells were labeled by calcein-AM (Red fluorescence) and preantral follicle viable (B) Green fluorescence. Bar = 50μm.

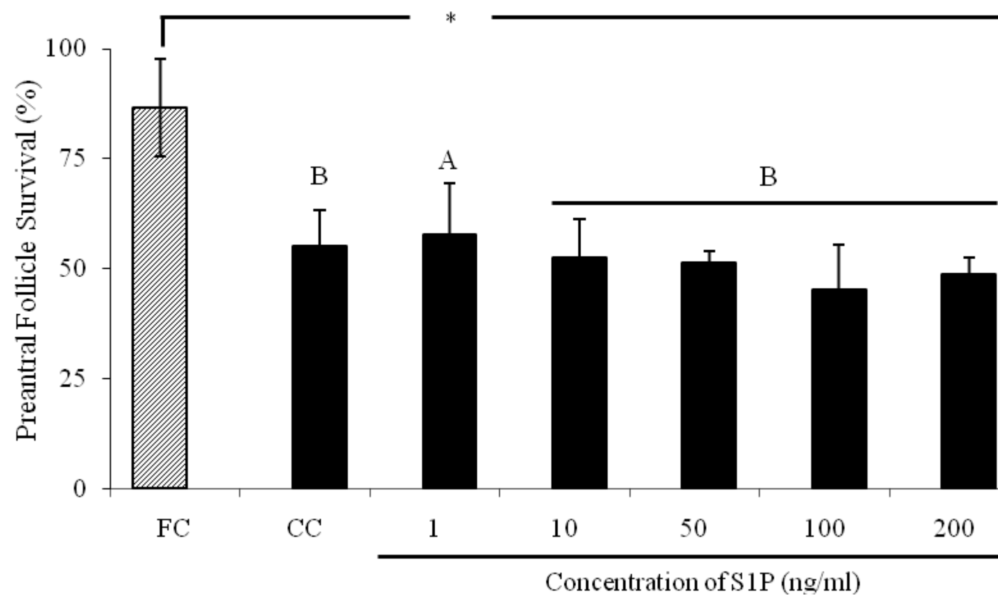

Figure 1

* differs significantly from control (P<0.05).

A, B differs significantly between each treatment (P<0.05).

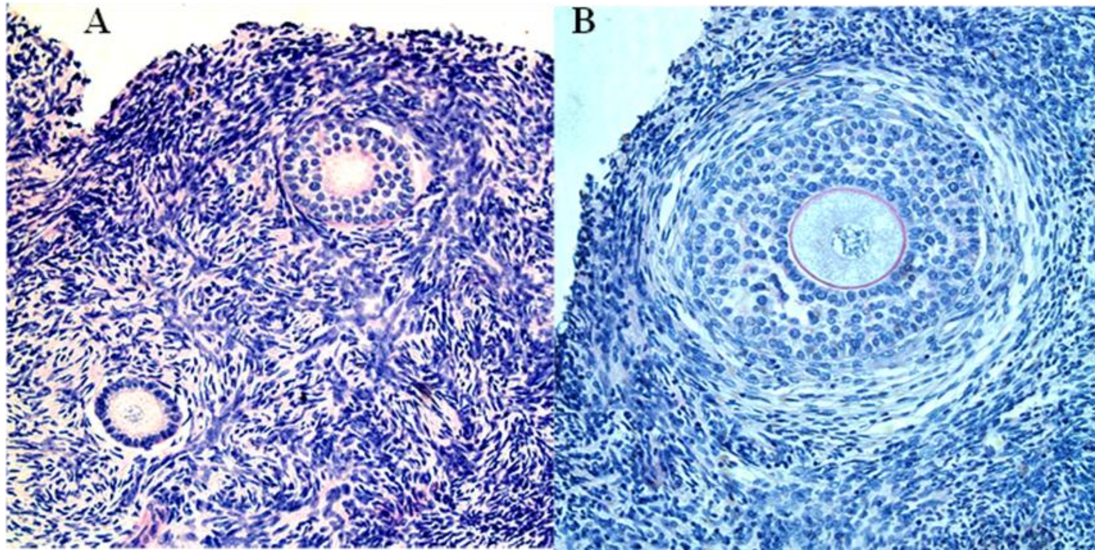

Figure 2

Preantral Follicle Stage	Primordial	Activated
Tratamento	D7	D7
FC	39,04 ± 13,12	60,96 ± 13,12
CC	21,76 ± 9,25*	78,24 ± 9,25 *
S1P.1 ng/ml	3,27 ± 4,05*♦	96,73 ± 4,05*♦
S1P.10 ng/ml	3,91 ± 6,23*♦	96,09 ± 6,23*♦
S1P. 50 ng/ml	5,00 ± 6,85 *♦	95,00 ± 6,85 *♦
S1P. 100 ng/ml	2,65 ± 3,71 *♦	97,35 ± 3,71 *♦
S1P. 200 ng/ml	2,87 ± 3,95 *♦	97,13 ± 3,95 *♦

Figure 3

* differs significantly FC (P<0.05).

♦ differs significantly from CC alone in each day of culture (P<0.05).

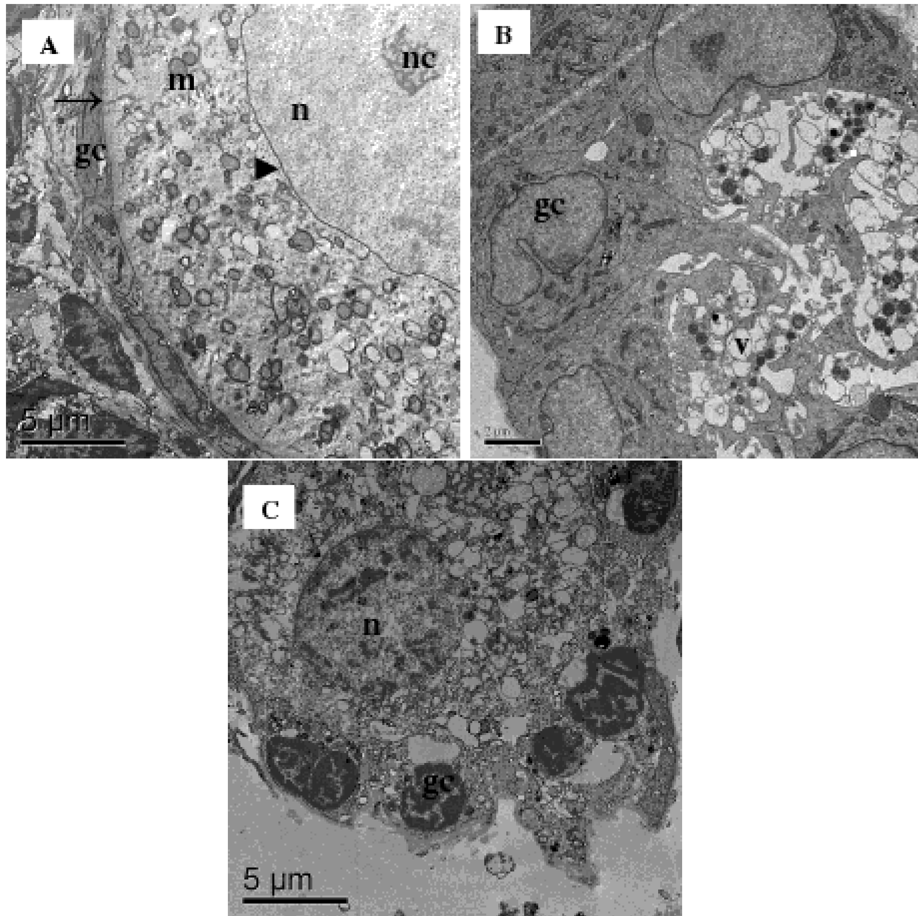

Figure 4

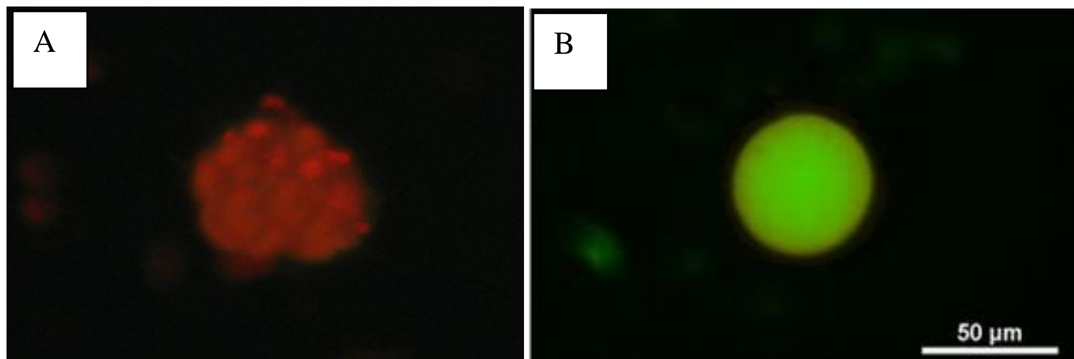

Figure 5

4. CAPÍTULO 2

TRABALHO ACEITO PARA PUBLICAÇÃO:

4.1. Leukemia Inhibitory Factor stimulates the transition of primordial to primary follicle and supports the goat primordial follicle viability in vitro

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Zygote, 2010

Leukemia Inhibitory Factor stimulates the transition of primordial to primary follicle and supports the goat primordial follicle viability *in vitro*

Running headline: LIF goat primordial follicle

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Leukemia Inhibitory Factor stimulates the transition of primordial to primary follicle and supports the goat primordial follicle viability in vitro.

Summary

The aim of this study was to evaluate the effect of Leukemia Inhibitory Factor (LIF) on the activation and survival of preantral follicles cultured *in vitro* enclosed in ovarian fragments (*in situ*). Goat ovarian cortex was divided into fragments to be used in this study. One fragment was immediately fixed (fresh control - FC) and the remaining fragments were cultured in supplemented Minimum Essential Medium (MEM) without (cultured control - CC) or with different concentrations of LIF (1, 10, 50, 100 or 200 ng/ ml) for 1 or 7 days, at 39°C in air with 5% CO_2. Fresh control, CC and treated ovarian fragments were processed for histological and fluorescence analysis. The percentage of histological normal preantral follicles cultured for 7 day with 1 ng/ ml (49.3%), 10 ng/ ml (58.6%) and 50 ng/ ml (58%) of LIF was higher than in the CC (32.6%; $p < 0.05$). After 7 days of culture, the percentage of primordial follicles *in situ* cultured with LIF decreased and primary follicles increased in all LIF concentrations compared to FC and CC ($p < 0.05$). In conclusion, Leukemia Inhibitory Factor induced primordial follicles activation and supported preantral follicle viability of goat ovarian tissues cultured for 7 days.

Keywords: Preantral follicles, leukemia inhibitory factor, ovary, primordial follicle activation, goat

Introduction

The mammalian ovary is constituted by thousands of primordial follicles at birth, which are considered the resting pool of follicles in the ovary. Throughout the female reproductive life span, a small number of primordial follicles are stimulated to grow into a process referred as follicular activation whereas the vast majority (99.9%) becomes atretic toward ovulation (SKINNER, 2005). Until recently, relatively little was known on how these primordial follicles are activated and stimulated to develop in a more advanced stage. Moreover, it is known that in mammalians, including primates, the early follicular development goes through a complex process, in which the oocytes grow and their surrounding somatic cells proliferate and differentiate through preantral follicle stages (GOUGEON, 1996; FORTUNE, 2003). However, few long-term *in vitro* culture studies achieved follicular activation in sheep (MURUVI et al., 2005), human (SADEU et al., 2006) and goat (ROSSETTO et al., 2009).

Leukemia inhibitory factor (LIF) is a glycoprotein with pleiotropic activity that exerts an important role on the follicular growth, oocyte maturation and a wide variety of cell types including somatic and follicular cells (DEMEESTERE et al., 2005). (SHEN & LEDER, 1992) suggested a specific role for LIF on mouse preimplantation development. Among its many activities, LIF can maintain embryonic stem cell monolayers in a pluripotent undifferentiated state. Recently, LIF is reported to inhibit myeloid leukemic cells and can enhance survival, migration, proliferation and meiosis resumption of primordial primitive cells in rodents (VAN DEN HURK & ZHAO, 2005).

In rats, the level of LIF in the follicular fluid increases as ovarian follicles develop. There are evidences that LIF is responsible for oocyte development and maturation and preantral follicle viability *in vitro* (HAIDARI et al., 2008). Reports on the development of rat preantral follicles cultured with LIF showed to promote the transition from primordial to primary follicles (NILSSON et al., 2002; HAIDARI et al., 2006). Although LIF was identified to be involved in mammalian folliculogenesis, the role of LIF on preantral follicle activation is an issue that remains far from understood. Therefore, the aim of the present study was to investigate the influence of different concentrations of LIF on the activation of primordial follicles and further follicular survival after *in vitro* culture of caprine ovarian tissue.

Materials and Methods

Unless mentioned otherwise, all chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO).

Ovaries collection

Ovaries (*n*= 10) were collected from five non-pregnant mixed-breed adult goats (1-3 years-old). All animals were in the follicular phase of the estrous cycle with absence of corpora lutea. Immediately after being slaughter, ovaries were dissected from the surrounding connective tissue and washed once in 70% alcohol, followed by two times in Minimum Essential Medium (MEM) supplemented with 100 µg/ ml penicillin and 100 µg/ ml streptomycin. Then, ovaries were transported to the laboratory at 4 °C within 1 h.

Experimental design

In the laboratory, ovaries were stripped of surrounding tissues and the medulla and visible growing follicles were manually removed. Ovarian tissue samples from each ovarian pair were cut in slices of 9 mm^3 (approximate size 3 mm x 3 mm x 1 mm thickness) using a 26-G needle and a scalpel blade under sterile conditions. The tissue pieces were then either directly fixed for histological analysis (fresh control - FC) or placed in culture for 1 or 7 days. The tissues were transferred to 24-well culture Petri dishes containing 1 ml of culture medium. Culture was performed at 39 °C in air with 5% CO_2 in a humidified incubator and medium were incubated for 1 h prior to use. The basic culture medium (cultured control - CC) consisted of αMEM supplemented with ITS (10 µg/ ml insulin, 5.5 µg/ ml transferrin and 5 ng/ ml selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine and 1.25 mg/ ml bovine serum albumin. For the experimental conditions, basic medium was supplemented or not with LIF at different concentrations (1, 10, 50, 100 or 200 ng/ ml). Each treatment was repeated five times and the culture media was replaced every other day throughout the 7 days period.

Histological assessment of *in vitro* follicular growth

For the evaluation of follicular morphology (survival), all the ovarian pieces were fixed in Carnoy's solution for 12 h and then dehydrated in increasing concentrations of ethanol at 70, 80, 95 and 100%, before culture (FC) and after 1 or 7 days of culture. After paraplast embedding, caprine tissues fragments were sliced into 5 µm sections, mounted on glass slides and stained by Periodic Acid Schiff - Hematoxylin. Follicle stage and viability were assessed microscopically on serial

sections. Coded anonymized slides were examined on a microscopy (Nikon, Japan) under 400X magnification (SILVA et al., 2002). Each follicle was examined in every section and matched with the same follicle on adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once, regardless of its size.

The developmental stages of follicles have been defined previously 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; (SILVA et al., 2004). Follicles were classified individually as histological normal when an intact oocyte was present and surrounded by a well organized of one or more layers of granulosa cells without the appearance of pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte or a pyknotic nucleus, and/or surrounded by disorganized granulosa cells, which were detached from the basement membrane. Overall, 1.950 follicles were evaluated for each treatment [30 follicles X 13 groups (1 or 7 days of culture) X 5 replicates]. To evaluate follicular activation, the percentages of healthy primordial follicles were calculated before FC and after culture in each medium with or without LIF.

Viability assessment of preantral follicles by fluorescence

Based on the results of histological analysis, the viability of follicles cultured with LIF that provided the best outcome (10 and 50 ng/ml) was further analyzed using a more accurate method of assessment based on fluorescent probes. Goat

preantral follicles were isolated using a mechanical method described by (LUCCI et al., 1999b). Ovarian fragments were cultured for 7 days with CC, 10 or 50 ng/ ml LIF. Briefly, using a Tissue Chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to a sectioning interval of 75 µm, samples were sliced into small fragments, placed in MEM and suspended 40 times using a large Pasteur pipette (diameter of about 1600 µm) and resuspended subsequently 40 times with a small Pasteur pipette (diameter of about 600 µm) to dissociate preantral follicles from stroma. The obtained material was passed through 100 µm nylon mesh filters, resulting in a suspension containing preantral follicles smaller than 100 µm in diameter. This procedure was carried out within 10 min at room temperature.

Thereafter, the viability of preantral follicles were analyzed using a two-color fluorescence cell assay based on the simultaneous determination of viable or degenerated cells by calcein-AM and ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), respectively. While the first probe detected intracellular esterase activity of viable cells, the second probe labeled nucleic acids of non-viable cells with plasmatic membrane disruption. Fluorescent analysis was performed by adding 4 µM calcein-AM e 2 µM ethidium homodimer-1 (LIVE/ DEAD Viability kit: L, Molecular Probes, Invitrogen, USA) to the suspension of isolated follicles, followed by incubation at 37 °C for 15 min in a dark chamber. After being labeled, follicles were centrifuged at 100 *g* for 5 min, washed once and resuspended in αMEM. Then, labeled follicles were mounted on a glass microscope slide using 5 µl of antifading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching and finally examined using a DMLB fluorescence microscope (Leica, Germany). The emitted fluorescent signals of calcein-AM, and ethidium homodimer

were collected at 488 and 568 µm, respectively. Oocytes and granulosa cells were considered viable if the cytoplasm was stained positively with calcein-AM (green) and chromatin was not labelled with ethidium homodimer (red); otherwise, they were classified as degenerated (LOPES et al., 2009).

Statistical analysis

The mean percentages of primordial, viable (at all stages) obtained after 1 or 7 days of culture were subjected to analysis of variance (ANOVA) using the GLM procedure of SAS/ STAT software. Data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. Dunnett´s test was applied to compare LIF-treated groups against FC and CC groups. Duncan's test was used to compare differences among LIF concentrations and days of culture. Data from fluorescent analysis were subjected to Qui-square Test. Differences were considered to be significant when $p < 0.05$ and data were expressed as mean ± standard error of means (S.E.M.).

Results

Follicular activation and development

The percentages of primordial and primary follicles during culture with different concentrations of LIF are shown in Table 1. After seven days of culture, the number of primordial follicles decreased (LIF from 9.8% to 15.2% vs. control groups FC 43.7% and CC 42.5%) and primary follicles increased (LIF from 38.9% to 53.4% vs control groups FC 15.4% and CC 22.8%) when LIF was present in culture ($p < 0.05$).

In the presence of LIF, formation and differentiation (from flattened to cuboidal cells) of a new cellular layer of granulosa cells were observed, giving origin to primary follicle. These findings are evidence of preantral follicle activation induced by LIF.

Follicular survival

The preantral follicle survival was histologically evaluated after 1 or 7 days of culture in the presence or absence of LIF. An example of viable preantral follicle is depicted in Fig. 1A. The degenerated follicles presented a retracted oocyte, pyknotic nucleus, and/ or cytoplasmic vacuolization (Fig. 1B). The viable preantral follicles were also labeled by calcein-AM green fluorescence (Fig. 1C) and the degenerated preantral follicles showed chromatin labelled by ethidium homodimer red fluorescence (Fig. 1D).

The preantral follicle survival did not differ among treatment and control groups after 24 h of culture. However, the survival of preantral follicles was improved when LIF was used in culture for 7 days (LIF treatments vs CC groups; $p < 0.05$; Fig. 2), which was confirmed by fluorescence method (Fig. 3). Furthermore, the highest percentages of viable preantral follicles were obtained at concentrations between 10 and 50 ng/ ml of LIF (from 58.6% to 58.0%), decreasing at 200 ng/ ml (46.7%; $p < 0.05$). The survival of isolated preantral follicles before culture (FC) was above 90%, which demonstrated follicular health at the time of follicular isolation.

Discussion

The effect of LIF on the activation of primordial and survival of preantral follicles was demonstrated in a 7-day culture system, using goat as a model. Our

results revealed that 1) LIF induced activation of primordial follicle, formation and differentiation of new cellular layer of granulosa cells, giving origin to primary follicle; and 2) LIF supported preantral follicle viability for 7 days in culture, based on histological and fluorescence analyses.

Slices of goat ovarian cortex were cultured in the absence and presence of different concentration of LIF to verify if LIF promotes primordial to primary follicle transition in ruminants. The percentages of primordial follicles decreased and primary follicles increased at all LIF concentration tested at 7 days of culture. The optimal LIF concentrations to maintain caprine preantral follicles viable and growing ranged from 10 to 50 ng/ ml. In fetal rat ovaries, LIF induced premature follicular development and meiosis resumption when used in a concentration of 100 ng/ ml (LYRAKOU et al., 2002). Recently, (HAIDARI et al., 2008) reported that LIF at 50 ng/ ml promoted follicular survival and preantral follicle development *in vitro* using rat follicles.

In the concentrations used in the present study, LIF induced increase in granulosa cell number and change from flattened to cuboidal shape, characterizing the transition to primary follicle. These results indicate that LIF can promote activation of primordial follicle and transition to primary follicle. Similarly, the dramatic decrease of primordial follicle and corresponding increase of primary follicle numbers after LIF treatment were observed in the rat ovary (NILSSON et al., 2002; HAIDARI et al., 2008). In the rat, LIF also promoted the transition from primordial to primary follicles and supported their viability for 14 days in culture. However, there is little information on the role of LIF in promoting preantral follicle activation, development and viability in domestic animals.

LIF is expressed in rat preantral follicle (NILSSON et al., 2002; HAIDARI et al., 2008) and in the ovarian stromal cells in human (ARICI et al., 1997). These last authors also demonstrated that LIF concentrations rise in periovulatory follicular fluid and regulate ovulation, estrogen production and early embryonic development. Despite being expressed in preantral follicles and promoting the development of primordial follicles, it appears that LIF has a role, but is not essential for activation and transition to primary follicles, considering that follicles reach ovulation in LIF knockout mice (STEWART et al., 1992). Therefore, there are strong evidences that the regulation of initial preantral follicle activation and growth is orchestrated by LIF and other signal factors (such as growth differentiation factor 9 - GDF9, kit ligand - KL, basic fibroblast growth factor 2 - bFGF2 and nerve growth factor - NGF), having a compensatory action when one factor is missing in the system.

The expression and function of several transcription factors and hormones responsible for the regulation of follicle development is species specific and differences have been demonstrate in rodents, ruminants and primates (SHIMASAKI et al., 2004; YOUNG & MCNEILLY, 2010). For example, the tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) activity is different between rodents and cattle during the periovulatory period (DOW et al., 2002). LIF and bone morphogenetic proteins (BMPs) are essential for mouse embryonic stem (ES) cells to maintain pluripotency (SMITH et al., 1992; YING et al., 2003); however, human ES requires activin/nodal (VALLIER et al., 2004; JAMES et al., 2005; VALLIER et al., 2005). LIF and other factors (such as bFGF, BMP, GDF9, hepatocyte growth factor-HGF, insulin like growth factor-IGF, IGF binding protein-IGFBP, interleukin-1-IL1, keratinocyte growth factor-KGF, luteinising hormone-LH, stem cell

factor/ kit ligand-SCF, transforming growth factor beta-TGFβ, tumour necrosis factor-TNFalpha) have specie specific pattern of expression and function (for review (YOUNG & MCNEILLY, 2010). Therefore, the investigation of the LIF function in ovarian preantral follicle development is imperative in ruminants, which have a high economic importance and have been used as an experimental model for humans.

In conclusion, LIF induced activation of primordial follicles, differentiation of granulosa cells from flattened to cuboidal shape and maintenance of preantral follicle viability for 7 days in culture. To our knowledge, this is the first evidence that LIF is involved in the initial follicle development and viability in ruminant.

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Table 1: Histological evaluation of *in situ* cultured fragments of goat ovarian cortex for 1 or 7 days using different concentrations of LIF (ng/ ml).

Follicular Stage	Primordial Follicle			Primary Follicle		
Groups	D0	D1	D7	D0	D1	D7
FC	43.7 ± 4.5	-	-	15.4 ± 5.1	-	-
CC	-	60.0 ± 2.1	42.5 ± 6.1	-	10.9 ± 2.7	22.8 ± 9.4
LIF 1	-	43.0 ± 6.4	13.6 ± 1.2 *†	-	22.4 ± 8.2	38.9 ± 7.6 *†
LIF 10	-	43.9 ± 14.3	15.1 ± 4.1 *†	-	21.9 ± 8.8	41.5 ± 9.5 *†
LIF 50	-	33.6 ± 10.3 †	12.6 ± 4.7 *†	-	28.1 ± 8.1 †	50.6 ± 6.1 *†
LIF 100	-	49.0 ± 11.2	15.2 ± 2.7 *†	-	26.9 ± 4.8 †	42.4 ± 3.1*†
LIF 200	-	42.2 ± 13.7	9.8 ± 5.7 *†	-	41.0 ± 5.2 *†	53,4 ± 6.1 *†

(D) Day of culture; (*) differs significantly from Fresh Control (FC) and (†) differs significantly from cultured control (CC) in each day of culture ($p < 0.05$). Data are shown as mean percentage ± standard error of means (S.E.M.) out of all preantral follicle stages (primordial and primary follicles). Data were pooled from five replicates.

Figure and Legends

Figure 1: Viability assessment of caprine preantral follicles using histological and fluorescent analyses. A) Histological section of preantral follicle cultured for 7 days showing the stroma and follicle integrity; B) Histological section of preantral follicle cultured for 7 days showing citoplasmic vacualization, disorganized stroma cells and pycnotic nucleus; C) An isolated viable preantral follicle labeled by calcein-AM (green fluorescence); D) Degenerated preantral follicle showing chromatin labelled by ethidium homodimer (red fluorescence). gc: granulosa cells, n: nucleus, o: ooplasm; arrow: pycnotic nucleus. Bars represent 50 μm.

Figure 2: Histological analysis of the viability of preantral follicles (%) after 7 days of culture. The results are presented as the mean ± SEM of five independent cultures, and bars with no common letters are significantly different ($p < 0.05$).

Figure 3: Viability of preantral follicles analyzed by a two-color fluorescence cell using calcein-AM and ethidium homodimer-1. The best results observed in the histological analysis were repeated using probes to detected intracellular esterase activity of viable cells and to label nucleic acids of non-viable cells with plasmatic membrane disruption. The results are presented as the mean ± SEM of five independent cultures, and bars with no common letters are significantly different ($p < 0.05$).

List of Tables

TABLE 1: Histological evaluation of *in situ* cultured fragments of goat ovarian cortex for 1 or 7 days using different concentrations of LIF (ng/ ml). (D) Day of culture; (*) differs significantly from Fresh Control (FC) and (†) differs significantly from cultured control (CC) in each day of culture (P < 0.05). Data are shown as mean percentage ± standard error of means (S.E.M.) out of all preantral follicle stages (primordial and primary follicles). Data were pooled from five replicates.

Figure 1

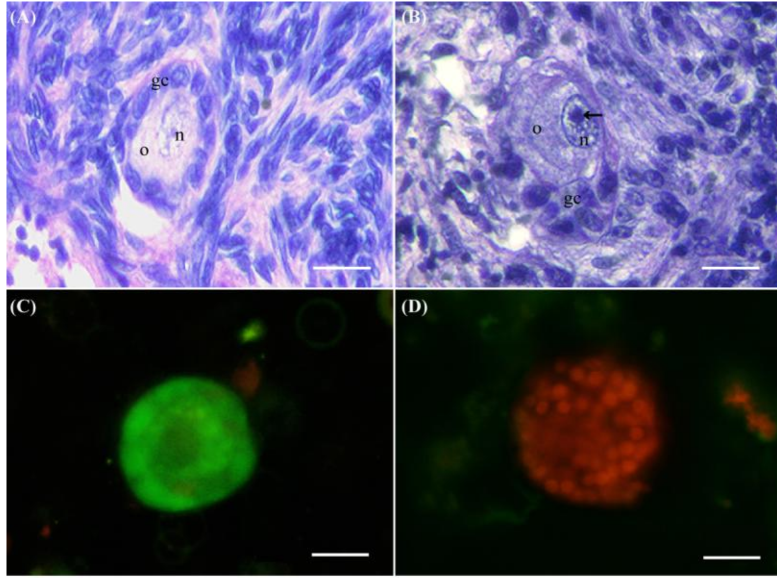


Figure 2

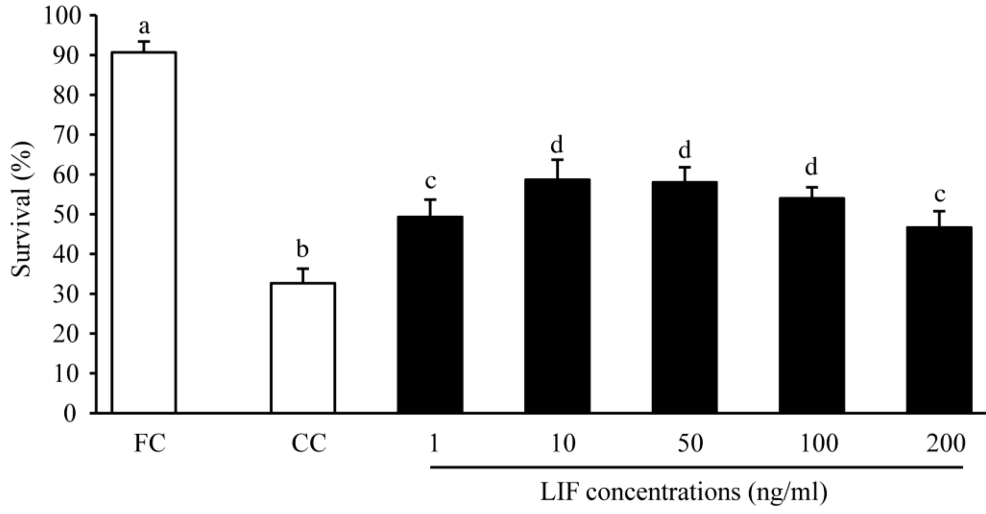
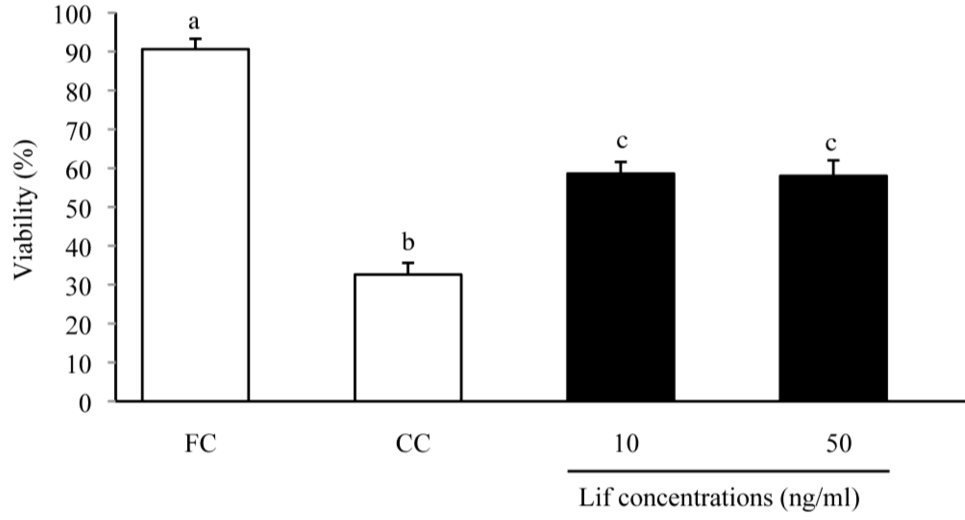


Figure 3



5. DISCUSSÃO

A MOIFOPA é uma alternativa para aumentar o potencial reprodutivo das fêmeas. Tendo em vista que a quantidade dos oócitos é limitada e, sua renovação em fêmeas adultas é discutida entre os cientistas. As perdas folículo-oocitária podem ser agradavas por fatores externos como : idade avançada, condição nutricional deficiente, exposição a radiações ionizantes ou quimioterapia e condições sanitárias desfavoráveis que potencializam a perda da reserva oocitária. Portanto os resultados com a manutenção da viabilidade dos FOPA cultivados *in situ* com S1P e LIF oriundos dos fragmentos do córtex ovariano, tendo cabras como modelo experimental, neste trabalho, poderá ser aproveitado e adaptado à outras espécies, incluindo humanos. Isso possui especial relevância em circunstância no qual o tecido ovariano é submetido a tratamentos altamente agressivos como radioterapia e quimioterapia. Além da manutenção da viabilidade folicular (EPPIG & SCHROEDER, 1989) conseguiram embriões *in vitro* em ratas, (WU et al., 2001) embriões de porcas e (GUPTA et al., 2008) obtiveram embriões de búfalas, todos apartir do cultivo FOPA *in vitro*. Os resultados obtidos com os FOPA de cabras, vêm a somar junto com outras pesquisas e sugerir que é possível manter a capacidade reprodutiva de mulheres mesmo submetidas aos tratamentos anti câncer.

A qualidade do FOPA e a manutenção da viabilidade após sua retirada dos ovários dependem de vários fatores, como o tempo de retirada, a temperatura de transporte e os meios empregados no cultivo, que são as condições essenciais para a manutenção da viabilidade folicular ao longo do cultivo *in vitro*. Várias técnicas têm sido empregadas para o isolamento de FOPA, algumas delas permitindo a recuperação de grande quantidade de Foliculos. Os métodos químicos ou enzimáticos muitas vezes são os eleitos (ITOH & HOSHI, 2000). Para que fossem mantidas as características do tecido ovariano, foi realizado o cultivo *in situ*, que

dentre as vantagens, dessa técnica, ela mimetizam as interações foliculares e oocitárias e destes com estroma ovariano.

Outra busca para o desenvolvimento folicular de FOPA *in vitro* é a obtenção de um meio de cultivo, que deve dispor e prover os elementos necessários que possibilitem o desenvolvimento e o crescimento folicular, além de manter sua viabilidade. Os resultados dessa pesquisa demonstram que é possível manter o desenvolvimento *in vitro* de FOPA de cabras *in vitro*, em que a adição de S1P 1ng/ml ao meio de cultivo, participou da ativação e crescimento dos FOPA até estagio secundário e a adição do LIF 10ng/ml manteve viabilidade folicular ao longo do cultivo participando da ativação oocitária. Estes são relatos inéditos empregando S1P e LIF em FOPA na espécie caprina.

A abordagem de cultivo utilizada nessa pesquisa possibilitou de forma artificial, condicionar a regulação entre os fatores estimulatórios e inibitórios presentes naturalmente no ovário. Alguns fatores acrescidos aos meios de cultivo podem contribuir para a ativação e outros apenas para o crescimento folículo-oocitário. Embora (RAJARAJAN et al., 2006) tenham demonstrado que a manutenção da viabilidade dos FOPA de cabras com adição vários fatores de crescimento ao meio de cultivo, em sua pesquisa todos os meios foram suplementados com FSH. Nessa pesquisa os FOPA de cabras foram cultivados *in situ* apenas com o S1P 1ng/ml ou LIF 10/ml, em meios livres de esteróides exógenos, demonstrando que as gonadotrofinas parecem não participar ou regular a ativação, manter a viabilidade ou o crescimento folicular. Por outro lado, é precoce afirmar a participação efetiva do FSH na foliculogênese em fase pré-antral.

Como não há relatos de cultivo *in situ* de FOPA isolados com S1P 1ng/ml ou LIF 10ng/ml e tendo em vista a resposta da ativação e crescimento folicular além da manutenção da viabilidade por 7 dias de cultivo, é possível especular que existem receptores para S1P (EDG1) e para o LIF (GP130) em FOPA de cabras. Os resultados obtidos em cabras reforçam

os resultados preliminares observados em ovários de ratas, em que a S1P manteve a viabilidade e estimulou o crescimento folicular em camundongas (SPIEGEL & KOLESNICK, 2002; KAYA et al., 2008). Em outros estudos, o LIF contribuiu para ativação dos folículos primordiais em mulheres, bem como, crescimento e ativação dos folículos de ratas (ABIR et al., 2004); (NILSSON et al., 2002; HAIDARI et al., 2006).

Compreender o sistema de sinalização intracelular que controla a manutenção e ativação folicular tem implicações significativas para melhorar a produtividade e longevidade reprodutiva das fêmeas, além de sua aplicação na criação de animais domésticos, no controle populacional de animais selvagens e infertilidade nas mulheres (MCLAUGHLIN & MCIVER, 2009).

Sabendo que os FOPA presentes nos ovários são formados em estágio fetal, podendo permanecer quiescentes por muitos anos, esse modelo proposto se apresenta como uma alternativa que pode ser adaptado à outras espécies. Para a espécie caprina, tanto a S1P 1ng/ml quanto o LIF 10ng/ml em cultivos com FOPA são fatores que propiciam a ativação do crescimento folicular. Assim essa pesquisa vem a contribuir para o entendimento e a compreensão de uma parte importante da foliculogênese, tendo como modelo experimental a espécie caprina.

6. CONCLUSÕES

Conforme os resultados obtidos e exposto neste trabalho, permitem afirmar que o meio de cultivo empregado nesta pesquisa, acrescido de S1P na concentração de 1ng/ml teve como resultado a ativação e o crescimento dos FOPA de cabras cultivados *in situ* por 7 dias, confirmado pela histologia, microscopia eletrônica de transmissão e microscopia de fluorescência a ativação e desenvolvimento FOPA da fase quiescente ou primordial, até seu desenvolvimento em estágio de FOPA secundário, mimetizando *in vitro* as mesmas condições fisiológicas do ovário.

Quanto a resposta do LIF adicionado ao meio de cultivo, suporta afirmar que sua adição ao meio de cultivo na concentração de 10ng/ml foi a que se destacou dentre as demais concentrações, tendo em vista a resposta dos FOPA inclusos no córtex ovariano de cabras contendo cultivados *in situ*, que foi capaz de manter a viabilidade e promover a ativação desses folículos após 7 dias de cultivo *in vitro*. Os resultados foram confirmados pelas diferentes técnicas de microscopia como histologia clássica e microscopia de florescência.

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