UNIVERSIDADE ESTADUAL DO CEARÁ
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

JULIANA JALES DE HOLLANDA CELESTINO

EXPRESSÃO DO RNAm DO KIT LIGAND (KL), PROTEÍNA MORFOGENÉTICA ÓSSEA 15 (BMP-15) E FATOR DE CRESCIMENTO EPIDERMAL (EGF) E EFEITO DAS RESPECTIVAS PROTEÍNAS SOBRE O DESENVOLVIMENTO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS CAPRINOS

FORTALEZA
2010

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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para obtenção do título de Doutor em Ciências Veterinárias.

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Ao meu marido e filha, Ricardo Antonio Rebouças Celestino e Júlia de Hollanda Celestino;
Aos meus pais, José Agenor Matos de Hollanda e Maria Auxiliadôra Jales Cartaxo;

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RESUMO

Os objetivos deste estudo foram: 1) quantificar os níveis de RNAm para o kit ligand (KL), proteína morfogenética óssea 15 (BMP-15) e fator de crescimento epidermal (EGF) em ovários caprinos através da técnica de RT-PCR em tempo real; 2) avaliar o efeito da adição de diferentes concentrações destas substâncias sobre a sobrevivência, ativação e crescimento *in vitro* de folículos pré-antrais caprinos cultivados *in situ* e 3) investigar os efeitos do EGF sozinho ou associado ao hormônio folículo estimulante (FSH) sobre a sobrevivência, formação de antro e crescimento de folículos secundários caprinos isolados, bem como sobre os níveis de RNAm para o EGF e receptor de FSH (FSH-R). Para o cultivo *in situ*, fragmentos de córtex ovariano foram cultivados *in vitro* por um ou sete dias em MEM^+ adicionado de diferentes concentrações (0, 1, 10, 50, 100 ou 200 ng/mL) de KL, BMP-15 e EGF. Antes e após cultivo, os fragmentos foram fixados e analisados por histologia, microscopia eletrônica de transmissão e/ou de fluorescência, e os folículos foram classificados em primordiais, transição, primários e secundários, bem como em normais ou atrésicos. Além disso, os diâmetros oocitário e folicular também foram avaliados. Com relação ao cultivo de folículos isolados, folículos secundários foram microdissecados e cultivados por seis dias em α-MEM^+ contendo ou não FSH (100 ng/mL) e suplementado ou não com EGF (10 ng/mL). Os resultados mostraram que os níveis de RNAm para KL, BMP-15 e EGF aumentaram com o desenvolvimento folicular, sendo significativamente superiores em folículos secundários. Além disso, os complexos cumulus-oócito de pequenos e grandes folículos antrais apresentaram maiores níveis de RNAm para BMP-15 e EGF do que as suas respectivas células granulosa/teca, acontecendo o mesmo para o KL somente nos grandes folículos antrais. Após sete dias de cultivo, 50 ng/mL de KL promoveu a manutenção da sobrevivência folicular, o crescimento e a transição para folículos primários. A adição de 100 ng/mL de BMP-15 ao meio manteve a viabilidade, promoveu a ativação e o crescimento *in vitro*, além do aumento do percentual de folículos secundários. E ainda, a utilização de 1 ou 10 ng/mL de EGF promoveu a sobrevivência e aumentou as taxas de folículos primários, mantendo a integridade ultraestrutural folicular. Já após cultivo dos folículos isolados, observou-se que o EGF sozinho ou associado ao FSH promoveu significativa formação de antro e crescimento folicular. Além disso, FSH, EGF ou ambos reduziram os níveis de RNAm para EGF, enquanto o EGF reduziu os níveis de RNAm para FSH-R. Concluindo, os resultados deste estudo mostraram que os RNAm para KL, BMP-15 e EGF foram detectados em todas as

categorias foliculares e tipos celulares investigados. A utilização de KL (50 ng/mL), BMP-15 (100 ng/mL) e EGF (1 ou 10 ng/mL) promoveu a manutenção da sobrevivência folicular, a ativação e o desenvolvimento dos folículos pré-antrais caprinos. Além disso, o EGF e o FSH promoveram o crescimento de folículos secundários caprinos, reduziram os níveis de RNAm para o EGF, e ainda, o EGF diminuiu os níveis de RNAm para FSH-R em folículos secundários caprinos cultivados.

Palavras-chave: KL. BMP-15. EGF. Cultivo *in vitro*. Folículos pré-antrais caprinos.

ABSTRACT

The objectives of this study were: 1) to quantify the mRNA levels of kit ligand (KL), bone morphogenetic protein 15 (BMP-15) and epidermal growth factor (EGF) in goat ovaries by real-time RT-PCR; 2) to evaluate the effect of adding different concentrations of those substances on the survival, activation and *in vitro* growth of caprine preantral follicles cultured *in situ* and 3) to investigate the effects of EGF alone or in combination with follicle stimulating hormone (FSH) on the survival, antrum formation and growth of isolated secondary follicles from goats, as well as on the EGF and FSH receptor (FSH-R) mRNA levels. To the *in situ* culture, fragments of ovarian cortex were cultured *in vitro* for one or seven days in MEM^+ supplemented with different concentrations (0, 1, 10, 50, 100 or 200 ng/mL) of KL, BMP-15 and EGF. Before and after culture, the fragments were fixed and analysed by histology, transmission electron microscopy and/or fluorescence, and the follicles were classified as primordial, intermediate, primary and secondary, as well as normal or atretic. Moreover, the oocyte and follicle diameters were also evaluated. With regard to isolated culture, secondary follicles were microdissected and cultured for six days in $\alpha\text{-MEM}^+$ with or without FSH (100 ng/mL) and supplemented or not with EGF (10 ng/mL). The results showed that KL, BMP-15 and EGF mRNA levels increased with follicular development and were significantly higher in secondary follicles. In addition, the cumulus-oocyte complexes from small and large antral follicles showed higher BMP-15 and EGF mRNA levels than their corresponding granulosa/theca cells, which also occurred for KL only in the large antral follicles. After seven days of culture, 50 ng/mL KL promoted the maintenance of follicular survival and growth, and the transition to primary follicles. The addition of 100 ng/mL BMP-15 to the medium maintained the viability and promoted the activation and growth *in vitro*, and also increased the percentage of secondary follicles. Moreover, the use of 1 or 10 ng/mL EGF promoted the follicular survival and increased the rates of primary follicles, maintaining the integrity of follicular ultrastructure. After culture of isolated follicles, it was observed that EGF alone or in combination with FSH promoted a significant antrum formation and follicular growth. Furthermore, FSH, EGF or both reduced the EGF mRNA levels, while EGF reduced the FSH-R mRNA levels. In conclusion, our results showed that KL, BMP-15 and EGF mRNA were detected in all categories and follicular cell types investigated. The use of KL (50 ng/mL), BMP-15 (100 ng/mL) and EGF (1 or 10 ng/mL) promoted the maintenance of survival, activation and development of caprine preantral follicles. Furthermore, EGF and

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Keywords: KL. BMP-15. EGF. *In vitro* culture. Caprine preantral follicles.

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LISTA DE ABREVIATURAS E SIGLAS

A	: Antrum (Antro)
Akt	: Protein kinase (Proteína kinase)
ALK-3, -6	: Receptor-like kinase-3, -6 (Receptor semelhante à kinase-3, -6)
AMH	: Anti-müllerian hormone (Hormônio anti-mülleriano)
ANOVA	: Analysis of variance (Análise de variância)
Apaf-1	: Apoptotic protease-activating factor (Fator ativador de protease apoptótica)
AR	: Amphiregulin (Anfiregulina)
as	: antisense (anti senso)
ATP	: Adenosine-5'-triphosphate (Adenosina-5'-trifosfato)
Bak	: BCL2 antagonist killer 1
Bax	: BCL2 associated X protein
Bcl-2	: B-cell lymphoma protein 2
Bcl-w	: BCL2 like 2 protein
Bcl-xL	: BCL2 related protein, long isoform
BDNF	: Brain-derived neurotrophic factor (Fator neurotrófico derivado do cérebro)
Bid	: BH3 interacting domain death agonist
BMP-4, -6, -7, -15	: Bone morphogenetic protein-4, -6, -7, -15 (Proteína morfogenética óssea-4, -6, -7, -15)
BMPR-IA, -IB, -II	: Type IA, IB, II bone morphogenetic protein receptor (receptor de proteína morfogenética óssea do tipo IA, IB, II)
BSA	: Bovine serum albumin (Albumina sérica bovina)
BTC	: Betacellulin (Betacelulina)
Ca^{2+}	: Calcium ion (Íon cálcio)
CAD	: Caspase-activated DNase (DNase ativada por caspase)
cAMP	: Cyclic adenosine-3',5'-monophosphate (Adenosina-3',5'-monofosfato cíclico)
CAPES	: Coordenação de Aperfeiçoamento do Pessoal de Nível Superior
Caspase 1-14	: Cysteinyl aspartic acid protease 1-14
CCND2	: Cyclin D2
cdc25	: cell division cycle 25 phosphatase

cDNA	: Complementary deoxyribonucleic acid (Ácido desoxirribonucléico complementar)
CETENE	: Centro de Tecnologias Estratégicas do Nordeste
CGP	: Células germinativas primordiais
c-kit	: kit ligand receptor (receptor para kit ligand)
CNPq	: Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	: Dióxido de carbono
COCs	: Cumulus–oocyte complexes (Complexos cúmulus oócito)
CPqAM	: Centro de Pesquisa Aggeu Magalhães
CT	: Cycle threshold
Cx43	: Connexin 43 (Conexina 43)
CXCL12	: Chemokine (C-X-C motif) ligand 12
DABCO	: 1,4-diazabicyclo[2.2.2]octane (Octano do diazabicyclo 1,4 [2.2.2])
DD	: Death domain (Domínio de morte)
DISC	: Death-inducing signaling complex (Complexo sinalizador indutor de morte)
DNA	: Deoxyribonucleic acid (Ácido desoxirribonucléico)
DNAse	: Desoxirribonuclease
dNTP	: Deoxy-nucleotide-triphophates (desoxinucleotídeo trifosfato)
DR-4, -5	: Death receptor-4, -5 (receptor de morte-4, -5)
DTT	: Dithiothreitol (Ditiotreitol)
EGF	: Epidermal growth factor (Fator de crescimento epidermal)
EGF-R	: Receptor of epidermal growth factor (Receptor do fator de crescimento epidermal)
ELISA	: Enzyme-linked imumunosorbent assay
EPR	: Epiregulin (Epiregulina)
ErbB 1/2/3/4	: EGF receptor tyrosine kinase family 1/2/3/4 (Família de receptor EGF do tipo tirosina kinase 1/2/3/4)
Erk 1/2	: Extracellular signal-regulated kinase 1/2
F	: Fluorescência
FADD	: Fas-associated death domain (Domínio de morte associado ao Fas)
Fas	: Fatty acid synthetase
Fas/CD95	: Fatty acid synthetase receptor

FasL	: Fatty acid synthetase ligand
FAVET	: Faculdade de Veterinária
FGF-2, -7, -10	: Fibroblast growth factor-2, -7, -10 (Fator de crescimento fibroblástico-2, -7, -10)
Fig α	: Factor in the germ-line alpha (Fator de linha germinal alfa)
Fig.	: Figure (Figura)
FINEP	: Financiadora de Estudos e Projetos
FIOCRUZ	: Fundação Oswaldo Cruz
FOXO	: Forkhead/winged helix transcription factor subfamily
FSH	: Follicle stimulating hormone (Hormônio folículo estimulante)
FSH-R	: Follicle stimulating hormone receptor (Receptor do hormônio folículo estimulante)
FUNCAP	: Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico
g	: gravidade
G	: Granulosa cells (Células da granulosa)
GAPDH	: Glyceraldehydes-2-phophate dehydrogenase (Gliceraldeído-2-fosfato desidrogenase)
GC	: Granulosa cells (Células da granulosa)
GDF-9, -9B	: Growth diferentiation factor-9, -9B (Fator de crescimento e diferenciação-9, -9B)
GDNF	: Glial cell-derived neurotrophic factor (Fator neurotrófico derivado da célula glial)
GFRα1	: Glial cell-derived neurotrophic factor receptor alpha1 (Receptor alfa1 do fator neurotrófico derivado da célula glial)
GH	: Growth hormone (Hormônio do crescimento)
GI	: GenInfo identifier
GLM	: General linear models
GT	: Granulosa/theca cells (Células da granulosa e teca)
h	: horas
HB-EGF	: Heparin-binding EGF (Fator de crescimento semelhante ao EGF ligado à heparina)
HC	: Histologia clássica

HER1 1/2/3/4	: EGF receptor tyrosine kinase family 1/2/3/4 (Família de receptor EGF do tipo tirosina kinase 1/2/3/4)
HGF	: Hepatocyte growth factor (Fator de crescimento de hepatócito)
IAA	: Indole-3-acetic acid (Ácido 3-indol-acético)
IC	: Interstitial cells (células intersticiais)
IGF-1, -2	: Insulin-like growth factor-1, -2 (Fator de crescimento semelhante à insulina -1, -2)
IGFBP-4	: Insulin-like growth factor-binding protein-4 (Proteína ligante 4 transportadora de fator de crescimento semelhante à insulina)
IL-1β, -6	: Interleucin-1β, -6 (interleucina-1β, -6)
I-Smads	: Inhibitory-Seven mothers against dpp gene da Drosophila
ITS	: Insulin, tranferrin and selenium (Insulina, transferrina e selênio)
IU	: International units (Unidades internacionais)
JAK2	: Janus-activated kinase 2
JNKs	: Jun NH2-terminal protein kinases
K^+	: Potassium ion (Íon potássio)
KGF	: Keratinocyte growth factor (Fator de crescimento keratinócito)
KL-1, -2	: Kit ligand-1, -2
kV	: quilovolts
l	: lipid droplets (gotas lipídicas)
L	: Litro
LABOVIR	: Laboratório de Virologia
LAMOFOPA	: Laboratório de Manipulação de Oócitos e Folículos Pré-Antrais
LH	: Luteinizing hormone (Hormônio luteinizante)
Lhx8	: LIM-homeobox protein 8
$Lhx8^{-/-}$	: Lhx8-deficient (Deficiência no gene Lhx8)
LIF	: Leukemia inhibitory factor (Fator inibidor de leucemia)
m	: mitochondria (mitocôndria)
M	: Molar
MA	: Massachusetts
MAPK	: Mitogen-activated protein kinase (Proteína kinase ativada por mitógenos)
MCGF	: Mast-cell growth factor (Fator de crescimento de mastócitos)

Mcl-1 : Myeloid cell leukemia-1
MEK : MAP Kinase/extracellular protein kinase
MEM : Minimal essential medium (Meio essencial mínimo)
MEM^+ : Supplemented minimal essential medium (Meio essencial mínimo suplementado)
MET : Microscopia eletrônica de transmissão
min. : minutos
mg : miligramas
mL : mililitros
mm : milímetros
mm^2 : milímetros quadrados
mm^3 : milímetros cúbicos
mM : milimolar
MO : Missouri
MOIFOPA : Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais
mOsm/L : miliosmol/litro
mRNA : Messenger ribonucleic acid (Ácido ribonucléico mensageiro)
mv : microvilli (microvilo)
Na^+ : Sodium ion (Íon sódio)
NaCl : Cloreto de sódio
nc : nucleolus (nucléolo)
ng : nanograma
NGF : Nerve growth factor (Fator de crescimento do nervo)
nm : nanômetros
no : nucleolus (nucléolo)
NOBOX : Newborn ovary homeobox gene
$NOBOX^{-/-}$: NOBOX-deficient (Deficiência no gene NOBOX)
NRG 1-4 : Neuregulins 1-4 (Neuregulinas 1-4)
NTF5 : Neurotrophin 5 (Neurotrofina tipo 5)
nu : nucleus (núcleo)
Nu : Oocyte nucleus (Núcleo do oócito)
NUBIS : Núcleo de Biotecnologia de Sobral

NuR	: Nuclear region (Região nuclear)
O	: Oocyte (Oócito)
OCT4	: Octamer-binding transcription factor 4
P<0.05	: Probabilidade de erro menor do que 5%
P>0.05	: Probabilidade de erro maior do que 5%
p.	: página
p34cd2	: Protein kinase p34 (cd2)
p38-MAPKs	: p38 mitogen-activated protein kinases
p53	: 53 protein (proteína 53)
PAS-H	: Periodic acid-Schiff and hematoxylin (Ácido periódico de Schiff e hematoxilina)
PBS	: Phosphate buffer saline (Tampão fosfato salino)
PCR	: Polimerase chain reaction (Reação em cadeia polimerase)
pFSH	: Pituitary follicle stimulating hormone (Hormônio folículo estimulante pituitário)
PGCs	: Primordial germ cells (Células germinativas primordiais)
pH	: potencial hidrogeniônico
PI3K	: Phosphoinositide 3-kinase (Fosfatidilinositol 3-kinase)
PK A, B, C	: Protein kinase A, B, C (Proteína kinase A, B, C)
POU5F1	: Pituitary octamer neural unc domain, class 5, transcription factor 1
PPGCV	: Programa de Pós-Graduação em Ciências Veterinárias
PTEN	: Phosphatase and tensin homolog deleted on chromosome 10 (Fosfatase e tensina homóloga com deleção no cromossomo 10)
rbFSH	: Recombinant bovine follicle stimulating hormone (Hormônio folículo estimulante recombinante bovino)
RENORBIO	: Rede Nordeste de Biotecnologia
RET	: Ubiquitous tyrosine kinase receptor
R-FSH	: Follicle stimulating hormone receptor (Receptor do hormônio folículo estimulante)
rFSH	: Recombinant follicle stimulating hormone (Hormônio folículo estimulante recombinante)
rhEGF	: Recombinant human epidermal growth factor (Fator de crescimento epidermal recombinante humano)

rhKL	: Recombinant human kit ligand (Kit ligand recombinante humano)
RNAm	: Ribonucleic acid messenger (Ácido ribonucléico mensageiro)
RNAse	: Ribonuclease
R-Smads	: Receptor-Seven mothers against dpp gene da Drosophila
RT	: Room temperature (Temperatura ambiente)
RT-PCR	: Reverse transcription-polimerase chain reaction (Transcrição reversa-reação em cadeia polimerase)
RT-qPCR	: Reverse transcription-quantitative polimerase chain reaction (Transcrição reversa-quantitativa reação em cadeia polimerase)
s	: sense (senso)
SAS	: Statistical analysis system
SBAC	: Solução à base de água de coco
SCF	: Stem cell factor (Fator de células tronco)
SD	: Standard deviation
Sec.	: Secunde (Segundos)
SEM	: Standard error of means (Erro padrão da média)
SF	: Steel factor
Smads 1/4/5/6/7/8	: Seven mothers against dpp gene da Drosophila 1/4/5/6/7/8
SNK	: Student–Newman–Keuls
SP	: São Paulo
Sohlh-1, -2	: Spermatogenesis and oogenesis helix-loop-helix-1, -2
STAT	: Signal transducer and activator of transcription (Transdutor de sinal e ativador de transcrição)
T 1, 2, 3	: Tratamento 1, 2, 3
TC	: Theca cells (Células da teca)
TEM	: Transmission electronic microscopy (Microscopia eletrônica de transmissão)
TGF-β, - α	: Transforming growth factor beta, alpha (Fator de crescimento transformante beta, alfa)
TNF	: Tumor necrosis factor (Fator de necrose tumoral)
TNFR1	: Tumor necrosis factor receptor 1 (Receptor tipo 1 do fator de necrose tumoral)
TNF-α	: Tumor necrosis factor alpha (Fator de necrose tumoral alfa)

TRADD	: TNF receptor-associated death domain
TRAIL	: TNF-related apoptosis-inducing ligand
TrkB	: Tyrosine kinase receptor B
TUNEL	: Terminal deoxynucleotidil transferase-mediated deoxyuridine triphosphate biotin nick end-labeling
UBQ	: Ubiquitin (Ubiquitina)
UECE	: Universidade Estadual do Ceará
UFC	: Universidade Federal do Ceará
UnB	: Universidade de Brasília
USA	: United States of America (Estados Unidos da América)
v	: vacuole or vesicles (vacúolo ou vesículas)
v.	: volume
VEGF	: Vascular endothelial growth factor (Fator de crescimento do endotélio vascular)
VEGFR-2	: Vascular endothelial growth factor receptor-2 (Receptor 2 para fator de crescimento do endotélio vascular)
VIP	: Vasoactive intestinal peptide (Peptídeo intestinal vasoativo)
Vol.	: Volume
X	: Eixo das abicissas
Y	: Eixo das ordenadas
ZP	: Zona pellucida (Zona pelúcida)
α-MEM	: Alpha minimal essential medium (Meio essencial mínimo alfa)
α-MEM$^+$	: Supplemented alpha minimal essential medium (Meio essencial mínimo alfa suplementado)
µg	: Microgramas
µL	: Microlitros
µm	: Micrômetros
µM	: Micromolar
%	: Percentage (Porcentagem)
~	: Aproximadamente
±	: Mais ou menos
°C	: Graus Celsius

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1 INTRODUÇÃO

Os caprinos estão presentes em todos os continentes e são vistos comercialmente como animais altamente atrativos, uma vez que eles têm sido utilizados para muitos propósitos, tais como produção de leite, carne e pele. Nas últimas duas décadas, desenvolvimentos significativos têm sido alcançados no campo da biotecnologia da reprodução assistida, tanto em animais como em humanos. Em animais, incluindo os caprinos, essas modernas biotecnologias estão sendo utilizadas para melhoria e preservação da genética dos animais, e aumento da sua eficiência reprodutiva (RAHMAN; ABDULLAH; WAN KHADIJAH, 2008).

É conhecido que os ovários das diferentes espécies mamíferas, como a espécie caprina, contêm milhares de oócitos imaturos inclusos predominantemente nos folículos pré-antrais, representando esses folículos uma fonte potencial de gametas fertilizáveis, e com isso, sendo de grande interesse assegurar o crescimento *in vitro* e permitir a aquisição da competência dos oócitos provenientes destes folículos (MCLAUGHLIN et al., 2010). Entretanto, mais de 99,9% dos oócitos inclusos em folículos pré-antrais não ovularão, mas sim serão eliminados por um processo natural conhecido como atresia. Dessa forma, o resgate dos folículos pré-antrais dos ovários, evitando assim a atresia folicular, seguido pelo desenvolvimento de sistemas de cultivo *in vitro* (ovário artificial) que permitam o crescimento e maturação de seus oócitos poderia trazer, no futuro, um maior impacto para a produção *in vitro* de embriões (FIGUEIREDO et al., 2007).

Estudos *in vitro* com o cultivo de folículos pré-antrais de camundongas demonstraram que é possível a obtenção de crias vivas produzidas de oócitos oriundos destes folículos cultivados *in vitro* (O'BRIEN; PENDOLA; EPPIG, 2003; HASEGAWA et al., 2006). Em outros animais, um número limitado de embriões tem sido produzido de oócitos crescidos e maturados *in vitro* (suínos - WU; EMERY; CARREL, 2001; WU; TIAN, 2007; bubalinos - GUPTA et al., 2008; ovinos - ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010 e caprinos - SARAIVA et al., 2010a; MAGALHÃES et al., 2011), consistindo assim em um grande desafio o nascimento de indivíduos vivos normais a partir de folículos pré-antrais crescidos *in vitro* nestas espécies. Sabendo-se então que o crescimento dos folículos presentes no ovário mamífero é regulado por gonadotrofinas e por fatores intra-ovarianos (FORTUNE, 2003), atualmente esforços têm sido concentrados para melhorar a identificação e compreensão das diferentes substâncias envolvidas na promoção do desenvolvimento folicular e no curso da atresia. Diante desse melhor conhecimento acerca da foliculogênese,

será possível tentar desenvolver um sistema de cultivo *in vitro* capaz de permitir o desenvolvimento de um grande número de folículos pré-antrais, melhorando assim no futuro a taxa de produção de embriões, e permitindo a obtenção de nascimentos a partir de folículos pré-antrais destas espécies. Dentre as substâncias reguladoras da foliculogênese, merecem destaque o kit ligand (KL), a proteína morfogenética óssea-15 (BMP-15) e o fator de crescimento epidermal (EGF).

Para uma melhor compreensão da importância deste trabalho, a revisão de literatura a seguir abordará aspectos relativos à regulação da foliculogênese em mamíferos, destacando a importância dos hormônios e fatores de crescimento; cultivo *in vitro* de folículos pré-antrais, especialmente na espécie caprina; estado atual do cultivo; e as principais técnicas para avaliação dos folículos cultivados *in vitro*.

2 REVISÃO DE LITERATURA

2.1 Foliculogênese

A foliculogênese é um evento iniciado na vida pré-natal na maioria das espécies, podendo ser definida como o processo de formação, crescimento e maturação folicular, iniciando-se com a formação do folículo primordial e terminando no estádio de folículo pré-ovulatório (VAN DEN HURK; ZHAO, 2005; GOUGEON, 2010).

O folículo é considerado a unidade morfológica e funcional do ovário mamífero, proporcionando um ambiente ideal para o crescimento e maturação do oócito (CORTVRINDT; SMITZ, 2001a), além de produzir algumas substâncias fundamentais para sua manutenção e desenvolvimento (ADASHI, 1994). Essa estrutura é composta por um oócito circundado por células somáticas (granulosa e/ou tecais), tendo a interação entre esses compartimentos celulares um papel crítico no decorrer da foliculogênese (VANDERHYDEN; TELFER; EPPIG, 1992; EPPIG; WIGGLESWORTH; PENDOLA, 2002; MATZUK et al., 2002). Durante o processo da foliculogênese, a morfologia folicular é alterada observando-se o crescimento oocitário, a diferenciação e a proliferação das células da granulosa e o aparecimento das células tecais (SILVA, 2005; BRISTOL-GOULD; WOODRUFF, 2006). Com base nessa mudança morfológica, os folículos podem ser divididos em: 1) folículos pré-antrais ou não cavitários, que abrangem os folículos primordiais, transição, primários e secundários e 2) folículos antrais ou cavitários, compreendendo os folículos terciários e de De Graaf ou pré-ovulatório (SILVA et al., 2004a). Vale ressaltar que os folículos pré-antrais representam mais de 90% da população folicular do ovário (SAUMANDE, 1981), sendo 95% destes folículos primordiais (ERICKSON, 1986), os quais constituem o *pool* de reserva de gametas femininos durante toda a vida reprodutiva (QU et al., 2000).

A foliculogênese pode ser dividida nas seguintes fases de desenvolvimento: 1) fase pré-antral, que é subdividida em ativação dos folículos primordiais e crescimento de folículos de transição, primários e secundários; 2) fase antral, subdividida em crescimento inicial e terminal dos folículos terciários e formação do folículo pré-ovulatório. Além disso, a foliculogênese pode ainda ser classificada de acordo com a dependência gonadotrófica (MCGEE; HSUEH, 2000; CRAIG et al., 2007; MCNATTY et al., 2007; ORISAKA et al., 2009) em: (1) fase independente e responsiva à gonadotrofina (crescimento folicular através dos estádios primordial, transição, primário, secundário e transição de pré-antral para o

estádio antral inicial) e (2) fase dependente de gonadotrofina (crescimento contínuo além do estádio antral inicial), a qual inclui recrutamento dos folículos, seleção, dominância e ovulação (KUMAR et al., 1997). Todas as fases citadas podem ser visualizadas com mais detalhes na Fig. 1.

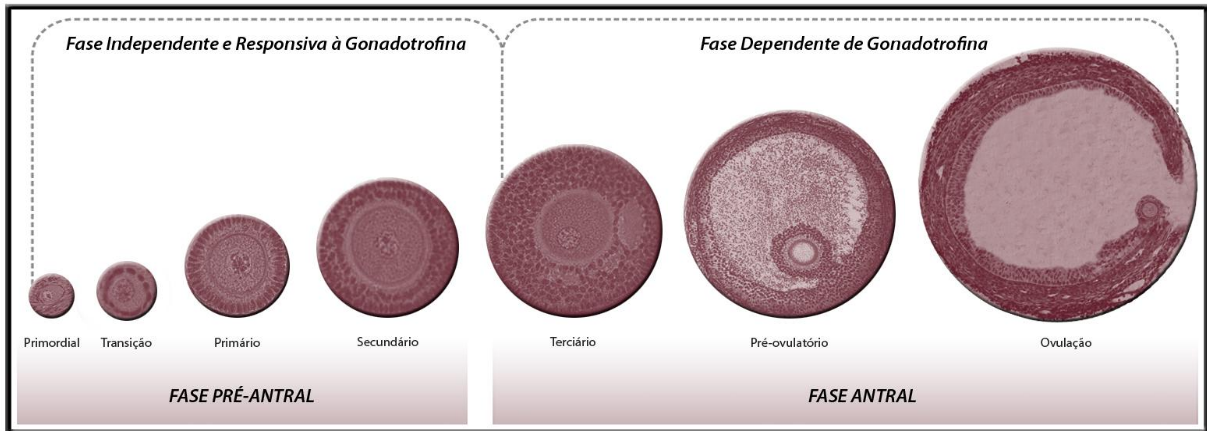

Figura 1. Fases da foliculogênese. Adaptado de: Orisaka et al., 2009.

O desenvolvimento folicular através dos estádios da foliculogênese é caracterizado por elevadas taxas de proliferação e atresia, as quais dependem de um balanço entre substâncias estimulatórias e inibitórias. Entretanto, a diminuição do número de folículos em cada estádio de desenvolvimento indica que o controle da sobrevivência ou da atresia folicular durante a foliculogênese não é o mesmo em cada estádio (MARKSTRÖM et al., 2002). A susceptibilidade à atresia, seja ela pelo processo degenerativo de necrose ou apoptose (ver detalhes sobre a atresia na revisão apresentada no Capítulo 1 da presente Tese) depende do estádio de desenvolvimento folicular, sendo predominante na fase antral.

2.2 Caracterização estrutural dos folículos ovarianos e regulação da foliculogênese

2.2.1 Formação do folículo primordial

Em mamíferos, os estádios iniciais do desenvolvimento ovariano são formados pela migração das células germinativas primordiais (CGP) a partir do saco vitelínico para a gônada primitiva, e sua posterior colonização (BRISTOL-GOULD; WOODRUFF, 2006). Esse processo é controlado e direcionado pela secreção de citocinas e fatores de crescimento pleiotrópicos, tais como o CXCL12 e o fator de células-tronco (Stem cell factor - SCF), também conhecido como kit ligand (KL) (MOLYNEAUX; WYLIE, 2004; KUNWAR;

SIEKHAUS; LEHMANN, 2006). Com a chegada das CGP na gônada primitiva, ocorre a formação dos cordões sexuais. A partir de então, as CGP perdem a sua motilidade e passam a se multiplicar por mitose, morrendo, entretanto, a grande maioria delas por apoptose (KIM; TILLY, 2004), fenômeno este responsável pela regulação do número de folículos primordiais presentes no ovário (BAKER, 1963). As demais CGP são então diferenciadas em oogônias (BAKER; FANCHI, 1967; SATHANANTHAN; SELVARAJ; TROUNSON, 2000) e uma vez diferenciadas, estas irão se dividir sucessivamente por mitose e irão formar ninhos de oogônias interligados por pontes intercelulares (PEPLING; SPRADLING, 1998, 2001; PEPLING, 2006). Com a parada da mitose, as oogônias entram nos estádios iniciais da meiose I diferenciando-se em oócitos primários (HIRSHFIELD, 1991). Em seguida, os oócitos perdem suas pontes intercelulares e são circundados por uma camada de células da pré-granulosa, as quais podem ser derivadas do mesonefron ou do epitélio da superfície ovariana (MCNATTY et al., 2000). Uma vez que o oócito é circundando pelas células somáticas, ocorre uma parada da meiose no estádio de diplóteno da prófase I, também conhecido como estádio de vesícula germinativa (BAKER; FRANCHI, 1967; PICTON; BRIGGS; GOSDEN, 1998), no qual as células da pré-granulosa param de se multiplicar e entram num período de quiescência (SAWYER et al., 2002) juntamente com o oócito, formando os folículos primordiais e dando início à foliculogênese. A progressão da divisão meiótica ocorre somente na puberdade, com a liberação do pico pré-ovulatório de FSH e LH, formação dos oócitos secundários e outra parada da meiose na fase de metáfase II (HUTT; ALBERTINI, 2007). A meiose será retomada novamente somente após a fecundação do oócito pelo espermatozóide, originando o oócito haplóide fecundado, e marcando assim o fim da oogênese (FIGUEIREDO et al., 2008).

Alguns fatores transcricionais envolvidos nos padrões de sinalização da oogênese e foliculogênese inicial têm sido identificados através de análises fenotípicas de camundongas. Um dos primeiros fatores encontrados com papel na formação do folículo primordial foi o fator de linha germinal α (*Fig α ou Figla*) (LIANG; SOYAL; DEAN, 1997), o qual é expresso pelo oócito (SOYAL; AMLEH; DEAN, 2000). Em camundongas, esse fator é expresso no estádio embrionário, mas sua expressão é reduzida após sete e 14 dias do nascimento; entretanto, transcritos residuais são encontrados no ovário adulto (SOYAL; AMLEH; DEAN, 2000). Estudos mais recentes têm revelado que o *Figla* regula a foliculogênese inicial aumentando diversos outros genes, incluindo o POU5F1. O POU5F1 (ou OCT4) é um fator transcricional cujo alvo preciso não é conhecido e o papel dele na oogênese tem sido

recentemente descrito (PANGAS; RAJKOVIC, 2006). Ele é expresso nas CGP até elas migrarem para a gônada primitiva e a expressão é então reprimida após o início da prófase I meiótica no oócito, voltando a ser re-expresso pelos oócitos após o nascimento, o que coincide com o período de crescimento destes (PARFENOV et al., 2003). Pouco foi conhecido sobre o papel do POU5F1 no ovário pós-natal, mas análises de nocaute desse fator nas CGP demonstraram a sua importância na sobrevivência dessas células, uma vez que na sua ausência nenhum folículo foi encontrado no ovário, o que foi atribuído a uma apoptose prematura das CGP antes da colonização da gônada (KEHLER et al., 2004). Outro fator que vem demonstrando importância neste processo é o LHX8. Este gene está envolvido no padrão de formação e sobrevivência do folículo primordial, sendo preferencialmente expresso no oócito de ovários de camundongas. Foi observado que camundongas sem esse fator falharam em manter os folículos primordiais e que estes desapareceram na primeira semana de vida (CHOI et al., 2008). Este achado parece ter sido causado por uma marcada redução na expressão do KL e de seu receptor (c-kit) em ovários de camundongas sem LHX8.

As neurotrofinas podem também estar envolvidas na sinalização entre CGP e células somáticas no momento da formação dos folículos primordiais. Essa afirmativa foi formulada com base nas mudanças observadas ao longo do desenvolvimento no padrão de expressão da neurotrofina 4 e do seu receptor de alta afinidade (TrkB) em oócitos de humanos e ovários de ratas (ANDERSON et al., 2002). Recentes estudos têm ainda sugerido que os níveis de progesterona e estradiol fetais e maternos regulam a formação do folículo primordial (KEZELE; SKINNER, 2003; BRITT et al., 2004; NILSSON; STANFIELD; SKINNER, 2006a; CHEN et al., 2007).

2.2.2 Transição de folículo primordial para primário

Como já relatado, os gametas femininos são estocados no ovário na forma de folículos primordiais (FAIR, 2003). Até pouco tempo atrás, prevalecia o dogma de que o *pool* de folículos primordiais representava uma reserva finita de gametas femininos (ZUCKERMAN, 1951). Entretanto, a noção de uma reserva fixa e não renovável de folículos primordiais no ovário mamífero tem sido questionada, sendo sugerido que células-tronco da linha germinativa extra e intraovariana poderiam reabastecer os oócitos e formar novos folículos primordiais (JOHNSON et al., 2004, 2005). Por outro lado, diferentes estudos não encontraram evidências para suportar a hipótese de que células progenitoras de origem

extragonadal possam renovar as células foliculares no ovário adulto (KERR et al., 2006; BEGUM; PAPAIOANNOU; GOSDEN, 2008).

Independente disso, logo após a formação, alguns folículos primordiais podem ser estimulados a crescer imediatamente ou, na maioria destes, as células da pré-granulosa param de se multiplicar e entram num período de quiescência até receberem sinais para entrar no *pool* de crescimento (MCGHEE; HSUEH, 2000). O início do desenvolvimento dos folículos primordiais pode ocorrer dias, meses ou anos após a sua formação (VAN DEN HURK; ZHAO, 2005), sendo considerado o maior evento biológico que controla o potencial reprodutivo das fêmeas (MCLAUGHLIN; MCIVER, 2009). A maioria desses folículos sofre atresia na vida pré- ou pós-natal, e nunca inicia o complexo padrão de desenvolvimento que pode ou não culminar na ovulação (KNIGHT; GLISTER, 2006).

O início do crescimento de folículos primordiais, também conhecido como ativação, é um processo que ocorre através da transição dos folículos do *pool* de reserva, ou folículos quiescentes, para o *pool* de folículos em crescimento (transição, primário, secundário, terciário e pré-ovulatório) (RÜSSE, 1983). As características morfológicas que marcam o início do crescimento dos folículos primordiais são: aumento do diâmetro oocitário e transformação da morfologia das células da granulosa de pavimentosa para cúbica. Durante esta fase, os folículos que apresentam células da granulosa pavimentosas e cúbicas são denominados folículos de transição (SILVA et al., 2004a). Quando o oócito é circundado por uma camada completa de células da granulosa de morfologia cúbica, os folículos são denominados primários (GOUGEON; BUSSO, 2000).

Os fatores e mecanismos responsáveis pela ativação de folículos primordiais são pouco conhecidos. Acredita-se que a ativação dos folículos primordiais seja regulada por um balanço entre fatores inibitórios e estimulatórios originários do ovário (VAN DEN HURK; ZHAO, 2005). Dessa forma, a caracterização dos fatores e mecanismos envolvidos no padrão de sinalização da ativação é fundamental para o conhecimento dos sistemas moleculares responsáveis por assegurar o conveniente e oportuno fornecimento de oócitos aptos à fecundação (MCLAUGHLIN; MCIVER, 2009).

As células da pré-granulosa que circundam o oócito de folículos primordiais expressam um grande número de fatores peptídicos, incluindo o KL e o fator inibidor de leucemia (LIF), os quais têm estimulado *in vitro* a transição de folículos primordiais para primários, o crescimento do oócito e o recrutamento e proliferação das células da teca do estroma circundante (NILSSON; KEZELE; SKINNER, 2002; NILSSON; SKINNER, 2003,

2004). O receptor para o KL (c-kit) está expresso no oócito e células intersticiais/tecais, capazes então de responder ao estímulo do KL. O KL é um dos poucos fatores que possui um papel bem definido sobre a ativação folicular. Recentes estudos têm demonstrado que este fator de crescimento atua na ativação folicular por meio do padrão de sinalização intracelular no oócito denominado PI3K-AKT-FKHRL1 e PTEN (LIU et al., 2007a,b; REDDY et al., 2008). A cascata de sinalização iniciada pelo c-kit na superfície do oócito, ativado pelo KL, é seguida por uma subsequente ativação da PI3K, podendo aumentar o crescimento do oócito e a produção de fatores locais capazes de estimular a proliferação e diferenciação das células da granulosa circundantes (MCLAUGHLIN; MCIVER, 2009). A revisão de literatura apresentada no Capítulo 2 desta Tese abordará os detalhes sobre a ação do KL na foliculogênese inicial.

Além disso, algumas das células mesenquimais que circundam os folículos primordiais (células precursoras da teca) produzem outros peptídeos, conhecidos como fator de crescimento de queratinócito (KGF, também chamado de fator de crescimento fibroblástico-7, FGF-7) e o fator de crescimento do hepatócito (HGF), que podem então atuar nas células da pré-granulosa e/ou células da granulosa aumentando a expressão do KL e amplificando assim seus efeitos positivos no desenvolvimento folicular (KEZELE; NILSSON; SKINNER, 2005; GUGLIELMO et al., 2010). Em adição, foi demonstrado que o FGF-2 (também conhecido como FGF básico), expresso nos oócitos de folículos primordiais, aumentou a expressão do KL nas células da pré-granulosa e promoveu a transição de folículo do estádio primordial para primário no cultivo de ovários de ratas neonatais (NILSSON; SKINNER, 2004). O KL e o FGF-2 têm efeitos estimulatórios mútuos no oócito e nas células da granulosa e também promovem o recrutamento das células da teca a partir da população de células do estroma/intersticial circundantes. Células do estroma/intersticiais e células da teca secretam as proteínas morfogenéticas ósseas-4 e -7 (BMP-4 e BMP-7), as quais também promovem a ativação e a sobrevivência folicular. O fator de crescimento e diferenciação-9 (GDF-9) e/ou a BMP-15, ambos secretados pelo oócito, promovem a proliferação das células da granulosa, expressão do KL e a formação das células da teca (KNIGHT; GLISTER, 2006).

Análises funcionais utilizando sistemas de cultivo *in vitro* de ovários neonatais têm confirmado que a taxa de ativação de folículos primordiais é diretamente proporcional ao aumento no número de citocinas e fatores de crescimento pleiotrópicos, que incluem o KL (HUTT; MCLAUGHLIN; HOLLAND, 2006), LIF (NILSSON; KEZELE; SKINNER, 2002), BMP-4 e BMP-7 (LEE et al., 2001; NILSSON; SKINNER, 2003; CRAIG et al., 2007), fator

de crescimento derivado de plaquetas (PDGF, NILSSON; DETZEL; SKINNER, 2006b), KGF (KEZELE; NILSSON; SKINNER, 2005), FGF-2 (NILSSON; PARROTT; SKINNER, 2001), fator neurotrópico derivado da glia (GDNF, DOLE; NILSSON; SKINNER, 2008) e as neurotrofinas (NGF, NTF5 e BDFN; DISSEN et al., 2002; ROMERO et al., 2002; SPEARS et al., 2003; PAREDES et al., 2004; DOLE; NILSSON; SKINNER, 2008).

A identificação de ligantes com multiplicidade de papéis na foliculogênese, como os ligantes da superfamília de Fator de Crescimento Transformante-β (TGF-β), como por exemplo, o TGF-β, GDF-9 e BMP-15, os quais são bem reconhecidos como proteínas regulatórias derivadas do oócito, freqüentemente têm um papel durante o desenvolvimento do folículo pré-antral, além do seu papel na ativação de folículos primordiais em algumas espécies. Enquanto camundongas com nocaute para BMP-15 são subférteis (YAN et al., 2001), ovelhas com esse tipo de mutação sofrem uma parada no desenvolvimento folicular no estádio de folículos primários (MCNATTY et al., 2007). A BMP-15, que será discutida em detalhes na revisão mostrada no Capítulo 3, também tem sido implicada na regulação do KL (HUTT; ALBERTINI, 2007), cujo efeito na ativação é bem conhecido.

Outro fator que tem ação documentada sobre o desenvolvimento de folículos primordiais é o fator de crescimento epidermal (EGF). No entanto, seu efeito sobre a ativação tem se mostrado controverso. O EGF é importante para a formação de folículos primários em experimentos *in vitro* com bovino (WANDJI; EPPIG; FORTUNE, 1996), folículos neonatais de camundongas (EPPIG; O'BRIEN, 1996) e ovino (ANDRADE et al., 2005). Entretanto, ele não foi capaz de ativar os folículos primordiais em cultivo de órgãos de ratas (KEZELE; NILSSON; SKINNER, 2002). Em caprinos, o RNAm e a proteína para o ligante EGF foram encontrados em todas as categorias foliculares e em todos os tipos celulares (SILVA et al., 2006), sugerindo assim uma possível importância desse fator na ativação e posterior desenvolvimento folicular inicial. Em outras espécies, o EGF foi localizado em oócitos de folículos unilaminares suínos (SINGH; RUTLEDGE; ARMSTRONG, 1995), de hamsters (ROY; GREENWALD, 1990) e humanos (MARUO et al., 1993; QU et al., 2000), e seu receptor (EGF-R/ ErbB1) em ambos, oócito e células da granulosa (SINGH; RUTLEDGE; ARMSTRONG, 1995; QU et al., 2000). Mais detalhes acerca das funções do EGF sobre a foliculogênese inicial serão mostrados a seguir, no Capítulo 4.

As células da granulosa de folículos em crescimento secretam o hormônio Anti-Mulleriano (AMH) que, segundo alguns estudos, atua como um inibidor do recrutamento de folículos primordiais (DURLINGER; VISSER; THEMMEN, 2002), embora um trabalho

mais recente em humanos tenha relatado que o AMH inicia o desenvolvimento folicular (SCHMIDT et al., 2005).

Uma das questões-chave de interesse no processo de ativação é o fato de que um folículo em particular é estimulado a crescer, enquanto outro imediatamente adjacente permanece quiescente. Uma proposta conhecida como hipótese da 'linha de produção' sugere que os primeiros oócitos a entrarem na parada meiótica durante o desenvolvimento da gônada embrionária são, de fato, também os primeiros a ativarem na vida adulta (HENDERSON; EDWARDS, 1968; MCLAUGHLIN; MCIVER, 2009). O crescimento inicial pode depender da proporção de cada tipo de célula que compõe o folículo, em que cada tipo celular pode ter um limiar para determinar o início da ativação de cada folículo (HIRSHIFIELD, 1992). Além disso, em humanos, estudos relacionados ao estoque de gametas sugerem que a proporção de folículos que ativam parece depender do tamanho da reserva folicular ovariana, e existe uma aparente correlação inversa entre a fração de folículos em crescimento e o tamanho do estoque de folículos primordiais (PETERS, 1979; GOUGEON, 1996).

2.2.3 Transição de folículo primário para secundário

Durante o crescimento dos folículos primários, ocorre o aumento do oócito e do seu conteúdo protéico, formação da zona pelúcida, grande proliferação das células da granulosa para formar multicamadas, formação de uma lâmina basal e condensação das células do estroma circundando a lâmina basal para formar a camada da teca interna (PICTON; BRIGGS; GOSDEN, 1998; KNIGHT; GLISTER, 2006). Quando duas ou mais camadas de células da granulosa se desenvolvem e as células da teca podem ser evidenciadas do estroma circundante, os folículos secundários são formados. O surgimento da camada de célula da teca no estádio de folículo secundário é um importante evento fisiológico para o desenvolvimento folicular inicial, evidenciado por: 1) concomitante organização da camada de célula da teca, aumento do crescimento do folículo e resposta esteroidogênica às gonadotrofinas (WANDJI; EPPIG; FORTUNE, 1996; GUTIERREZ et al., 2000); 2) aumento no suprimento sanguíneo pela camada da célula da teca contendo reguladores ovarianos para o desenvolvimento do folículo (GOUGEON, 1996; BRAW-TAL; YOSSEFI, 1997); e 3) aumento na produção de andrógenos pela aromatização tecal para biossíntese de estrógeno pelas células da granulosa, e aumento no crescimento folicular inicial por produtos androgênicos das células da teca (VENDOLA et al., 1998; WEIL et al., 1999; WANG et al., 2001).

Inevitavelmente, há diferenças entre as espécies com relação ao tempo de progressão folicular. Em roedores, folículos adquirem uma camada da teca bem definida em um estádio muito precoce em relação aos folículos de ruminantes ou primatas. Além disso, folículos de roedores parecem se tornar dependentes de gonadotrofinas no estádio pré-antral tardio, enquanto em ruminantes e primatas essa dependência não ocorre até a metade do estádio antral. Embora existam grandes evidências que as gonadotrofinas influenciam a progressão do desenvolvimento dos folículos pré-antrais iniciais (DUFOUR; CAHILL; MAULEON, 1979; CORTVRINDT; SMITZ; VAN STEIRTEGHEM, 1997), seu papel não é considerado essencial. Ao invés disso, evidências indicam que fatores locais regulam a transição de folículos primários para secundários, além do posterior crescimento do folículo até o estádio antral inicial. Dentre esses fatores, destacam-se o GDF-9 e a BMP-15 originários do oócito, ativinas originárias das células da granulosa, BMP-4 e BMP-7 oriundas das células da teca, e o TGF-β oriundo de células da granulosa e da teca. Em contraste, outros estudos apontam o papel negativo para o AMH no desenvolvimento de folículos pré-antrais (KNIGHT; GLISTER, 2006). Evidências fisiológicas consolidam os achados de estudos funcionais *in vivo* e *in vitro*, em animais com deleções alvo ou mutações inativantes de alguns dos genes relevantes para expressão desses fatores (MATZUK, 2000; MCNATTY et al., 2001, 2005). O GDF-9 e a BMP-15 têm se mostrado essenciais para formação de folículos secundários, uma vez que animais geneticamente deficientes para esses fatores mostraram um bloqueio do desenvolvimento folicular além do estádio de primário (DONG et al., 1996; MCNATTY et al., 2007). Além disso, na ausência de GDF-9, os folículos são incompetentes para emitir o sinal que recruta os precursores das células da teca para circundar os folículos.

Outras substâncias ainda têm se mostrado importantes para essa fase. YANG e FORTUNE (2006, 2007) mostraram após o cultivo *in vitro* de folículos pré-antrais bovinos, que a testosterona e o fator de crescimento do endotélio vascular (VEGF) também podem influenciar a transição de folículos primários para secundários. Dentre as neurotrofinas, o NGF parece ser um bom candidato para estimular o desenvolvimento folicular inicial, inclusive de folículos secundários. Na presença de FSH em níveis normais no soro, ovários de camundongas deficientes para o NGF exibiram uma marcada redução no número de folículos primários e secundários, sendo a proliferação das células da granulosa extremamente reduzida após cultivo *in vitro* de biópsias ovarianas dessas camundongas (DISSEN et al., 2001).

2.2.4 Transição de folículo secundário para antral

Com a intensa proliferação das células da granulosa dos folículos secundários, uma área preenchida por fluido folicular é identificada na camada granulosa e, a partir de então, os folículos passam a ser classificados como antrais (RÜSSE, 1983). Esse fluido antral pode servir como uma importante fonte de substâncias regulatórias ou moduladoras derivadas do sangue ou secreções de células foliculares, como por exemplo, gonadotrofinas, esteróides, fatores de crescimento, enzimas, dentre outras substâncias. Durante o desenvolvimento folicular, a produção de fluido antral é intensificada pelo aumento da vascularização folicular e permeabilidade dos vasos sanguíneos, os quais estão fortemente relacionados com o aumento do folículo antral. Contudo, embora alguns dos mecanismos de desenvolvimento da cavidade antral sejam conhecidos, os sinais para formação do antro ainda não foram plenamente elucidados (VAN DEN HURK; ZHAO, 2005).

A progressão dos folículos através do estádio antral de desenvolvimento é associada com uma contínua proliferação das células da granulosa e da teca, aumento da vascularização tecal, crescimento adicional do oócito e um aumento relativamente rápido no diâmetro e volume folicular. O aumento no tamanho e na complexidade histotípica do folículo irá limitar a transferência por difusão de moléculas sinalizadoras secretadas entre células de diferentes compartimentos intrafoliculares (KNIGHT; GLISTER, 2006). Nesta fase, as células da teca sofrem alterações morfológicas e funcionais, e aquelas células localizadas próximas à membrana basal passam a ser denominadas teca interna, enquanto que as localizadas perifericamente são classificadas como teca externa.

O crescimento dos folículos secundários é primariamente controlado pelos reguladores intraovarianos (exemplo: fatores de crescimento, citocinas e esteróides gonadais) e não requerem gonadotrofinas (CATTANACH et al., 1977; HALPIN et al., 1986), embora estes folículos possuam RNAm para receptores de FSH nas células da granulosa (VAN DEN HURK; ZHAO, 2005). Estudos *in vitro* com folículos de roedores mostraram que a ativina (ZHAO et al., 2001) e o KL (DRIANCOURT et al., 2000) são possíveis candidatos para transição de folículos secundários para antrais.

Vários outros fatores derivados dos folículos secundários, isto é, EGF, TGFs, fatores de crescimento semelhantes à insulina (IGFs), FGF-2, FGF-7, BMPs e ativina têm sido associados com a sobrevivência e o desenvolvimento de folículos secundários em várias espécies de roedores e animais domésticos, já que a supressão destes favorece a apoptose

celular e interfere na proliferação e diferenciação das células da granulosa em estudos *in vitro* (VAN DEN HURK; BEVERS; BECKERS, 1997; MCNATTY et al., 1999; VAN DEN HURK et al., 2000a; ZHAO, 2000; CAMPBELL, 2009). O EGF e o TGF-α são estruturalmente semelhantes agindo sobre o receptor de EGF e primariamente exercem efeito mitogênico sobre as células da granulosa de várias espécies (MULHERON; SCHOMBERG, 1993; ROY, 1999). Em hamsters, o EGF estimulou a síntese de DNA e a produção de progesterona em folículos secundários cultivados (ROY; GREENWALD, 1991). Além disso, trabalhos *in vitro* utilizando o EGF em folículos secundários bovinos mostraram um efeito estimulatório deste fator na formação antral (GUTIERREZ et al., 2000). O neurotransmissor peptídeo intestinal vasoativo (VIP) tem sido indicado como importante regulador do desenvolvimento de folículos secundários de ratas, bovinos e primatas (MCGHEE; HSUEH, 2000; VAN DEN HURK et al., 2000a), e tem sido implicado na função esteroidogênica de células da granulosa em estádios iniciais e mais avançados em roedores (DISSEN; LES DEE; OJEDA, 1993).

Logo após a formação do antro, os folículos passam por um rápido período de crescimento caracterizado pela alta proliferação celular devido aos altos índices mitóticos em folículos com até 2 mm (CAHILL; MAULEON, 1980; FATET; PELLICER-RUBIO; LEBOEUF, 2010). Com a progressão do desenvolvimento folicular em direção à fase pré-ovulatória, os folículos tornam-se criticamente dependentes do suporte de FSH, e um grupo de folículos antrais dependentes de FSH com diâmetro entre 2-3 mm é recrutado e entram em seu crescimento terminal. Somente dois a três folículos alcançam 4 mm de diâmetro e são selecionados para entrar na fase de dominância. Sob influência do LH, eles alcançam o estádio pré-ovulatório (6-9 mm), enquanto os folículos subordinados degeneram (FATET; PELLICER-RUBIO; LEBOEUF, 2010).

Na fase final do desenvolvimento folicular, observa-se a formação do folículo pré-ovulatório, o qual é caracterizado por um oócito circundado por células da granulosa especializadas, denominadas de células do cumulus. As células da granulosa de folículos pré-ovulatórios param de se multiplicar em resposta ao hormônio luteinizante (LH) e iniciam o programa final de diferenciação, sendo a ovulação do oócito circundado pelas células do cumulus também resultante da ação do LH (pico pré-ovulatório). Em todas as espécies, a formação de folículos pré-ovulatórios ocorre geralmente durante a puberdade (DRIANCOURT, 2001).

No início da fase de seleção, os níveis de IGF-1 intrafoliculares estão elevados, o que promove a inibição da produção de IGFBP-4 (proteína ligante 4 transportadora de fator de crescimento semelhante à insulina), resultando no aumento da disponibilidade de IGFs. Esses elevados níveis de IGF-1 e inibina estimulam a esteroidogênese, resultando em níveis superiores de estrógeno. Além disso, os elevados níveis de IGF-1 aumentam também a formação de receptores para FSH e LH nas células da granulosa, os quais determinam uma maior sensibilidade dos folículos a baixos níveis de FSH e aumentam a responsividade ao LH. Essa maior sensibilidade ao LH resulta no estímulo de fatores angiogênicos como o VEGF, que por sua vez estimula a vascularização, fornecendo ao folículo um maior aporte de nutrientes, oxigênio e fatores de crescimento estimulatórios que permitirão o desenvolvimento adequado do folículo até o estádio pré-ovulatório (VAN DEN HURK; ZHAO, 2005).

Diante dessa complexidade de eventos durante a foliculogênese, em que é possível observar a participação de diferentes substâncias, diversas pesquisas têm sido realizadas com diferentes espécies. Estas pesquisas priorizam o desenvolvimento de meios de cultivo eficientes que promovam a maturação de milhares de oócitos oriundos dos folículos pré-antrais, o que possibilitaria a produção *in vitro* de embriões em larga escala. Nesse contexto, e com o intuito de estudar o efeito de importantes fatores de crescimento no desenvolvimento folicular em caprinos, experimentos com cultivo *in vitro* de folículos pré-antrais foram realizados nesta espécie utilizando as substâncias KL, BMP-15 e EGF e serão abordados nos Capítulos 5 a 8 desta tese.

2.3 Cultivo *in vitro* de folículos pré-antrais

O cultivo *in vitro* de folículos pré-antrais, também conhecido como ovário artificial, é uma importante etapa da biotécnica de Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais (MOIFOPA). O objetivo desta biotécnica é criar um sistema de cultivo *in vitro* adequado que permita a sobrevivência, crescimento, maturação e posterior fecundação de oócitos oriundos de folículos pré-antrais, prevenindo a atresia folicular que ocorre abundantemente nos ovários (FIGUEIREDO et al., 2008).

O cultivo *in vitro* de folículos pré-antrais é uma técnica importante uma vez que poderá fornecer um grande número de oócitos, os quais poderão ser utilizados para diversas biotecnologias, como a produção *in vitro* de embriões, transferência nuclear, produção de animais transgênicos, desenvolvimento de células-tronco embrionárias, dentre outras,

podendo ainda ser utilizada para auxiliar na preservação da fertilidade de mulheres jovens sujeitas à quimioterapia. Além disso, o cultivo *in vitro* de folículos pré-antrais é uma ferramenta que permite aperfeiçoar o conhecimento básico sobre os mecanismos envolvidos na foliculogênese ovariana (SÁNCHEZ et al., 2009; ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010). Dessa forma, diversos sistemas de cultivo têm sido desenvolvidos no sentido de promover o crescimento e garantir a manutenção da viabilidade de folículos pré-antrais *in vitro* (VAN DEN HURK et al., 2000b). Nesses sistemas de cultivo *in vitro*, os folículos ovarianos podem ser cultivados inclusos no próprio tecido ovariano (cultivo *in situ*) ou na forma isolada (cultivo isolado). Aliado a isto, pode ainda ser realizado um cultivo de dois passos associando esses dois sistemas de cultivo, em que primeiro é realizado o cultivo *in situ*, e em seguida o cultivo dos folículos isolados crescidos *in vitro* (O'BRIEN; PENDOLA; EPPIG, 2003; TELFER et al., 2008). Em roedores, devido à pequena dimensão da gônada feminina, os ovários são cultivados inteiros, permitindo estudar os fatores que podem afetar o início do crescimento de folículos primordiais quiescentes, ou seja, a ativação folicular (FORTUNE, 2003). Por outro lado, em animais domésticos de médio e grande porte, devido às grandes dimensões dos ovários, alguns autores têm realizado o cultivo de pequenos fragmentos de córtex ovariano, rico em folículos primordiais, com o objetivo de estudar a ativação folicular e o crescimento de folículos primários (bovinos: BRAW-TAL; YOSSEFI, 1997; humanos: ZHANG et al., 2004; caprinos: SILVA et al., 2004a). Além da praticidade, o cultivo de fragmentos de córtex ovariano tem a vantagem de manter o contato celular e a integridade tridimensional dos folículos (ABIR et al., 1999, 2006).

Com relação aos folículos secundários, esses são cultivados mais frequentemente na forma isolada (PICTON et al., 2008). Desta forma, métodos mecânicos e/ou enzimáticos têm sido desenvolvidos para isolar um grande número de folículos primários e/ou secundários intactos de diferentes espécies (vacas: FIGUEIREDO et al., 1993; cabras: LUCCI et al., 1999; ovelhas: CECCONI et al., 1999; ratas: ZHAO, 2000; camundongas: LENIE et al., 2004; PESTY et al., 2007). Os folículos isolados podem ser cultivados em modelo bidimensional, no qual são cultivados diretamente sobre o suporte de plástico (placa de cultivo) ou sobre uma matriz extracelular como o colágeno, ou ainda em modelo tridimensional (DEMEESTERE et al., 2005), que possui como vantagem a manutenção da integridade folicular. Esse sistema de folículos isolados tem sido adotado sem soro (ovinos: NEWTON; PICTON; GOSDEN, 1999; humano: TELFER et al., 2008), utilizando membranas hidrofóbicas que previnem a aderência

folicular (camundongo: NAYUDU; OSBORN, 1992), em gel de colágeno (murinos: CARROL; WHITTINGHAM; WOOD, 1991; suínos: HIRAO et al,. 1994) ou de alginato (camundongo: XU et al., 2006; WEST et al., 2007). O cultivo de folículos isolados apresenta como vantagens permitir o acompanhamento individual dos folículos durante o cultivo, além de favorecer a maior perfusão do meio para o folículo (ABIR et al., 2006).

A eficiência do cultivo *in vitro* de folículos pré-antrais pode ser afetada por diversos fatores, incluindo a espécie animal, tipo de sistema de cultivo (bi ou tridimensional), pH, temperatura, tensão de oxigênio e composição do meio. A composição do meio é um importante fator para a obtenção de bons resultados durante o cultivo *in vitro* de folículos pré-antrais. Alguns meios de base têm sido utilizados para o cultivo folicular de diferentes espécies, como: Meio Essencial Mínimo (MEM - CORTVRINDT; SMITZ; VAN STEIRTEGHEM, 1996; NEWTON; PICTON; GOSDEN, 1999; MARTINS et al., 2005); meio Waymouth (EPPIG; O'BRIEN, 1996; MURUVI et al., 2005) e meio McCoy's 5a (TELFER et al., 2008). Esses meios geralmente possuem componentes com funções vitais para garantir a viabilidade e o crescimento dos folículos, como eletrólitos, antioxidantes, aminoácidos, substratos energéticos e vitaminas (PICTON et al, 2008), podendo ainda serem acrescidos de antibióticos/antifúngicos, preparações comerciais de insulina, transferrina e selênio (WRIGHT et al., 1999), ou ainda de hormônios e/ou fatores de crescimento (EPPIG; SCHROEDER, 1989; BOLAND; GOSDEN, 1994; FORTUNE, 2003).

Devido à importância da composição do meio, nosso grupo de pesquisa tem se focado em estabelecer um meio de cultivo *in vitro* capaz de assegurar a sobrevivência e o desenvolvimento de folículos pré-antrais caprinos. Os primeiros estudos realizados pelo nosso grupo, e a maioria dos resultados obtidos até o presente momento, foram envolvendo o cultivo *in vitro* de folículos pré-antrais caprinos inclusos em fragmentos de córtex ovariano, que será descrito a seguir.

2.3.1 Cultivo *in vitro* de folículos pré-antrais caprinos inclusos em fragmentos de córtex ovariano

Utilizando o cultivo de pequenos fragmentos de córtex ovariano (~ 3 x 3 x 1 mm), o efeito de diversas substâncias já tem sido avaliado no cultivo *in vitro* de folículos pré-antrais caprinos, incluindo a solução à base de água de coco, antioxidantes, soro, diferentes tipos de hormônios e fatores de crescimento.

Nos primeiros estudos de nossa equipe, foram comparados os efeitos do Meio Essencial Mínimo (MEM) com diferentes concentrações (5, 10, 20, 25, 50, 75, 80, 90, 95 ou 100%) da solução à base de água de coco (SBAC) sobre o crescimento de folículos primordiais caprinos, sobrevivência e proliferação das células da granulosa. Nesses estudos, observou-se que, após 5 dias de cultivo, as melhores taxas de sobrevivência e ativação foram alcançadas com a utilização do meio de base MEM sozinho, ou quando utilizada a água de coco em baixas proporções (5 ou 10%). Além disso, houve um aumento na degeneração folicular quando utilizada a SBAC pura (SILVA et al., 2004b; MARTINS et al., 2005). Ainda nesse estudo de SILVA et al. (2004b), foi verificada a influência da adição de suplementos como insulina-transferrina-selênio, piruvato, glutamina, hipoxantina e albumina sérica bovina (BSA) aos meios de base MEM ou SBAC, e constatou-se que quando adicionado tais suplementos aos meios de cultivo, houve uma diminuição significativa da degeneração folicular.

Em um estudo posterior, foi testado um dos componentes da água de coco, uma auxina conhecida como Ácido 3-Indol-Acético (IAA), em diferentes concentrações (0, 10, 20, 40 ou 100 ng/mL) sobre a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos, visualizados através da análise histológica e ultraestrutural. Os resultados mostraram que houve uma elevada percentagem de folículos histologicamente normais no MEM sozinho ou MEM suplementado com IAA (20 ng/mL), quando comparados aos outros tratamentos. O IAA, quando adicionado ao meio na concentração de 20 ou 40 ng/mL, aumentou a proporção de folículos primordiais que entraram na fase de crescimento após 5 dias, e nas concentrações de 10 ou 20 ng/mL aumentou o diâmetro folicular. Entretanto, os estudos ultraestruturais não confirmaram a manutenção da integridade morfológica nos folículos caprinos cultivados por 1 ou 5 dias em MEM suplementado com IAA (20 ng/mL), mostrando que o aumento do diâmetro observado era provavelmente devido à degeneração folicular (MATOS et al., 2006).

Também tem sido investigada a importância de alguns antioxidantes como o α-tocoferol, a ternatina e o ácido ascórbico. Quando testados o α-tocoferol e a ternatina nas concentrações de 0, 5, 10 ou 15 μM, verificou-se que esses antioxidantes não mantiveram a integridade ultraestrutural de folículos pré-antrais caprinos cultivados por 5 dias, bem como não tiveram efeito adicional sobre a ativação e o crescimento folicular (LIMA-VERDE et al., 2009). Entretanto, o ácido ascórbico mostrou ser um importante antioxidante para o cultivo *in vitro* de folículos caprinos por um longo período (14 dias). Quando testado o ácido ascórbico em diferentes concentrações (0, 50 ou 100 μg/mL), sozinho ou associado ao FSH (50 ng/mL),

foi verificado que a adição de 50 µg/mL de ácido ascórbico associado ao FSH não somente manteve a integridade folicular, mas também promoveu a ativação e o crescimento de folículos pré-antrais caprinos (ROSSETTO et al., 2009).

Devido ao fato de o soro ser uma substância rica em nutrientes, hormônios e fatores de crescimento, foi investigada a importância de diferentes origens (soro fetal bovino, soro de cabra em estro e em diestro) e concentrações (0, 10 ou 20%) desta substância, no cultivo *in vitro* de folículos pré-antrais caprinos. Independente da origem ou concentração, o soro não manteve a integridade ultraestrutural de folículos pré-antrais caprinos cultivados *in vitro* por 7 dias, além de não ter mostrado efeitos adicionais sobre a ativação e o crescimento folicular (BRUNO et al., 2008).

Diante da importância dos hormônios e fatores de crescimento ovarianos para a regulação da foliculogênese, seus efeitos também foram investigados em muitos experimentos com o cultivo *in vitro* de folículos pré-antrais caprinos. Uma das substâncias mais estudadas pelo nosso grupo é a gonadotrofina FSH. No primeiro estudo, foram testadas três diferentes concentrações do pFSH (Stimufol®) (10, 50 ou 100 ng/mL) e verificou-se que, quando utilizado na concentração de 50 ng/mL, o pFSH manteve a integridade morfológica de folículos pré-antrais caprinos cultivados por 7 dias, e estimulou a ativação de folículos primordiais e o crescimento dos folículos ativados (MATOS et al., 2007a). Posteriormente, foram avaliados os efeitos de diferentes pFSH (Stimufol® e Folltropin®) sobre a sobrevivência e o crescimento *in vitro* de folículos pré-antrais caprinos cultivados também por 7 dias. Os resultados mostraram que as preparações de FSH afetaram de diferentes formas os folículos pré-antrais caprinos cultivados *in vitro*. O Stimufol® foi melhor para preservar a ultraestrutura folicular, enquanto que o Folltropin® foi mais eficiente para promover o crescimento folicular (MAGALHÃES et al., 2009a). Em outro estudo, as diferentes origens de FSH (pituitário e recombinante) em diferentes concentrações foram comparadas. Verificou-se que o FSH recombinante (FSHr) foi mais eficiente do que pFSH, uma vez que 50 ng/mL de FSHr manteve a integridade ultraestrutural de folículos pré-antrais caprinos, e ainda promoveu a ativação dos folículos primordiais e o posterior crescimento dos folículos cultivados por 7 dias (MAGALHÃES et al., 2009b).

Outra gonadotrofina importante e que também já foi testada no cultivo *in vitro* de folículos pré-antrais caprinos é o hormônio luteinizante (LH). Essa gonadotrofina foi avaliada em diferentes concentrações (0, 1, 5, 10, 50 ou 100 ng/mL), na ausência ou presença de FSH suíno (50 ng/mL). Nesse estudo, verificou-se que a utilização de 1 ng/mL de LH sozinho ou

associado ao FSH, mantém a integridade folicular e também promove o crescimento *in vitro* de folículos pré-antrais caprinos (SARAIVA et al., 2008).

Além das gonadotrofinas, também já foi testada a influência do hormônio esteróide estradiol em folículos caprinos (LIMA-VERDE et al., 2010a). Nessa pesquisa, avaliando o estradiol (0, 1, 5, 10, 20, 40 pg/mL) sozinho ou associado ao FSH suíno, na mesma concentração utilizada no estudo anterior, foi verificado que a utilização de estradiol (1 pg/mL) associado ao FSH, manteve a integridade morfológica e ultraestrutural após 7 dias de cultivo, bem como estimulou a ativação e o crescimento *in vitro* de folículos pré-antrais. Entretanto, quando utilizado outro hormônio esteróide, a androstenediona, também em diferentes concentrações (0, 1, 10, 50, or 100 ng/mL), sozinha ou associada ao FSH (50 ng/mL), tal hormônio não apresentou efeito adicional sobre a sobrevivência de folículos pré-antrais caprinos em relação ao meio de base utilizado (MEM) (LIMA-VERDE et al., 2010b).

Além dos hormônios, no nosso grupo também foi investigada a influência de fatores intraovarianos através do cultivo de folículos pré-antrais caprinos. Muitas das moléculas sinalizadoras implicadas na regulação do início da foliculogênese são pertencentes à superfamília do fator de crescimento transformante β (TGF-β). Dessas moléculas, foram estudadas a ativina, a folistatina, as BMPs - 6, - 7 e - 15, e o GDF-9.

SILVA et al. (2006a) investigaram os efeitos da ativina-A e da folistatina em diferentes concentrações (0, 10 ou 100 ng/mL) sobre o desenvolvimento *in vitro* de folículos primordiais e primários caprinos. Os resultados desse estudo mostraram que os folículos primordiais foram ativados e se desenvolveram até estádios mais avançados, por exemplo, até folículos de transição e primários durante o cultivo *in vitro*, mas nem a ativina-A nem a folistatina afetaram o número de folículos primordiais que entraram em fase de crescimento. O tratamento com a ativina-A (100 ng/mL) aumentou o número de folículos morfologicamente normais e estimulou seu crescimento durante o cultivo de córtex ovariano por 5 dias. Esses efeitos, entretanto, não foram alterados pela presença da folistatina.

Após cultivo *in vitro* de folículos pré-antrais caprinos em diferentes concentrações (1, 10, 50 ou 100 ng/mL), as BMPs- 6 e - 7 se comportaram de maneira diferente. No estudo com a BMP-7, verificou-se que baixas concentrações deste fator podem promover a sobrevivência e o crescimento de folículos pré-antrais caprinos. Além disso, a BMP-7 na concentração de 1 ng/mL manteve a integridade ultraestrutural destes folículos pré-antrais após 7 dias de cultivo (ARAÚJO et al., 2010a). Entretanto, a BMP-6 afetou negativamente a sobrevivência e a ultraestrutura de folículos pré-antrais caprinos após cultivo *in vitro*

(ARAÚJO et al., 2010b). Quando a BMP-15 foi testada em diferentes concentrações (0, 1, 10, 50, 100 ou 200 ng/mL), observou-se que a utilização de 100 ng/mL de BMP-15 não somente manteve a integridade morfológica dos folículos pré-antrais caprinos cultivados por 7 dias, mas também promoveu o crescimento e a transição para o estádio de folículo secundário. Mais detalhes acerca desse experimento e dos resultados obtidos serão mostrados no Capítulo 6.

Outro fator testado dessa superfamília foi o GDF-9, tendo este fator alcançado resultados bastante satisfatórios. Quando adicionado nas mesmas concentrações utilizadas para a BMP-15 no cultivo de folículos pré-antrais caprinos por 7 dias, o GDF-9 na concentração de 200 ng/mL, promoveu a manutenção da ultraestrutura normal de folículos pré-antrais, a ativação de folículos primordiais, além de ter promovido a transição de folículos primários para secundários (MARTINS et al., 2008). O GDF-9 (200 ng/mL) também foi testado em associação com o IGF-1 e com o hormônio do crescimento (GH), nas concentrações de 50 e 10 ng/mL, respectivamente. Verificou-se que a associação dessas três substâncias mantém a viabilidade de folículos pré-antrais, bem como a promove a ativação e o crescimento folicular (MARTINS et al., 2010).

Em estudos adicionais foram investigados os efeitos de outras substâncias, dentre elas algumas mitogênicas, como membros da família FGF (FGF-2 e -10) e o EGF, além de outras substâncias como o KL, VEGF, VIP e a neurotrofina NGF. MATOS et al. (2007b) demonstraram que a concentração de 50 ng/mL de FGF-2 manteve a integridade morfológica de folículos pré-antrais caprinos cultivados por 5 dias, além de estimular a ativação de folículos primordiais e o crescimento dos folículos ativados. Também foi observada uma interação positiva entre o FGF-2 e o FSH em promover o início do crescimento de folículos primordiais e o crescimento do oócito, bem como na manutenção da integridade ultraestrutural folicular (MATOS et al., 2007c; MATOS et al., 2010). Já a utilização do FGF-10, também na concentração de 50 ng/mL, manteve a integridade morfológica dos folículos pré-antrais caprinos, e estimulou o crescimento dos folículos ativados (CHAVES et al., 2010a).

Contrariamente ao que foi observado com o FGF-2, um trabalho prévio utilizando uma única concentração de EGF (100 ng/mL) associada ao pFSH (100 ng/mL), não mostrou interação positiva entre essas duas substâncias no que diz respeito ao crescimento folicular (SILVA et al., 2004a). CELESTINO et al. (2009) investigaram os efeitos de diferentes concentrações de EGF (0, 1, 10, 50, 100, ou 200 ng/mL) sobre a sobrevivência e o

crescimento de folículos pré-antrais caprinos. Estes autores demonstraram que as baixas concentrações de EGF (1 ou 10 ng/mL) mantiveram a integridade folicular caprina e promoveram a transição de folículos primordiais para primários após 7 dias de cultivo. O capítulo 7 da presente Tese mostrará a metodologia e os resultados desse experimento mais detalhadamente. Após testar as mesmas concentrações reportadas para o EGF, resultados similares, que serão mostrados no Capítulo 5, foram observados utilizando KL (50 ng/mL) (CELESTINO et al., 2010).

Fatores angiogênicos, como o VEGF, também desempenham importante papel no desenvolvimento folicular *in vitro*. BRUNO et al. (2009) investigaram o efeito do VEGF sobre a sobrevivência e o crescimento de folículos pré-antrais caprinos após cultivo *in vitro*, e verificaram a expressão do receptor de VEGF (VEGFR-2) em ovários caprinos. Os estudos imunohistoquímicos demonstraram a expressão de VEGFR-2 nos oócitos e células da granulosa em todos os estádios foliculares, exceto nas células da granulosa de folículos primordiais. Quanto aos resultados do cultivo *in vitro*, as melhores concentrações de VEGF para promover o crescimento e a manutenção da viabilidade folicular após 7 dias de cultivo foram 10 e 200 ng/mL, respectivamente (BRUNO et al., 2009). Em um estudo posterior, o VIP mostrou efeitos similares quando adicionado ao meio de base na concentração de 10 ng/mL (BRUNO et al., 2010).

Utilizando a neurotrofina NGF nas concentrações 0, 1, 10, 50, 100 ou 200 ng/mL, observou-se que a concentração mais baixa (1 ng/mL) mostrou efeitos positivos na sobrevivência e manutenção da viabilidade folicular após 7 dias, mas nenhum efeito sobre a ativação e o crescimento (CHAVES et al., 2010b). No Quadro 1, podem ser observados os principais resultados obtidos por nossa equipe com o cultivo *in situ* de folículos pré-antrais caprinos.

Quadro 1. Principais resultados obtidos pelo LAMOFOPA com o cultivo *in situ* de folículos pré-antrais caprinos.

Substância	Duração do cultivo	Forma de avaliação folicular	Efeito	Conclusão	Referência
Água de coco	5 dias	HC	-	Ausência de efeito	Silva et al., 2004b
Água de coco	5 dias	HC	-	Ausência de efeito	Martins et al., 2005
IAA	5 dias	HC e MET	Ativação e crescimento	Ativação: 20 ou 40 ng/mL Crescimento: 10 ou 20 ng/mL	Matos et al., 2006
α-tocoferol e ternatina	5 dias	HC e MET	-	Ausência de efeito	Lima-Verde et al., 2009
Ácido ascórbico sem ou com FSH Stimufol	14 dias	HC, MET e F	Viabilidade, ativação e crescimento	50 µg/mL de ácido ascórbico + FSH	Rossetto et al., 2009
Soro fetal bovino, de cabra em estro ou diestro	7 dias	HC e MET	-	Ausência de efeito	Bruno et al., 2008
FSH Stimufol	7 dias	HC e MET	Viabilidade, ativação e crescimento	50 ng/mL de FSH	Matos et al., 2007a
FSH Stimufol x FSH Folltropin	7 dias	HC e MET	Viabilidade e ativação	Viabilidade: 50 ng/mL Stimufol Ativação: 100 ng/mL Folltropin	Magalhães et al., 2009a
FSH Folltropin x FSH recombiante	7 dias	HC e MET	Viabilidade, ativação e crescimento	50 ng/mL de FSH recombiante	Magalhães et al., 2009b
LH sem ou com FSH Stimufol	7 dias	HC e MET	Viabilidade e crescimento	1 ng/mL de LH sozinho ou com FSH	Saraiva et al., 2008
Estradiol sem ou com FSH Stimufol	7 dias	HC e MET	Viabilidade, ativação e crescimento	1 pg/mL de estradiol + FSH	Lima-Verde et al., 2010a
Ativina/folistatina	5 dias	HC	Viabilidade e crescimento	100 ng/mL de ativina sozinha ou com folistatina	Silva et al., 2006a
GDF-9	7 dias	HC e MET	Viabilidade e ativação (folículos secundários)	200 ng/mL de GDF-9	Martins et al., 2008
GDF-9 + IGF-1 + GH	7 dias	HC e F	Viabilidade, ativação e crescimento	200 ng/mL de GDF-9, 50 ng/mL de IGF-1 e 10 ng/mL de GH	Martins et al., 2010
FGF-2	5 dias	HC e MET	Viabilidade, ativação e crescimento	50 ng/mL de FGF-2	Matos et al., 2007b
FGF-2 + FSH Stimufol	7 dias	HC e MET	Viabilidade, ativação e crescimento	50 ng/mL de FGF-2 e 50 ng/mL de FSH	Matos et al., 2007c
FGF-10	7 dias	HC, MET e F	Viabilidade e crescimento	50 ng/mL de NGF	Chaves et al., 2010a
EGF	7 dias	HC e MET	Viabilidade e ativação (folículos primários)	1 ou 10 ng/mL de EGF	Celestino et al., 2009
EGF + FSH Stimufol	5 dias	HC e IHQ	-	Ausência de efeito	Silva et al., 2004a
KL	7 dias	HC e MET	Viabilidade, ativação (folículo primários) e crescimento	50 ng/mL de KL	Celestino et al., 2010
VEGF	7 dias	HC, MET e F	Viabilidade e crescimento	Viabilidade: 200 ng/mL Crescimento: 10 ng/mL	Bruno et al., 2009
VIP	7 dias	HC e MET	Viabilidade e crescimento	10 ng/mL de VIP	Bruno et al., 2010

HC: Histologia Clássica; MET: Microscopia Eletrônica de Transmissão; F: Fluorescência.

2.3.2 Cultivo *in vitro* de folículos pré-antrais caprinos isolados

Existem poucos trabalhos com o cultivo *in vitro* de folículos pré-antrais isolados em caprinos. Embora o número de resultados obtidos até o momento com esse sistema nesta espécie ainda seja pequeno, bons resultados têm sido alcançados, incluindo a manutenção da sobrevivência folicular, crescimento folicular e oocitário, formação de antro, obtenção de oócitos competentes para retomar a meiose, e ainda, a obtenção de embriões a partir de oócitos provenientes de folículos pré-antrais cultivados *in vitro*.

ZHOU e ZHANG (2000) reportaram o desenvolvimento *in vitro* de folículos primários e secundários caprinos isolados (50 a 150 µm de diâmetro), demonstrando que folículos primários desenvolvem até o estádio de folículos secundários, embora poucos destes folículos oriundos do estádio primário desenvolvam-se até antrais. Além disso, foi observado que a sobrevivência dos folículos secundários foi maior do que a dos folículos primários, e que uma grande quantidade dos folículos secundários atingiram o estádio antral.

RAJARAJAN et al. (2006) investigaram o efeito do TGF-α (10 ng/mL), fator de crescimento semelhante à insulina II (IGF-II; 20 ng/mL), EGF (50 ng/mL) e FSH (1 µg/mL) após 6 dias de cultivo *in vitro* de pequenos (40–60 µm) e grandes (61–100 µm) folículos pré-antrais caprinos. Nesse estudo, eles mostraram que o EGF, TGF-α e IGF-II estimularam o crescimento de pequenos e grandes folículos pré-antrais, enquanto o FSH foi mais eficiente em estimular o desenvolvimento apenas de pequenos folículos pré-antrais.

Focando em oócitos, foi estudado o efeito do EGF (50 mg/L) e IGF-I (100 mg/L), ambos individualmente ou em associação, sobre a viabilidade e o crescimento *in vitro* de oócitos de folículos pré-antrais caprinos (ZHOU; ZHANG, 2005a). Os resultados mostraram elevadas taxas de sobrevivência e crescimento do oócito quando o EGF foi associado ao IGF-I. Além disso, ambos os fatores de crescimento, quando utilizados individualmente, melhoraram a sobrevivência e o crescimento do oócito quando comparado ao controle. Em outro trabalho desses mesmos autores, além de ter sido observado os efeitos benéficos da associação do EGF e IGF-I conforme descrito anteriormente, o FGF-2 (50 ng/mL) estimulou a sobrevivência do oócito, embora não tenha causado nenhum efeito no seu crescimento (ZHOU; ZHANG, 2005b).

Os primeiros trabalhos do nosso grupo com o cultivo *in vitro* de folículos secundários isolados foram realizados mais recentemente, envolvendo condições básicas de cultivo, como por exemplo, tensão de oxigênio ideal, regime de troca de meio, bem como a forma do cultivo

de folículos isolados, individual ou em grupo, com ou sem FSH, na presença ou ausência de folículos antrais.

Ao testar duas diferentes tensões de oxigênio (5 ou 20%) observou-se que, na concentração de 20% de O_2, os folículos pré-antrais caprinos crescidos *in vitro* após 30 dias de cultivo apresentaram um maior crescimento, e um destaque para a retomada da meiose (SILVA et al., 2010).

Em outro estudo foram avaliados os efeitos de diferentes intervalos de troca de meio (2 ou 6 dias) sobre a viabilidade, formação da cavidade antral, crescimento e maturação *in vitro* de oócitos caprinos e ovinos oriundos de folículos pré-antrais isolados e cultivados por 24 dias. Os folículos pré-antrais caprinos e ovinos comportaram-se de maneira completamente diferente. De acordo com os resultados obtidos, para melhorar a eficiência dos sistemas de cultivo, o meio deve ser trocado a cada 2 e 6 dias para folículos pré-antrais caprinos e ovinos, respectivamente (MAGALHÃES et al., 2010b).

Outro estudo testou ainda a influência de diferentes regimes de troca sobre o desenvolvimento *in vitro* de folículos pré-antrais caprinos cultivados por 18 dias. Nesse estudo foi testado três diferentes regimes de troca: T1 (remoção de meio seguida da adição do mesmo volume de meio fresco a cada troca); T2 (somente adição de meio fresco a cada troca) e T3 (remoção de meio seguida da adição de 5 µl a mais de meio fresco a cada troca, ou seja, aumento de 5 µl do volume final em cada troca). Como conclusão, foi observado que embora o T3 tenha sido tão bom quanto o T2 no parâmetro de crescimento folicular, o T2, ou seja, de adição periódica de meio foi o tratamento recomendado por ser mais prático, manter a sobrevivência, além de promover o desenvolvimento *in vitro* de folículos pré-antrais caprinos, com uma superior percentagem de oócitos destinados para MIV quando comparado aos demais tratamentos (ARAÚJO et al., 2010c).

Também foram testadas diferentes formas de cultivo, por exemplo, individual ou em grupo, com ou sem FSH, na presença ou na ausência de folículos antrais. Nesse estudo, na ausência de FSH, o cultivo por 24 dias de folículos pré-antrais em grupo mostrou taxas superiores de sobrevivência e crescimento em relação aos folículos cultivados individualmente. Por outro lado, na presença de FSH, esses parâmetros não foram afetados, mas foi observado um grande número de oócitos crescidos e que retomaram a meiose quando os folículos foram cultivados em grupo. Finalmente, o co-cultivo de folículos antrais com folículos pré-antrais afetou negativamente a sobrevivência e o crescimento folicular, enquanto que resultados opostos foram observados quando os folículos foram cultivados em grupo

(DUARTE et al., 2010). Utilizando ainda esse hormônio, em outro estudo foi verificado que o FSH na presença de 10% de soro fetal bovino teve um importante papel no crescimento de folículos pré-antrais, não só na espécie caprina, mas também na espécie ovina, após 18 dias de cultivo (RODRIGUES et al., 2010).

Em outro estudo realizado com FSH, em que foi verificada a influência desse hormônio sobre o desenvolvimento *in vitro* de folículos caprinos isolados cultivados por 18 dias, foram testadas concentrações fixas de 100 ou 1000 ng/mL, ou adições do FSH ao meio de cultivo de modo seqüencial, ou seja, T1: FSH 100 ng/mL (do dia 0 ao dia 6), FSH 500 ng/mL (do dia 6 ao dia 12) e FSH 1000 ng/mL (do dia 12 ao dia 18), e T2: FSH 500 ng/mL (do dia 0 ao dia 9) e 1000 ng/mL (do dia 9 ao dia 18). Nesse estudo foi verificado que a utilização do Tratamento 1 melhorou a sobrevivência, formação de antro e reduziu a extrusão do oócito, e ambos os meios sequenciais promoveram uma elevada taxa de retomada da meiose (SARAIVA et al., 2010b).

Empregando ainda esse sistema de cultivo, foi observada a influência do EGF sozinho ou associado ao FSH sobre o desenvolvimento *in vitro* de folículos pré-antrais caprinos isolados cultivados por 6 dias. Além disso, foi verificada a influência da adição dessas substâncias ao meio de cultivo sobre a expressão do RNAm para o EGF e FSH-R. Nele observou-se que o EGF e o FSH promoveram o crescimento de folículos secundários caprinos, reduziram os níveis de RNAm para o EGF, e ainda, o EGF diminuiu os níveis de RNAm para FSH-R em folículos secundários caprinos cultivados. O capítulo 8 da presente Tese mostrará a metodologia e os resultados desse experimento mais detalhadamente.

Foi testado ainda o efeito do momento da adição de LH (100 ng/mL) ao meio de cultivo sobre o desenvolvimento *in vitro* de folículos pré-antrais caprinos. Nele observou-se que quando adicionado tal hormônio desde o início do cultivo, ou seja, é feita uma exposição prolongada, há um detrimento na retomada da meiose desses folículos cultivados *in vitro*. Já em um cultivo de 18 dias, quando este hormônio foi adicionado a partir do dia 6, houve uma elevada sobrevivência e os oócitos foram hábeis para retomar a meiose (SILVA et al., aceito para publicação).

Por último, os resultados mais relevantes são relativos à obtenção de embriões caprinos a partir de oócitos oriundos de folículos pré-antrais isolados crescidos, maturados e fecundados *in vitro* (SARAIVA et al., 2010a e MAGALHÃES et al., 2011). Tais resultados foram alcançados com a adição de alguns hormônios, como o FSH de forma sequencial (100 ng/mL do dia 0 ao dia 6, 500 ng/mL do dia 6 ao dia 12 e 1000 ng/mL do dia 12 ao dia 18), LH

(100 ng/mL) e GH (50 ng/mL), bem como com a adição do fator de crescimento EGF (100 ng/mL). No Quadro 2, podem ser observados os principais resultados obtidos com o cultivo de folículos pré-antrais caprinos isolados.

Quadro 2. Principais resultados obtidos pelo LAMOFOPA com o cultivo isolado de folículos pré-antrais caprinos.

Condições de cultivo	**Duração do cultivo**	**Forma de avaliação folicular**	**Efeito**	**Conclusão**	**Referência**
5 ou 20% de oxigênio	30 dias	Morfológica (subjetiva) e Fluorescência	Formação de antro, crescimento e retomada da meiose	20% de oxigênio	Silva et al., 2010
Regime de troca (a cada 2 ou 6 dias)	24 dias	Morfológica (subjetiva) e Fluorescência	Viabilidade, formação de antro e oócitos crescidos para MIV	Troca a cada 2 dias	Magalhães et al., 2010b
Cultivo folicular individual ou em grupo, sem ou com FSH recombinante, na ausência ou presença de folículo antral	24 dias	Morfológica (subjetiva) e Fluorescência	Viabilidade, crescimento e retomada da meiose	Viabilidade: grupo sem FSH e na presença de folículos antral Crescimento: grupo sem ou com FSH e na presença de folículos antral Retomada da meiose: grupo com FSH	Duarte et al., 2010
Concentrações fixas de FSH (100 ou 1000 ng/mL) ou adição seqüencial de FSH (T1: 100, 500 e 1000 ng/mL e T2: 500 e 100 ng/mL)	18 dias	Morfológica (subjetiva) e Fluorescência	Viabilidade, formação de antro e retomada da meiose	Sobrevivência: T1 Formação de antro: T1 Retomada da meiose: ambos seqüenciais (T1 e T2)	Saraiva et al., 2010b
FSH seqüencial (T1) + EGF e LH 50 ou 100 ng/mL	18 dias	Morfológica (subjetiva) e Fluorescência	Obtenção de embrião após FIV	Retomada da meiose: EGF (100) + LH (50 ou 100) Embrião: EGF (100) + LH (100)	Saraiva et al., 2010a
FSH seqüencial (T1) + GH 10 ou 50 ng/mL	18 dias	Morfológica (subjetiva) e Fluorescência	Obtenção de embrião após FIV	Retomada da meiose e embrião: GH (50)	Magalhães et al., 2011

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

É indiscutível a ocorrência de grandes progressos no cultivo *in vitro* de folículos pré-antrais em diferentes espécies animais. Em gatas (JEWGENOW; STOLTE, 1996), gambás (BUTCHER; ULLMAN, 1996) e macacas (FORTUNE et al., 1998) foi observado o crescimento de folículos ovarianos pré-antrais isolados após o cultivo *in vitro*, porém, sem a formação de antro. Em humanos (ROY; TREACY, 1993; TELFER et al., 2008), bovinos (GUTIERREZ et al., 2000; MCCAFFERY et al., 2000) e cadelas (SERAFIM et al., 2010), folículos pré-antrais isolados foram cultivados *in vitro* e desenvolveram-se até o estádio antral. Com relação aos estudos com outras espécies domésticas, os resultados mais relevantes foram obtidos com suínos (WU; EMERY; CARREL, 2001; WU; TIAN, 2007), búfalos (GUPTA et al., 2008), ovinos (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010) e caprinos (SARAIVA et al., 2010a; MAGALHÃES et al., 2011), os quais obtiveram a produção de embriões após o cultivo de folículos pré-antrais e posterior maturação e fecundação *in vitro* dos oócitos.

Apesar do grande avanço no cultivo *in vitro* de folículos pré-antrais com as espécies supracitadas, os resultados mais satisfatórios têm sido observados em animais de laboratório. CARROLL et al. (1990) obtiveram o nascimento de camundongos *in vitro* após congelação e descongelação, crescimento, maturação e fecundação *in vitro* de oócitos oriundos de folículos primários. Posteriormente, EPPIG e O'BRIEN (1996) obtiveram o nascimento de um camundongo a partir de folículos primordiais crescidos, maturados e fecundados *in vitro*. Mais recentemente, esta mesma equipe, aperfeiçoando o protocolo utilizado anteriormente, relatou a produção de embriões e o nascimento de 59 camundongos saudáveis a partir de folículos pré-antrais cultivados, maturados e fecundados *in vitro* (O'BRIEN; PENDOLA; EPPIG, 2003).

2.5 Técnicas para análise folicular durante o cultivo *in vitro*

Diferentes técnicas podem ser utilizadas para análise folicular durante o cultivo *in vitro* de folículos pré-antrais, auxiliando assim no sucesso do cultivo. Dentre elas, podemos destacar aquelas que permitem a avaliação da qualidade folicular, como por exemplo, a histologia clássica (HC), a microscopia eletrônica de transmissão (MET) e a microscopia de fluorescência. Além disso, existem ainda as técnicas de biologia molecular que permitem o

estudo da expressão de genes que codificam ligantes e/ou receptores de diferentes substâncias importantes para a foliculogênese, contribuindo para uma melhor elucidação desse processo. A seguir, será abordada brevemente cada uma dessas técnicas.

2.5.1 Histologia Clássica

A histologia clássica (HC) é uma técnica importante para avaliação dos folículos pré-antrais cultivados *in vitro*, pois além de permitir uma análise quantitativa, ou seja, de um grande número de folículos cultivados, permite ainda verificar o número e a mudança na morfologia das células da granulosa de pavimentosa para cúbica, no momento da ativação folicular, além de analisar a integridade morfológica do oócito e das células da granulosa. Tais avaliações permitem, portanto, a classificação dos folículos quanto ao seu estádio de desenvolvimento (primordial, transição, primário ou secundário), e ainda quanto às suas características morfológicas (normais ou atrésicos). Entretanto, a HC possui como desvantagens não permitir a avaliação da integridade de membranas e das organelas citoplasmáticas. Vale salientar que tal técnica pode ser realizada tanto em folículos isolados, como naqueles inclusos em fragmentos de córtex ovariano (MATOS et al., 2007d).

Alguns autores mostraram que, na análise histológica, as alterações indicativas de atresia em folículos pré-antrais ocorrem primariamente no oócito, sendo a picnose nuclear o primeiro sinal de atresia (JORIO; MARIANA; LAHLOU-KASSI, 1991; WOOD; MONTALI; WILDT, 1997; SILVA et al., 2002). Outros estudos realizados mostraram que a HC pode dar resultados similares aos de viabilidade folicular utilizando marcadores fluorescentes como a calceína e o corante azul de tripan (CORTVRINDT; SMITZ, 2001b; AMORIM et al., 2003). Além disso, outro estudo relatou ainda que a HC (coloração com hematoxilina-eosina ou ácido períodico de Schiff-hematoxilina) pode ser utilizada para avaliar a morte da célula por necrose, através da visualização da presença de vacúolos (MARTINEZ-MADRID et al., 2007).

2.5.2 Microscopia Eletrônica de Transmissão

A MET é considerada uma técnica qualitativa e acurada, capaz de permitir a avaliação da integridade de membranas celulares e organelas citoplasmáticas (SALEHNIA; MOGHADAM; VELOJERDI, 2002). Ela se mostra como uma ferramenta valiosa para

detectar modificações morfológicas iniciais devido à atresia, as quais podem ser observadas apenas em nível ultraestrutural, antes de se tornarem mais visíveis e possíveis de serem identificadas por microscopia óptica. Tal técnica é, portanto, capaz de discernir a qualidade do oócito e das células da granulosa (LUCCI et al., 2001; LOPES et al., 2009), sendo deste modo, comumente utilizada como uma técnica complementar à HC. Com esta técnica é possível ainda avaliar a atresia, ou seja, a morte do folículo tanto por apoptose (STALDEMANN; LASSMAN, 2000) como pelo processo degenerativo de necrose (MARTINEZ-MADRID et al., 2007).

No que se refere à utilização da MET após o cultivo, ela serve para confirmar se realmente houve a manutenção da integridade morfológica após análise histológica. MATOS et al. (2006) embora tenham encontrado uma elevada percentagem de folículos histologicamente normais no MEM suplementado com IAA (20 ng/mL) após 5 dias de cultivo, os estudos ultraestruturais não confirmaram a manutenção da integridade morfológica desses folículos, mesmo após 1 dia de cultivo. Já em outros estudos com o cultivo *in vitro* de folículos pré-antrais, os resultados da histologia clássica puderam ser confirmados por MET (MATOS et al., 2007a,b; MARTINS et al., 2008, ROSSETTO et al., 2009; LIMA-VERDE et al., 2010a).

2.5.3 Microscopia de Fluorescência

Na microscopia de fluorescência são utilizados marcadores fluorescentes, que quando excitados por certos comprimentos de onda, absorvem energia e emitem luz de maior comprimento de onda (JUNQUEIRA; CARNEIRO, 1999). A microscopia de fluorescência é considerada uma técnica confiável, prática e rápida para analisar a viabilidade folicular (CORTVRINDT; SMITZ, 2001b; LOPES et al., 2009), tendo sido, portanto, empregada para avaliação da viabilidade de folículos pré-antrais após o cultivo *in vitro* em diversos trabalhos (BRUNO et al., 2009; SILVA et al., 2010; MAGALHÃES et al., 2010b). Para tanto, os folículos pré-antrais foram analisados pelo teste de viabilidade celular por fluorescência baseado na detecção simultânea de células vivas e mortas marcadas por calceína-AM e pelo etídio homodímero-1, respectivamente. Enquanto a primeira sonda detecta atividade da esterase intracelular, enzima característica de células viáveis, a outra cora ácidos nucléicos em células não-viáveis, com ruptura na membrana plasmática (LOPES et al., 2009). Dessa forma, essa técnica pode oferecer uma nova maneira para investigações metabólicas e aspectos do

desenvolvimento folicular *in vitro* (BRUNO et al., 2009). As sondas fluorescentes calceína-AM e etídio homodímero-1 já haviam sido utilizadas com sucesso para avaliação da viabilidade de folículos bovinos em estádios iniciais (SCHOTANUS et al., 1997; VAN DEN HURK et al., 1998), tendo sido a sonda calceína-AM também já empregada para determinar a densidade e o número de folículos presentes em biópsias ovarianas humanas (CORTVRINDT; SMITZ, 2001b).

Outro marcador que pode ser utilizado na microscopia de fluorescência para avaliar os folículos cultivados *in vitro* é o Hoescht 33342. Esse marcador penetra em células vivas e marca a cromatina, permitindo assim a avaliação do estádio meiótico folicular (JEWGENOW, 1998; MATOS et al., 2007d).

2.5.4 Biologia Molecular

As técnicas de biologia molecular podem ser utilizadas antes, durante e após o cultivo *in vitro* de folículos pré-antrais, com o intuito de identificar e quantificar o local de atuação e produção de cada substância envolvida nas diferentes etapas do desenvolvimento folicular. De fato, muitos papéis das células foliculares ovarianas relacionados à sobrevivência, ao crescimento e à diferenciação são refletidos na alteração dos padrões da expressão gênica. Desta forma, a capacidade de quantificar os níveis de transcrição de genes específicos é fundamental para garantir a completa investigação das funções foliculares (ZAMORANO; MAHESH; BRANN, 1996). Dentre as técnicas de biologia molecular, podem ser citadas aquelas que detectam, localizam ou identificam os ácidos nucléicos (hibridização *in situ* Southern e Northern Blotting) ou proteínas (Western Blotting), as que podem efetuar a quantificação do DNA (Reação em Cadeia de Polimerase - PCR) ou do RNA (Reação de Transcriptase Reversa em Cadeia de Polimerase - RT-PCR), as que permitem a quantificação da expressão do RNAm, mesmo em uma mistura complexa de RNA total (ensaios de proteção de ribonuclease), ou que possibilitem a análise da expressão de milhares de genes simultaneamente (Microarranjos de DNA).

No que diz respeito ao estudo da foliculogênese, as técnicas de biologia molecular aplicadas isoladamente ou em associação podem fornecer dados essenciais para fundamentar a base do conhecimento sobre a expressão de RNAm, tanto de ligantes quanto de receptores associados a esse processo. Atualmente, a técnica mais utilizada para quantificar a expressão de RNAm é a RT-PCR em tempo real (RT-qPCR) (KREUZER; MASSEY, 2002). A qPCR

uma variante da RT-PCR convencional, permite uma análise precisa da quantificação da expressão gênica em determinado tecido ou amostra biológica. Esse método utiliza um sistema fluorescente em plataforma, capaz de detectar a luz oriunda da reação de amplificação de um determinado gene no momento real da amplificação (BUSTIN, 2002). Através da técnica de RT-qPCR e da RT-PCR convencional, já foi possível identificar a presença de diferentes substâncias, como hormônios e fatores de crescimento, presentes em folículos ovarianos caprinos (SILVA et al., 2004c, 2005, 2006b,c; SARAIVA et al., 2010b).

3 JUSTIFICATIVA

Os folículos primordiais constituem o *pool* de reserva de folículos quiescentes e compreendem cerca de 95% de toda população folicular presente no ovário mamífero. No entanto, para que estes folículos possam entrar em fase de crescimento é preciso que sejam ativados. Neste contexto, sabendo-se do grande valor econômico que a espécie caprina representa em especial para o Nordeste Brasileiro, é de extrema importância o desenvolvimento de um sistema de cultivo capaz de ativar esses folículos e assegurar seu posterior crescimento *in vitro,* otimizando o aproveitamento do potencial oocitário desses animais e incrementando a eficiência da reprodução animal. Os oócitos oriundos destes folículos crescidos *in vitro* poderiam ser utilizados em programas de produção *in vitro* e transferência de embriões e/ou criopreservação. Além disso, o desenvolvimento de um sistema de cultivo eficiente poderá fornecer subsídios para uma melhor compreensão sobre os fatores que regulam a foliculogênese na fase pré-antral, necessários para a sobrevivência, a ativação e o início do crescimento folicular.

Estudos referentes aos fatores e mecanismos envolvidos na regulação e ativação dos folículos primordiais são escassos, especialmente em animais de produção, como os caprinos. Neste contexto, diversos autores têm investigado o efeito de diferentes fatores de crescimento no cultivo de folículos pré-antrais de animais de laboratório e de animais domésticos como vaca, ovelha e cabra. Entretanto, os efeitos de diferentes concentrações dos fatores como o KL, BMP-15 e EGF, que são importantes reguladores da foliculogênese, ainda não haviam sido testados no cultivo *in vitro* de folículos pré-antrais caprinos. Para este fim, além da técnica de histologia clássica, foi empregada a microscopia eletrônica de transmissão e/ou microscopia de fluorescência para determinar a qualidade de folículos pré-antrais caprinos cultivados *in vitro* e, consequentemente, melhor avaliar a eficiência dos meios de cultivo testados. Na tentativa de melhor desvendar a foliculogênese caprina, foi ainda realizado um estudo de quantificação da expressão gênica do RNAm para o KL, BMP-15 e EGF por meio da técnica de RT-PCR em tempo real (RT-qPCR), permitindo dessa forma, estabelecer estratégias para adição destes componentes em momentos ideais do cultivo.

Com o desenvolvimento de um eficiente sistema de cultivo, será possível, no futuro, a utilização dos oócitos oriundos de uma numerosa população de folículos pré-antrais crescidos *in vitro* em diversas biotécnicas reprodutivas, dentre elas a fecundação *in vitro*, contribuindo assim para a produção de embriões em larga escala.

4 HIPÓTESES CIENTÍFICAS

Diante do exposto, foram formuladas as seguintes hipóteses científicas:

1) A expressão dos níveis de RNAm para o KL, BMP-15 e EGF varia de acordo com o estádio de desenvolvimento folicular e com os tipos celulares que compõem o folículo.

2) As substâncias KL, BMP-15 e EGF afetam positivamente o desenvolvimento *in vitro* de folículos pré-antrais caprinos inclusos em fragmentos de tecido ovariano de uma maneira concentração-dependente.

3) O EGF, associado ou não ao FSH, influencia positivamente o crescimento *in vitro* de folículos secundários caprinos isolados, bem como os níveis de expressão do RNAm para o EGF e FSH-R.

5 OBJETIVOS

5.1 OBJETIVO GERAL

1) Verificar a expressão do RNAm para o KL, BMP-15 e EGF em folículos ovarianos caprinos.

2) Avaliar o efeito do KL, BMP-15 e EGF sobre o desenvolvimento de folículos pré-antrais caprinos cultivados *in vitro.*

5.2 OBJETIVOS ESPECÍFICOS

1) Quantificar, através da técnica de RT-PCR em tempo real (RT-qPCR), os níveis de RNAm para KL, BMP-15 e EGF nas diferentes categorias (folículo primordial, primário e secundário) e compartimentos de folículos antrais (oócito, células da granulosa e da teca) em ovários caprinos.

2) Estabelecer a curva concentração-resposta de KL, BMP-15 e EGF, tendo como parâmetros a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos inclusos em tecido ovariano e cultivados *in vitro* por 1 ou 7 dias.

3) Analisar morfológica e ultraestruturalmente os folículos pré-antrais caprinos inclusos em tecido ovariano e cultivados *in vitro* por 1 ou 7 dias com KL, BMP-15 e EGF, e ainda a viabilidade dos folículos cultivados com BMP-15.

4) Verificar o efeito do EGF, na presença ou ausência de FSH, sobre a sobrevivência, formação de antro e crescimento de folículos secundários caprinos isolados e cultivados *in vitro* por 6 dias, bem como sobre os níveis de RNAm para o EGF e FSH-R após cultivo folicular.

Nas páginas seguintes, serão apresentados os quatro primeiros capítulos desta Tese, correspondendo a dois artigos de revisão que já foram publicadas em periódico incluso no

Qualis CAPES categoria “B3”, e as outras duas estão sob revisão. Além disso, em seguida, serão apresentadas as metodologias e os resultados desta Tese na forma de quatro capítulos referentes a quatro artigos científicos. Vale salientar que dois artigos já foram publicados em periódico incluso no Qualis CAPES categoria “A1” e outros dois encontram-se em fase de julgamento.

6 CAPÍTULO 1

Mecanismos de atresia em folículos ovarianos

Mechanisms of atresia in ovarian follicles

Periódico: *Animal Reproduction* 6: 495-508, 2009.

Resumo

Existem milhares a milhões de folículos no ovário mamífero, e a grande maioria deles (99,9%) é eliminada pelo processo conhecido como atresia. Esse fenômeno ocorre em qualquer estádio de desenvolvimento folicular, através da apoptose ou do processo degenerativo de necrose. Assim, um melhor conhecimento dos mecanismos envolvidos na atresia é necessário para evitar a grande perda folicular que ocorre *in vivo* e maximizar o potencial reprodutivo das fêmeas. A presente revisão foca nos aspectos relacionados à população folicular e atresia, mecanismos de atresia (apoptose ou processo degenerativo de necrose), técnicas utilizadas para analisar a atresia em folículos ovarianos, e a ocorrência do processo atrésico durante os diferentes estádios foliculares.

Palavras-chave: Apoptose. Atresia. Folículo. Necrose. Ovário.

Mechanisms of atresia in ovarian follicles

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Abstract

There are thousands to millions of follicles in the mammalian ovary, and the majority (99.9%) are eliminated by a process known as atresia. This phenomenon occurs in any stage of follicular development, through an apoptotic manner or the degenerative process of necrosis. Thus, a better understanding of the mechanisms involved in atresia is necessary to avoid the great follicular loss that occurs *in vivo* and to maximize female reproductive potential. The present review focuses on aspects related to follicular population and atresia, mechanisms of atresia (apoptosis or the degenerative process of necrosis), techniques used to analyze atresia in ovarian follicles, and the occurrence of the atretic process during different follicular stages.

Keywords: apoptosis, atresia, follicle, necrosis, ovary.

Introduction

The mammalian ovary represents a dynamic organ that provides an adequate environment for the production of several substances, such as hormones and growth factors, and releases viable female gametes (Johnson, 2003). There are thousands to millions of ovarian follicles that are the structural and functional units of the mammalian ovary, which support an appropriate environment for oocyte growth and maturation (Cortvrindt and Smitz, 2001). Despite the great number of follicles present in the ovary, most do not reach ovulation

(about 99.9%), and instead die by atresia during growth and maturation (Markström et al., 2002).

Although atresia results in the loss of many ovarian follicles, this is a crucial event for the maintenance of mammalian ovarian homeostasis, which assures animal cyclicity (Amsterdam et al., 2003). In follicles, this process can occur through the apoptosis or the degenerative process of necrosis (for details see session 3). However, apoptosis is the most frequent form of physiologic cellular death (van Cruchten and van den Broeck, 2002) leading to significant follicular loss. Further knowledge of the intracellular mechanism, as well as the factors that regulate atresia, will contribute to a better comprehension of this process, which can facilitate the development of strategies to minimize the great follicular loss that occurs *in vivo*.

This review outlines the current understanding of these aspects related to follicular population and atresia, the mechanism of atresia (apoptosis or degenerative process of necrosis), techniques used to analyze atresia in ovarian follicles, and the occurrence of the atretic process during different stages of follicular development.

Follicular population and atresia

Folliculogenesis is an event that begins in prenatal life for most species and can be defined as the process of follicular assembly, growth, and maturation, beginning with the formation of the primordial follicle and finishing with the preovulatory follicle. The ovarian follicle is composed of an oocyte surrounded by somatic cells (granulosa and theca cells). According to the degree of development, follicles can be classified as preantral (primordial, intermediate, primary, and secondary) and antral follicles (tertiary and preovulatory), as shown in Fig. 1.

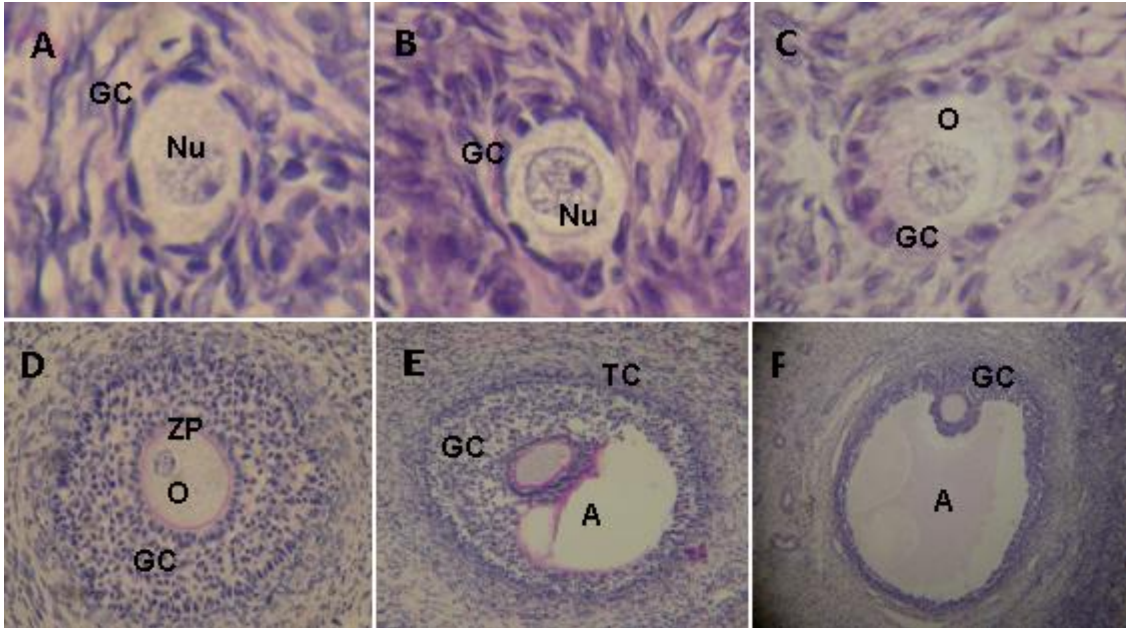

Figure 1. Histological sections containing ovarian follicles after staining with PAS-haematoxylin (400x). Preantral follicles: (A) primordial; (B) intermediate; (C) primary and (D) secondary. Antral follicles: (E) tertiary and (F) preovulatory. Nu: oocyte nucleus; O: oocyte; GC: granulosa cells; ZP: zona pelucida; A: antrum; TC: theca cells.

The female gametes are stocked in the ovary, especially as primordial follicles, which are composed of an immature oocyte surrounded by a single layer of flattened pre-granulosa cells. Primordial follicles remain relatively inactive in ovaries until recruitment into the growing follicle population (van den Hurk and Zhao, 2005), a process known as follicular activation (Nilsson and Skinner, 2004). The follicles enter into a pre-programmed course of development and maturation after activation, which is necessary for the success of ovulation and fertilization. Alternatively, the follicles can die by atresia.

Although there is a great follicular population present in the mammalian ovary (for example: 1,500 follicles in mouse - Shaw et al., 2000; 35,000 in goat - Lucci et al., 1999; 114,000 in domestic cat - Lima, 2006; 160,000 in sheep - Driancourt, 1991; 235,000 in cow - Betteridge et al., 1989; 2,000,000 in woman - Erickson, 1986), almost all follicles do not reach ovulation (99.9%), but rather die by a process called atresia. Even as a natural phenomenon, atresia significantly reduces the number of oocytes ovulated, decreasing the female reproductive potential. Thus, a better understanding of the mechanisms of atresia in ovarian follicles is necessary in order to minimize this great follicular loss.

Mechanisms of atresia in ovarian follicles

Apoptosis

Apoptosis is a form of programmed cell death and has also been implicated in a spectrum of processes associated with normal functions of the ovary and follicular development, such as atresia and corpus luteum regression (Hussein, 2005). This process is observed in ovarian follicles throughout fetal and adult life. Apoptosis is a genetically determined and active event, i.e., dependent on the balance of pro-and anti-apoptotic genes and requires energy (Hussein, 2005). Apoptosis is mediated by active intrinsic mechanisms and extrinsic factors (Johstone et al., 2002), such as oxidative stress, irradiation, activation of gene promoters of apoptosis, damage to DNA, cytokines, viral coat proteins, or the withdrawal of cell growth factors (Johnson, 2003).

The initiation, execution, and regulation of apoptosis involve various biochemical factors, and the caspase family of enzymes plays a central role in the apoptosis-signaling network. Caspases are members of the highly conserved family of cysteine proteases with aspartate specificity. Caspases are expressed as pro-enzymes that undergo proteolytic processing to generate the activated form after apoptotic stimulus. There are 14 types of caspases identified as caspase-1 to caspase-14 (Tibbets et al., 2003). Some members of the family function specifically in cellular death by apoptosis and are subdivided into initiator (caspases-2, -8, -9, and -10) and executor or effector caspases (caspase-3, -6, and -7; Strasser et al., 2000). Expression of caspase-3 has been found in ovarian leukocytes and in follicular cells of atretic follicles (Berardinelli et al., 2004; Tsai et al., 2005). Initiator caspases are cleaved in response to apoptotic stimuli and activate the effector caspases (Green, 2003). During apoptosis, the effector caspases cleave numerous proteins located in the cell membrane, nucleus, and cytoplasm. The activation of caspase-activated DNase (CAD) to facilitate DNA degradation is one of the important functions mediated by caspases in the apoptotic process (Nagase et al., 2003).

There are two main apoptotic pathways: (1) the extrinsic or death receptor pathway and (2) the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). The initial apoptotic stimulus strongly influences the pathway that is activated. The extrinsic and intrinsic pathways converge on the same terminal, or execution

pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Elmore, 2007). An overview of the two cellular death signaling pathways is presented in the following sections and is schematically illustrated in Fig. 2.

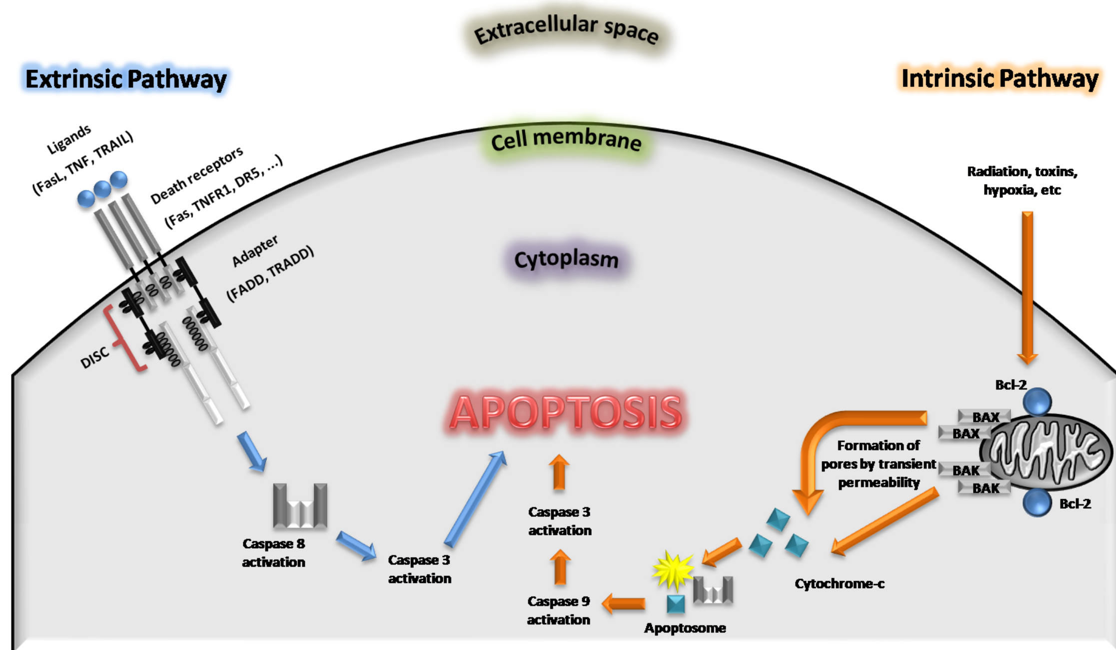

Figure 2. Two apoptotic pathways: membrane receptors (extrinsic) and mitochondrial (intrinsic). The extrinsic pathway can be induced by members of the TNF family of cytokine receptors, such as Fas, TNFR1 and DR5. These proteins recruit adapter proteins, including FADD, TRADD, which then binds pro-caspases. The intrinsic pathway can be induced by release of cytochrome-c from mitochondria, induced by various stimuli, including elevations in the levels of pore-forming pro-apoptotic Bcl-2 family proteins such as Bax. In the cytosol, formation of apoptosome occurs. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the executioner caspase 3. Adapted from: Elmore, 2007.

Extrinsic apoptotic pathway (membrane receptors)

Cells activate extrinsic apoptotic machinery in response to several conditions, such as signaling through apoptosis-related membrane receptors, which send apoptotic messages after binding to their respective cell death ligands. Caspase activation through cell death receptors is mediated by a subset of the tumoral necrosis factor (TNF) receptors superfamily, which

includes TNF receptor type 1 (TNFR1), Fas/CD95 (a membrane-associated polypeptide), and the TNF-related apoptosis-inducing Ligand (TRAIL) receptors, DR-4 and DR-5 (Slot, 2004; Contassot et al., 2007). The expression of Fas and FasL, as well as their functions in the ovary have been shown by Fujino et al. (2008) and Manabe et al. (2008), suggesting that the Fas system is involved in apoptosis in this organ. Additionally, Porter et al. (2001) showed higher concentrations of Fas and FasL in bovine atretic follicular cells than in healthy follicles.

Subsequent apoptotic signaling is mediated by the cytoplasmic domain of the death receptor, which contains a region termed the death domain (DD). Adapter molecules, like Fas-associated protein with death domain (FADD) or TNF receptor-associated protein with death domain (TRADD), bind to the activated death receptor, forming the death-inducing signaling complex (DISC). When bound to the DISC, several pro-caspase-8 molecules are recruited, resulting in cross-activation by (auto) proteolysis. Caspase-8 is a key initiator caspase that activates the downstream caspase cascade, and initiates the apoptotic program in the death receptor pathway (Reed, 2000), following activation in the DISC (Slot, 2004). In some cell types, a high concentration of caspase-8 in the DISC can process downstream effect or caspase-3 directly. This latter caspase leads the signaling of apoptosis to the nucleus, where an endonuclease is released and degrades DNA at each 180-200 kilobase pair fragments (Hussein, 2005).

Intrinsic pathway of apoptosis (mitochondria)

Mitochondria are not only important for amplifying extrinsic apoptotic pathway, but also for transmittance of death signals caused by intrinsic stress stimuli and apoptosis developmental instructions (Joza et al., 2002). Mitochondrial integrity and the release of cytochrome-c into the cytosol are primarily under the control of the Bcl-2 family members. The Bcl-2 family of proteins can be divided into anti-apoptotic members, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Boo, survivin, and pro-apoptotic members, such as Bax, Bak, Bok, and Diva. The Bcl-2 expression is found in the granulosa cells of both fetal and adult ovaries (Hussein, 2005; Hussein et al., 2006). Choi et al. (2004) showed a correlation between decreases in the level of Bcl-2 mRNA with the incidence of apoptosis in isolated granulosa cells which were cultured under different hormonal treatment conditions. They considered Bcl-2 to have a critical role in inhibiting the granulosa cell apoptosis pathway. Survivin is one member of the apoptosis inhibitor protein family that has been shown to bind and inhibit the

cell death effectors, caspase-3 and -7 (Shin et al., 2001). In the ovary, survivin acts in granulosa cells as a bifunctional protein associated with the regulation of the cell cycle and inhibition of apoptosis (Johnson and Bridgham, 2002). Bax is a pro-apoptotic protein involved in granulosa cell apoptosis (Tilly et al., 1995) and is an important regulator of follicle growth, but is dispensable for follicle atresia in mice. In addition, a defect in folliculogenesis was shown following Bax deletion (Greenfeld et al., 2007).

The key functions related to the cytochrome-c release from the mitochondrial intermembrane compartment into the cytosol have not been elucidated (Suzuki et al., 2000). One hypothesis is that Bcl-2 family members may insert inside the outer mitochondrial membrane to form large pore channels that allow the passage of molecules (Reed and Kroemer, 2000). Cytosolic cytochrome-c triggers the formation of the mitochondrial apoptosome, which is indicative of apoptosis and consists of cytochrome-c, Apaf-1 (Apoptotic Protease Activating Factor), and caspase-9 (Joza et al., 2002). The caspase-9 serves as the apical caspase of the mitochondrial pathway (Reed, 2000). Cytochrome-c binds to the Apaf-1 adapter protein, which recruits pro-caspase-9. This pro-caspase is cleaved into the active form, caspase-9, which proteolytically activates caspase-3, resulting in cell death. The link between the death receptor-activated caspase-8 (extrinsic pathway) and mitochondrial cytochrome-c release (intrinsic pathway) is created by a pro-apoptotic member, termed Bid. This member is cleaved by caspase-8 and is transferred to the mitochondria, where Bid acts in concert with the other apoptotic members of Bcl-2 family (Bax and Bak) to induce the release of cytochrome-c (Slot, 2004).

Another protein which is expressed in the apoptotic granulosa cells of atretic follicles is 53 protein (p53; Tilly, 1996). This protein functions as a transcription factor in response to DNA damage, inducing either growth, arrest or apoptosis (Slot, 2004). The p53 is known to activate the transcription of genes as Bax, Apaf-1, Fas, as well as to repress transcription of Bcl-2 genes (Bourdon et al., 2003). However, independent of its transcription control of these genes, p53 has also been shown to engage the apoptotic program by directly activating Bax to permeabilize mitochondria (Bras et al., 2005).

Morphological alterations in apoptotic cells

Independent of the particular stimulus and pathway, the morphologic features that characterize apoptosis are pycnosis and karyorrhexis in the nucleus, as well as condensation,

swelling, loss of cytoplasmic detail, and fragmentation in the cytoplasm (Zeiss, 2003). The condensed chromatin appears as crescents along the periphery of the nuclear membrane or as spherical bodies within the nucleus. The cytoplasmic condensation induces shrinking of the cell (Hussein, 2005). Subsequently, the nuclear and plasma membranes become convoluted, and small masses of condensed chromatin undergo fragmentation along with condensed cytoplasm to form "apoptotic bodies". The apoptotic bodies are bound to the plasma membrane, and often contain functional mitochondria and other organelles. The phosphatidyl serine residues that are normally localized to the inner membrane are relocated to the outside of the cell membrane prior to fragmentation. These residues of phosphatidyl serine on the apoptotic bodies serve as a signal to the neighboring healthy cells to perform phagocytosis and remove the cellular debris (Bhatia, 2004). There is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and (3) the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003).

Necrosis

Generally, necrosis is initiated by non-cellular mechanisms, such as ischemia, ATP depletion (Bhatia, 2004), and traumatic insults, which lead to irreversible cellular damage (McCully et al., 2004). In addition to passive mechanisms, studies have suggested that "active" mechanisms, such as Na^{+} overloading, Ca^{2+} accumulation, and changes in mitochondria permeability, can also participate in the necrotic process (Barros et al., 2001b; Padanilam, 2003).

The biochemical pathway that leads to necrotic cell death is not well known. In ischemic or hypoxic injury, energy depletion occurs by defective ATP production associated with the rapid consumption of ATP by Na^{+}-K^{+} pumps and through hydrolysis as well as ATP loss. The necrotic volume increase associated with necrotic cell death is initiated by an influx of Na^{+} and release of ATP due to membrane leakage (Padanilam, 2003). The increased Na^{+} levels in the cytosol activate the Na^{+}-K^{+}-ATPase pump, resulting in dissipation of ATP. In the early stages of the injury, a simultaneous efflux of K^{+} maintains ion homeostasis. Severe

depletion of ATP leads to failure in the pump-leak balance mechanism, leading to an influx of Na^+ and water that results in swelling and collapse of cell.

In addition, the reactive oxygen species have been suggested to mediate necrotic volume increase, and Na^+ influx is initiated by the binding of free radicals to ion channels, including nonselective Ca^{2+} channels (Barros et al., 2001b). The increased levels of Na^+ activate the Na^+-K^+-ATPase pump and consume ATP, activating the nonselective Ca^{2+} channels that result in massive cytosolic Ca^{2+} accumulation. High levels of Ca^{2+} can participate in ATP depletion by activating the Ca^{2+} ATPase pump and mitochondrial depolarization. The increased levels of Ca^{2+} activate endonucleases to degrade DNA and cellular proteases to degrade several structural and signalizing proteins (Wang, 2000). Figure 3 illustrates the biochemical events occurring in passive necrosis.

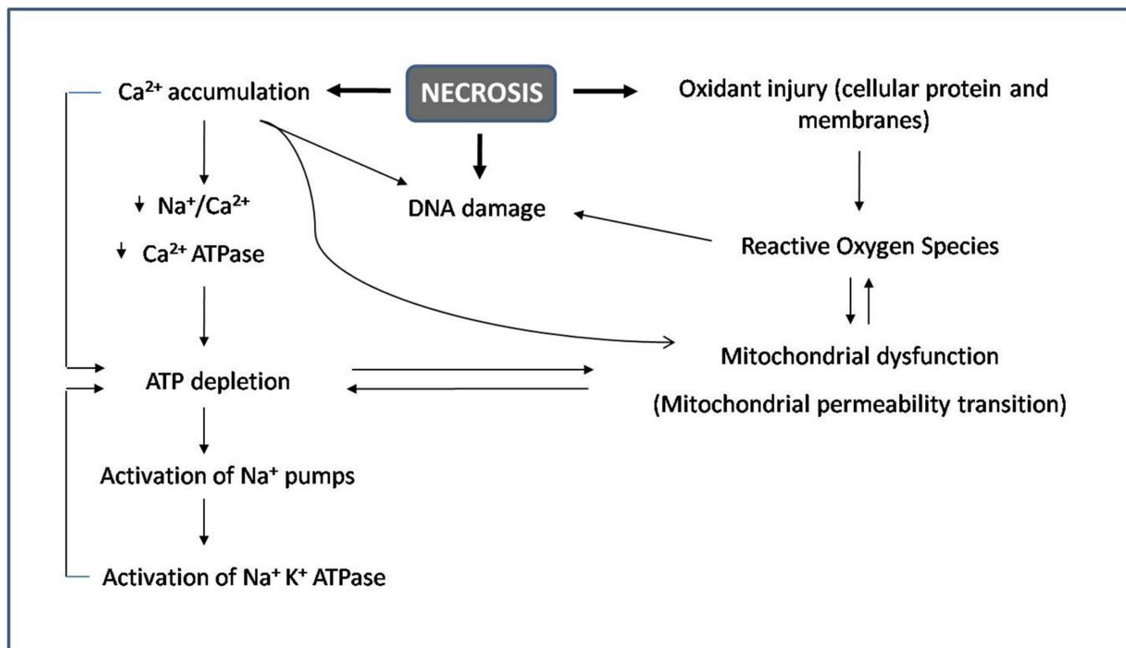

Figure 3. Cell death by necrosis caused by a sequence of bioquimical events. Adapted from: Bhatia, 2004.

Morphological alterations in necrotic cells

The morphology of a necrotic cell is very distinct from that of a cell undergoing classic apoptosis, with ultrastructural changes occurring in both the cytoplasm and the nucleus. The main features are chromatin flocculation, swelling and degeneration of the entire

cytoplasm and the mitochondrial matrix, blebbing of the plasma membrane, and eventual shedding of the cytoplasmic content into the extracellular space (Scaffidi et al., 2002). In contrast to apoptosis, the chromatin is not packed into discrete membrane-bound particles, but may form unevenly textured and irregularly shaped clumps. During necrosis, mitochondria undergo inner membrane swelling and disintegration (Barros et al., 2001a). Polyribosomes are dissociated and dispersed throughout the cytoplasm, imparting a dense and granular appearance of the cytoplasmic matrix. Moreover, dilation and fragmentation of the cisterns of the rough endoplasmic reticulum and Golgi apparatus are frequently observed (Berridge et al., 2000). It is also important to note that cytomorphological changes like karyolysis, pyknosis and karyorrhexis can occur in necrosis, which is not exclusive to apoptosis (Cotran et al., 1999).

Although the mechanisms and morphologies of apoptosis and necrosis differ, there is overlap between these two processes. Evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical network described as the "apoptosis-necrosis continuum" (Zeiss, 2003). For example, two factors that will convert an ongoing apoptotic process into a necrotic process include a decrease in the availability of caspases and intracellular ATP (Denecker et al., 2001). Whether a cell dies by necrosis or apoptosis depends in part on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and the physiologic milieu (Zeiss, 2003). Table 1 illustrates the differences between the processes of apoptosis and necrosis.

Techniques used for analysis of follicular atresia

Different techniques have been used to detect follicular atresia caused by either apoptosis or necrosis after cryopreservation and/or *in vitro* culture of ovarian follicles during the different stages of development. However, each technique has advantages and disadvantages which may make it acceptable to use for one application but inappropriate for another (Otsuki et al., 2003).

For apoptosis detection, different techniques may be utilized, such as: 1) morphological analysis (laser confocal microscopy and Transmission Electronic Microscopy – TEM; Staldemann and Lassman, 2000); 2) evaluation of DNA fragmentation (enzyme-linked imumunosorbent assay - ELISA and terminal deoxynucleotidil transferase-mediated deoxyuridine triphosphate biotin nick end-labeling – TUNEL; Linde et al., 2000); 3) analysis

of DNA content (flow cytometry); 4) evaluation of the translocation of phosphatidilserin residues located in the inner mitochondrial membrane (Wiegele et al., 1998); 5) analysis of gene expression and caspases involved with apoptosis (RT-PCR, northern and western blot, and immunohistochemistry; Kiechle and Zhang, 2002). With respect to techniques for cytomorphological alterations, the TEM is considered the gold standard to confirm apoptosis, which better defines the subcellular changes. However, the main disadvantages of TEM are that it is costly, time consuming, and the ability to only assay a small region at a time. The TUNEL has been used as a principal method to identify and quantify the apoptotic cells in atretic follicles during luteal regression (Zhang et al., 2008) because it is very sensitive and fast (it takes about 3 hours). The disadvantages are cost and the unknown parameter of how many DNA strand breaks are necessary for detection by this method. This method is also subject to false positives from necrotic cells and cells in the process of DNA repair and gene transcription (Elmore, 2007). For membrane alterations detection, the externalization of phosphatidyl serine residues on the outer plasma membrane of apoptotic cells allows detection via Annexin V in tissues, embryos or cultured cells (Bossy-Wetzel and Green, 2000), which can be visualized with fluorescent microscopy. The advantages are sensitivity (can detect a single apoptotic cell) and the ability to confirm the activity of initiator caspases. The disadvantage is that the membranes of necrotic cells are labeled as well (Elmore, 2007). Another method for detecting apoptosis is through the detection of some factors such as the caspase activation (Gurtu et al., 1997). The major disadvantage is that the integrity of the sample is destroyed thereby eliminating the possibility of localizing the apoptotic event within the tissue or determining the type of cell that is undergoing apoptosis. Another disadvantage is that caspase activation does not necessarily indicate that apoptosis will occur (Elmore, 2007). Among these techniques, the most used for apoptosis detection is TUNEL (evaluation of nuclear alterations, such as DNA fragmentation) and immunohistochemistry.

For evaluation of cell death by necrosis, several techniques are used, such as classical histology (staining with hematoxilin-eosin or Periodic Schiff Acid-hematoxilin), laser confocal microscopy, TEM (Martinez-Madrid et al., 2007), that allow for visualization of the presence of vacuoles, especially with TEM, which can also analyze organelle damage and the integrity of basal and nuclear membranes. Some vital fluorescent staining can also be used, like propidium iodide and Lucifer Yellow, which penetrate damaged membranes of necrotic follicles (Thomas et al., 2001; Choi et al., 2007).

Atresia during the different stages of follicular development

Ovarian follicles of all growth stages undergo atresia due to either apoptosis or necrosis (Chen et al., 2005; Valdez et al., 2005). According to the follicular stage, there is different susceptibility of follicular compartments to atresia. In preantral follicles, the atresia is most commonly observed in the oocyte. However, during the advanced stages of development (e.g., in antral follicles), atresia occurs both in the oocyte and the granulosa cells (Silva et al., 2002).

The balance of several substances may influence the decision of cell death during the different follicular stages, such as endocrine factors (Follicle Stimulating Hormone - FSH and Luteinizing Hormone - LH) and paracrine factors (Kit Ligand - KL, Insulin-Like Growth Factor-1 - IGF-1, Epidermal Growth Factor - EGF, Fibroblast Growth Factor-2 - FGF-2, Vascular Endothelial Growth Factor - VEGF, and activin). Additionally, there are atretogenic factors, which include TNF-α, androgens, IL-6, and free radicals (Markström et al., 2002). Furthermore, the importance of several pro- and anti-apoptotic genes, such as p53, Bcl-2, Bax, Fas, FasL and survivin, and their roles in follicular atresia have been shown in some reports (Fujino et al., 2008; Pru et al., 2009). Thus, we will focus on investigations in the following sections, which report the process of atresia after *in vitro* culture or cryopreservation of ovarian follicles from different stages of development.

Atresia in preantral follicles

Several human studies have demonstrated that apoptosis occurs in ovaries even before birth, and has been identified in 13 to 32 week old fetuses during pregnancy (Markström et al., 2002; Albamonte et al., 2008). The vast majority of oogonia and oocytes are lost during embryonic, neonatal and adult life through apoptosis (Kim and Tilly, 2004) and are not destined to produce mature oocytes for fertilization. Arguments that this is a selection mechanism designed to remove abnormal oocytes from the follicle pool are cogent but observations of single cell atresia in oocyte nests during primordial follicle formation (Pepling and Spradling, 2001) indicates that other factors such as somatic cell support may regulate this process.

Studies performed with animal ovarian models have suggested that the viability of primordial (quiescent) and primary (initial growth) follicles is determined by survival factors

derived from the oocyte. During these stages of development, cell death results specially from an insufficient availability of growth factors, such as KL, EGF, IGF-1, leukemia inhibitor factor (LIF), or growth and differentiation factor-9 (GDF-9). In primordial follicles, oocyte apoptosis is probably responsible for further follicular degeneration. This oocyte apoptosis was demonstrated by Reynaud and Driancourt (2000) in rodents, which described the importance of KL and the interaction of KL with other factors, such as EGF and FGF-2 in preventing apoptosis. Another study demonstrated that the interaction between KL and the KL receptor, c-kit, is important to prevent follicular degeneration and the rescue of the follicles, thus avoiding oocyte death (Driancourt et al., 2000). *In vitro* studies have demonstrated that KL inhibits apoptosis in oocytes of mouse primordial follicles by increasing the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-cL, and reducing the expression of the pro-apoptotic factor Bax (Jin et al., 2005). Other studies have demonstrated that the oocytes had no visible signs of degeneration after addition of 50 or 100 ng/ml of KL even after 20 days of culture (Klinger and De Felici, 2002).

The EGF is also known as an *in vivo* and *in vitro* survival factor (Markström et al., 2002). Some authors showed that low concentrations of EGF promote an inhibition of granulosa cells apoptosis or the reduction of follicular atresia levels in swine, bovine and caprine (Gutierrez et al., 2000; Mao et al., 2004, Zhou and Zhang, 2005). Furthermore, EGF was tested at different concentrations (1 or 10 ng/ml) for *in vitro* culture of caprine preantral follicles, leading to increased follicular survival after 7 days of culture which was demonstrated by the maintenance of follicular ultrastructure (Celestino et al., 2009). With respect to IGF-1, Mao et al. (2004) observed that the percentage of apoptotic granulosa cells in swine preantral follicles treated with 10 and 100 ng/ml of IGF-1 was lower than those treated with 0 and 1 ng/ml of IGF-1. Moreover, addition of EGF, IGF-1, or EGF + IGF-1 inhibited apoptosis in granulosa cells and stimulated proliferation of these cells to antrum formation after 8 days of culture. Furthermore, FGF-2 can also influence follicular atresia through the inhibition of apoptosis after culture of rat preantral follicles (McGee et al., 1999). Another factor derived from the oocyte that is important for small follicle survival is GDF-9. In mice, lack of this factor prevents the development of the follicles from the primary to early secondary stages, causing follicular atresia (Dong et al., 1996). In an *in vitro* study which tested different concentrations of GDF-9 in the culture of caprine preantral follicles, the concentration of 200 ng/ml maintained follicular survival after 7 days, without signs of atresia after analyzing ultrastructural integrity by TEM (Martins et al., 2008).

Matos et al. (2007a, b) verified that ultrastructural integrity of caprine preantral follicles was maintained after using FSH or the association between FSH and FGF-2 in the culture medium (Fig. 4A). Nevertheless, follicles cultured only in control medium (without addition of any hormone or growth factor) exhibited a high rate of degeneration by necrosis, which was characterized by a large vacuolization in the cytoplasm (Fig. 4B). However, another study showed that in the early stage of development, FSH and its mediator cAMP did not have any effect on apoptosis of the rat isolated preantral follicles cultured *in vitro* (McGee et al., 1997). In the same way, the Anti-Müllerian Hormone (AMH) stimulated follicular growth but did not inhibit apoptosis (McGee et al., 2001). Nevertheless, Visser et al. (2007) recently demonstrated that AMH had an important role in follicular growth and death, serving as a survival factor for small follicles.

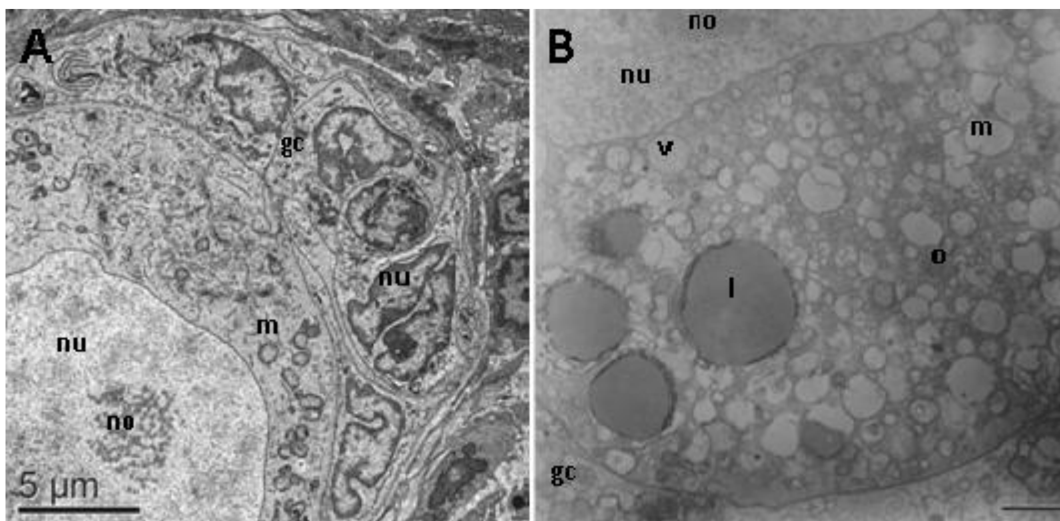

Figure 4. Electron micrograph of normal (A; 6000×; scale bar: 5 µm) and degenerated follicle (B; 7000×; scale bar: 2µm) after culture of caprine ovarian tissue in medium containing FSH + FGF-2 and control medium (Minimal Essential Medium), respectively. In Figure 4A, note the homogeneous cytoplasm with numerous rounded mitochondria and the basement membrane integrity. In Figure 4B, note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration. gc, granulosa cell; l, lipid droplet; m, mitochondria; no, nucleolus; nu, nucleus; o, oocyte; v, vesicles. (Reproduced with permission from Matos et al., 2007a).

The susceptibility to atresia, besides the relation to the stage of follicular development, depends on the conditions of *in vitro* culture, thus varying the pathway of cellular death, i.e., apoptosis or necrosis. After using the TUNEL technique to detect apoptosis in preantral

follicles cultured *in vitro*, Silva et al. (2006) demonstrated that addition of 100 ng/mL of activin to the medium significantly reduced the number of atretic follicles enclosed in ovarian tissue, but not for isolated follicles. Among the atretic follicles cultured in the ovarian cortex, less than 30% exhibited DNA fragmentation (Fig. 5), while this phenomenon occurred especially in granulosa cells of isolated preantral follicles. The authors suggested that this incidence could occur most likely due to a reduced access to oxygen and nutrient in follicles cultured in ovarian cortex, thus favoring the occurrence of necrosis as a pathway of degeneration. Contrarily, isolated follicles which have better access to nutrients and oxygen died via an apoptotic pathway.

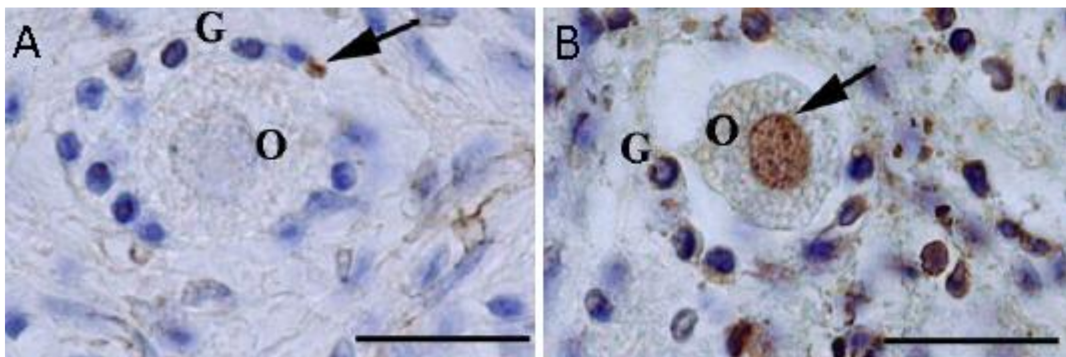

Figure 5. DNA fragmentation detected using TUNEL technique in caprine ovarian follicles cultured *in vitro* for 5 days in the presence of activin. Arrows represent DNA fragmentation in granulosa cell (A) and oocyte (B). O, oocyte, G, granulosa cells. Bars: 25 µm. (Reproduced with permission from Silva et al., 2006).

In addition to *in vitro* culture, cryopreservation studies have also been performed with ovarian follicles to observe the percentage of atresia and the most predominant pathway of cell death. Martinez-Madrid et al. (2007) showed that after cryopreservation of human ovaries with their vascular pedicle, no primordial or primary follicle was found to be positive for TUNEL or active caspase-3. Nevertheless, TEM revealed that some follicles exhibited morphological alterations that were suggestive of necrosis, such as oocyte nuclear membrane rupture and mitochondrial swelling. In another study, a high apoptosis rate was observed after cryopreservation of mouse ovaries followed by graft, with loss of approximately half of the number of primordial follicles present in the graft tissue (Liu et al., 2002). Apoptosis also may be involved in follicular damage during freezing and thawing of the ovary (Rimon et al., 2005). In addition, Tirelli et al. (2005) observed a significant increase in apoptosis of sow granulosa cells that were frozen slowly. They suggested the increase in apoptosis was

probably caused by physical alterations due to low temperature, high salt concentration and impairment of antioxidant metabolism. Similar results were reported by Rimon et al. (2005), who demonstrated a higher incidence of apoptosis in frozen–thawed human ovarian tissue compared with fresh ovarian tissue. In contrast, other investigations and previous report have shown that the incidence of apoptosis in cryopreserved tissue was not significantly different from fresh controls (Hussein et al., 2006; Mazoochi et al., 2008).

Choi et al. (2007) demonstrated a significantly higher proportion of apoptosis and necrosis in cryopreserved ovaries than in fresh ones after 1 and 5 days of culture, respectively. Most likely, the absence of apoptosis after 5 days of culture was due to the phagocytosis of apoptotic cells by healthy neighboring cells. Nevertheless, the cryopreservation procedure may lead to a death process by cooling through degeneration (necrosis), and not by an apoptotic pathway with caspase activation. Haidari et al. (2008) showed no significant differences between the survival rates or the ultrastructural changes of vitrified and non-vitrified, cultured, isolated follicles. In a more recent study in which apoptosis was assessed in the preantral follicles of vitrified mice and then cultured for 10 days, no signs of apoptosis were found by morphological and ultrastructural studies. However, real-time RT-PCR demonstrated that the vitrification affected the expression of some genes related to apoptosis (Mazoochi et al., 2009).

Atresia in antral follicles

In antral follicles, atresia in granulosa cells close to the antrum is an important sign of cell death (Amsterdam et al., 2003). In addition, the follicular selection depends largely on granulosa cell apoptosis (Rung et al., 2006). It has been established that granulosa cell death during follicular atresia and luteolysis results from apoptosis (Chen et al., 2005).

Among the factors locally produced that are important to the survival of rat, early antral follicles include IGF-1, EGF, FGF-2, VEGF, activin, and cytokine IL-1β. However, some locally produced factors are the most potent survival factors in the latest stages of follicular development. For instance, the IL-1β is more efficient in preventing apoptosis in preovulatory follicles than in early antral stages. In addition, early antral follicles express FSH receptors and are dependent on this stimulus for survival. Due to a lack of FSH support, several follicles never progress beyond this stage of development (Hirshfield, 1991). On the contrary, the stimulation of LH receptors has a limited effect on the survival of rat follicles in

this stage (Chun et al., 1996). Studies with rats, sheep, cows (Palumbo and Yeh, 1994; Yang and Rajamahendran, 2000) and goats (Yu et al., 2003) demonstrated that FSH alone or synergistically with other factors, such as IGF-1 and VEGF, reduced the rates of apoptotis in granulosa cells cultured *in vitro* (Kosaka et al., 2007). In rats, both gonadotropins FSH and LH inhibited the apoptosis level in isolated preovulatory follicles, as this apoptosis suppression is mediated by endogenous IGF-1 (Chun et al., 1994). Moreover, GH inhibited apoptosis in preovulatory follicles through the stimulation of endogenous IGF-1 production (Eisenhauer et al., 1995).

In addition to growth factors and hormones, the involvement of determinant genes in follicular atresia has been reported, especially in those follicles subordinate in a specific follicular wave. For example, death of subordinate and nonovulatory dominant follicles is mediated via proapoptotic pathways and granulosa cell survival is mediated in part by protein kinases: A (PKA), B (PKB) and C (PKC), and by another group of serine/threonine kinases: mitogen-activated protein kinases (MAPKs; Johnson, 2003; Ryan et al., 2007). A study performed by Forde et al. (2008) verified important roles of some genes in antral follicle survival, such as EphA4, CCND2, and GADD45. EphA4 is a receptor tyrosine kinase and a class A type receptor. It is a member of the ephrin family and binds both class A and class B ephrins (Frisen et al., 1999). EphA4 has a role to play in cell-cell adhesion via the cadherin family of molecules (Cheng et al., 2002). Another study demonstrated that enhanced expression of the proliferative gene CCND2 and the anti-apoptotic gene GADD45B in granulosa cells may support further growth of the dominant follicle (Mihm et al., 2008). Similarly, the gene product GADD45 may act as a potential survival factor in the growing dominant follicle, as it is involved in DNA damage repair and control of genomic stability, and has also anti-apoptotic properties (Sheikh et al., 2000; De Smaele et al., 2001).

Gonadotropins and growth factors mediate their biological effects through binding to cell surface receptors, which results in enzymatic phosphorylation cascades (signal transduction pathways) that transmit signals from outside the cell to the nucleus. One major survival pathway involves the activation of Akt (protein kinase B), a serine/threonine kinase which is a common mediator of cell survival and proliferation. Activation of the Akt pathway causes general inhibition of pro-apoptotic factors, such as the forkhead transcription factors, Bad and caspase 9, all of which are known to mediate apoptosis (Datta et al., 1999; Cardone, 2000). One of the most characterized pathways of the MAPK group is the extracellular-regulated kinase (Erk), which also regulates cell proliferation, differentiation, and survival,

depending on the cellular context (i.e., the type and duration of stimulus, cell type, and any additional signaling pathways: Zhang and Liu, 2002). Ryan et al. (2007) showed that higher levels of the genes Akt and Erk may confer a developmental advantage on the future dominant follicles by promoting survival at the time when circulating FSH concentrations decline and by regulating key processes, such as follicle growth and estradiol production. Furthermore, both Akt and Erk pathways have been implicated in promoting granulosa cell proliferation and survival.

The incidence of apoptosis in cumulus cells may be a good indicator of oocyte developmental competence (Corn et al., 2005; Yuan et al., 2005) due to the bidirectional communication established between cumulus cells and oocytes through gap junctions (De Loos et al., 1991). It was shown in different cell lines as well as various organs that gap junctions play a negative role in apoptosis (Lin et al., 1998; Krutovskikh et al., 2002). Specifically, the connexin 43 (Cx43) was shown to inversely correlate with apoptosis, acting in fact as a survival factor (Lin et al., 2003). The phenomenon of reduced expression of Cx43 in apoptotic follicles was also observed in the porcine, bovine and avian granulosa cells (Johnson et al., 1999; Krysko et al., 2004; Chang et al., 2005). Cumulus cells play an important role in regulating the maturation of the nucleus and cytoplasm in the oocyte (Tanghe et al., 2002) and in protecting oocytes against oxidative stress-induced apoptosis (Tatemoto et al., 2000). Some authors consider that cumulus oocyte complexes (COCs) with signs of early atresia are more developmentally competent (Bilodeau-Goeseels and Panich, 2002) because of the similarity between structural changes during oocyte degeneration and those occurring in the oocyte of the dominant follicle prior to the LH surge (Hyttel et al., 1997). Others authors, however, have reported that COCs with no signs of atresia yield higher blastocyst rates (Corn et al., 2005; Yuan et al., 2005). In prepubertal goats, apoptosis in cumulus cells was negatively related to the morphology of COCs (Anguita et al., 2009).

Final considerations

This review evidenced the complexity of the mechanisms regulating atresia in ovarian follicles during different stages of follicular development. Clearly, regulation of follicular atresia involves some mechanisms of extra and intra-ovarian control, which are dependent on each phase of follicular development. Elucidation of these mechanisms can contribute to a better comprehension of the processes involved in the ovarian folliculogenesis and,

consequently, provide large number of viable and mature oocytes, which can be destined for different *in vitro* techniques of reproduction. In addition to these applications in reproduction, a better knowledge regarding the mechanism of cell death can contribute to therapies for neoplasic and degenerative diseases.

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7 CAPÍTULO 2

Regulação da foliculogênese ovariana pelo sistema Kit Ligand e c-Kit em mamíferos

Regulation of ovarian folliculogenesis by Kit Ligand and the c-Kit system in mammals

Periódico: *Animal Reproduction* 6: 431-439, 2009.

Resumo

O sistema composto por Kit Ligand (KL) e seu receptor c-Kit tem mostrado desempenhar um papel na reprodução normal de fêmeas e na fertilidade de mamíferos. Estudos de expressão de genes têm revelado que atividades biológicas dos ligantes e receptores do sistema KL/c-Kit são importantes no controle da apoptose e proliferação celular nos tecidos reprodutivos. Coletivamente, esses estudos têm fornecido uma melhor compreensão da fisiologia ovariana e fertilidade das fêmeas através do estabelecimento do conceito de que o sistema KL/c-Kit regula a viabilidade das células germinativas primordiais e dos folículos, o início do crescimento do folículo primordial, e posterior desenvolvimento oocitário e folicular através de diferentes proteínas sinalizadoras. O objetivo desse artigo é revisar a importância do sistema KL/c-Kit no desenvolvimento folicular ovariano, especialmente na fase pré-antral da foliculogênese.

Palavras-chave: Sistema c-Kit. Folículo. Kit Ligand. Ovário. Vias de sinalização.

Regulation of ovarian folliculogenesis by Kit Ligand and the c-Kit system in mammals

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Abstract

The system comprised of Kit Ligand (KL) and its receptor c-Kit has proven to play a role in normal female reproduction and fertility in mammals. Gene expression studies have revealed that biological activities of ligands and receptors of the KL/c-Kit system are important in controlling apoptosis and cellular proliferation in reproductive tissues. Collectively, these studies have provided a better understanding of ovarian physiology and female fertility through the establishment of the concept that the KL/c-Kit system regulates the viability of primordial germ cells and follicles, initiation of primordial follicle growth, and further oocyte and follicular development through different signaling proteins. The purpose of this article is to review the importance of the KL/c-Kit system in ovarian follicular development, especially in the preantral phase of folliculogenesis.

Keywords: c-Kit system, follicle, Kit Ligand, ovary, signaling pathways.

Introduction

During the last decade, the role of growth factors in ovarian folliculogenesis has been extensively studied in several species, including rodents, domestic animals, and humans. In particular, Kit Ligand (KL), which was one of the first growth factors identified in the ovarian follicle, plays a key role in mammalian oogenesis and folliculogenesis (Thomas and Vanderhyden, 2006). Since its identification in 1990, *in vivo* and *in vitro* studies have shown that the functions of this system in the ovary include the establishment of primordial germ cells (PGCs), activation of primordial follicles, oocyte survival and growth, proliferation of granulosa cells, recruitment of theca cells, and maintenance of meiotic competence (Hutt *et al*., 2006b; Thomas *et al*., 2008), as shown in Fig. 1. This review will focus on the role of the

KL/c-Kit system in ovarian follicle development, especially in the preantral phase of folliculogenesis.

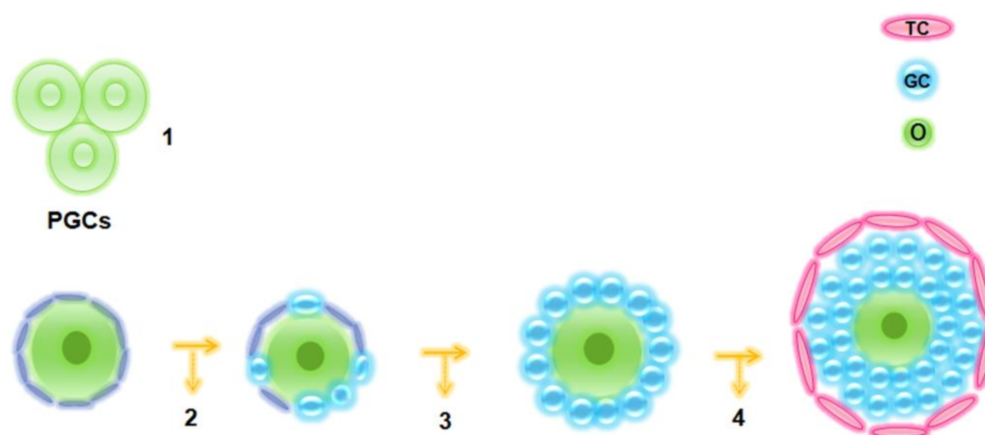

Figure 1. Several functions of this KL/c-Kit system in the ovary: 1) Establishment of primordial germ cells; 2) Activation of primordial follicles; 3) Oocyte survival and growth; 4) Proliferation of granulosa cells and recruitment of theca cells. PGCs: primordial germ cells; TC: theca cells; GC: granulosa cells; O: oocyte.

Expression of Kit Ligand and c-Kit in the ovary

Kit Ligand (KL), also known as Stem Cell Factor (SCF), Steel Factor (SF), and Mast Cell Growth Factor (MCGF), is a locally produced factor that has many roles in ovarian function from embryogenesis onward (Yoshida *et al.*, 1997; Driancourt *et al.*, 2000). In follicles, the expression of the mRNA for KL has been demonstrated in the granulosa cells of several species (rat: Ismail *et al.*, 1996; ovine: Tisdall *et al.*, 1999; mouse: Doneda *et al.*, 2002; human: Hoyer *et al.*, 2005 and caprine: Silva *et al.*, 2006). Depending on how the mRNA is spliced, KL can be expressed as a soluble protein (KL-1) or as membrane-associated protein (KL-2; Huang *et al.*, 1992). When translated, both transcripts yield membrane-associated products; however, KL-1 is efficiently cleaved and released as a soluble product due to a proteolytic cleavage site encoded by an 84-base pair exon. The other form, KL-2, lacks this cleavage site and therefore remains membrane-bound (Huang *et al.*, 1992). KL-2 is the main isoform required for the growth and survival of oocytes (Thomas *et al.*, 2008). Both isoforms are present in rodent (Ismail *et al.*, 1997) and goat (Silva *et al.*, 2006) ovaries. In goats, KL protein and mRNA are expressed in granulosa cells during all stages of

follicular development as well as in corpus luteum, epithelium surface, and medullar tissue (Silva *et al*., 2006). KL affects target cells through binding to its receptor c-Kit, a member of the tyrosine kinase receptor family. During postnatal ovarian development, c-Kit mRNA and protein are found in oocytes of all stages of follicular development. In addition, c-Kit is expressed in interstitial and thecal cells of antral follicles (rodent: Motro and Bernstein, 1993; ovine: Clark *et al*., 1996; caprine: Silva *et al*., 2006). Fig. 2 illustrates action and expression of KL and its receptor c-Kit.

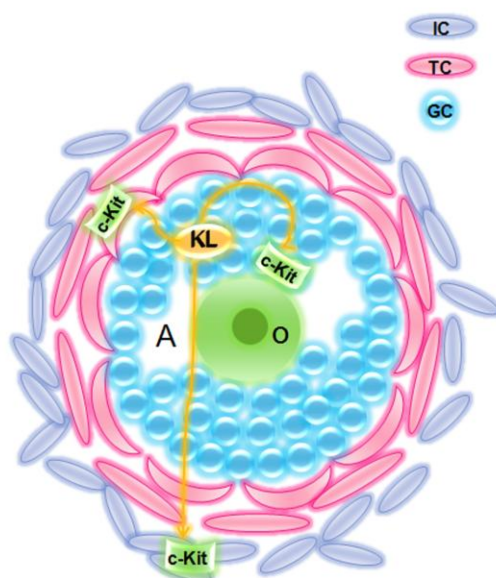

Figure 2. Autocrine action of Kit Ligand, expression in the granulosa cells and its role on the oocytes, interstitial and theca cells after binding to its receptor c-Kit, the tyrosine-kinase type. KL: Kit Ligand; c-Kit: receptor of Kit Ligand; A: antrum; O: oocyte; IC: interstitial cells; TC: theca cells; GC: granulosa cells.

Signaling pathways of the KL/c-Kit system in the regulation of oogenesis and folliculogenesis

The interaction between KL and its receptor is important for the development and differentiation of ovarian follicles in different species (Carlsson *et al*., 2006). KL produced by the granulosa cells in the oocyte acts by binding to c-Kit and may activate different signaling pathways. Currently, several studies have helped elucidate the pattern of signaling of KL/c-Kit via Phosphoinositide 3-Kinase (PI3K)-Akt-FKHRL1 and PTEN. The PI3K pathway is a fundamental signaling pathway for the regulation of cell proliferation, survival, migration, and metabolism, and it still plays an important role in regulating the activation of primordial

follicles (Cantley, 2002). Primordial follicle activation (i.e., the transition from primordial to primary follicles) is a process that occurs very slowly *in vivo* (Fortune, 2003) and is influenced by positive and negative feedback loops. These loops are likely mediated by factors (John *et al*., 2008) that have not yet been definitively identified (Skinner, 2005). Some potential candidates include growth and differentiation factor-9 (GDF-9; Gilchrist *et al*., 2004) and bone morphogenetic protein-15 (BMP-15; Otsuka *et al*., 2000).

Studies by Reddy *et al*. (2005) using postnatal mouse and rat ovaries revealed that the oocyte PI3K pathway is regulated by KL from granulosa cells, and this pathway is of great importance for early follicular development. These studies suggested that actions of KL on the primordial to primary follicle transition and subsequent follicle development may involve phosphorylation of the serine/threonine kinase Akt and the transcription factor FKHRL1, actions that most likely trigger Akt and inhibit FKHRL1 activities in oocytes. Akt is a signaling molecule that enhances cellular proliferation, survival, and glycogen and protein synthesis (Blume-Jensen and Hunter, 2001). FKHRL1(Foxo3a) is a member of the FOXO subfamily of forkhead transcription factors, which consists of Foxo3a, Foxo1 (FKHR), and Foxo4 (AFX); all of these are downstream effectors of the PTEN/PI3K/Akt pathway (Tran *et al*., 2003). Moreover, FKHRL1 is a substrate of Akt as well as a transcriptional factor that leads to apoptosis and cell cycle arrest. Therefore, it is suggested that Akt stimulates oocyte development, whereas FKHRL1 inhibits it.

Recently, John *et al*. (2008) also showed that the PI3K–Akt pathway has a key role in the initiation of oocyte growth (and hence in the maintenance of oocytes) and acts via Foxo3. Oocyte-specific ablation of the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) resulted in Akt hyperactivation, Foxo3 hyperphosphorylation, and Foxo3 nuclear export, culminating in global primordial follicle activation and premature ovarian failure. Surprisingly, oocyte-specific ablation of PTEN and Foxo3 resulted in virtually identical phenotypes of global primordial follicle activation, suggesting that Foxo3 is the primary if not sole effector of PI3K–Akt signaling in this physiologic context. Moreover, genetic evidence from mice lacking PTEN (a major negative regulator of PI3K) in oocytes demonstrates that the entire primordial follicle pool becomes activated. Subsequently, all primordial follicles become depleted in early adulthood, causing premature ovarian failure. This shows that the mammalian oocyte serves as the headquarters of follicle activation programming and that the oocyte PTEN-PI3K pathway governs follicle activation through control of the initiation of oocyte growth (Castrillon *et al*., 2003; Reddy *et al*., 2008). Figure 3

schematically illustrates the signaling pathway of KL/c-Kit via Phosphoinositide 3-Kinase (PI3K)-Akt-FKHRL1 and PTEN.

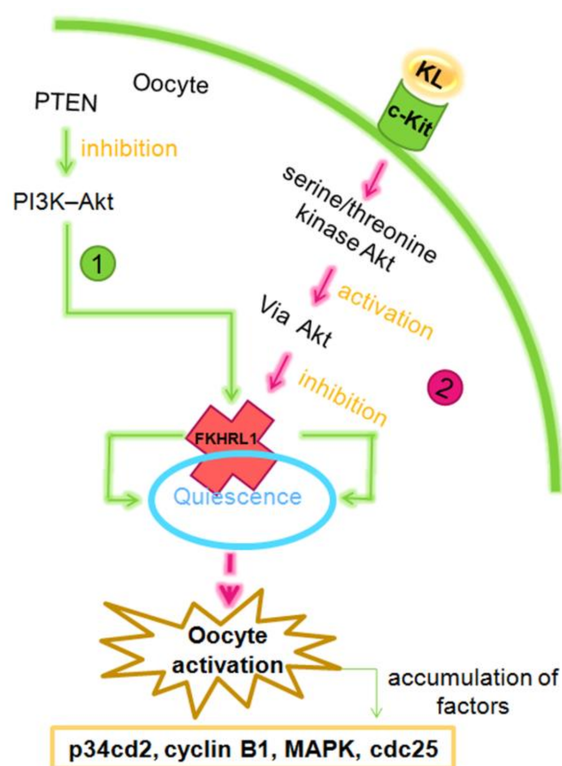

Figure 3. Pattern of signaling of KL/c-Kit system via Phosphoinositide 3-Kinase (PI3K)-Akt-FKHRL1 and PTEN. The oocyte PTEN-PI3K pathway governs follicle activation through control of initiation of oocyte growth, since it inhibits the PI3K-Akt pathway, which then allows the FKHRL1 to keep quiescent oocytes (1). The binding of KL to its receptor c-Kit phosphorylate serine/threonine kinase Akt group and activates Akt pathway, thus inhibiting the activity of FKHRL1 in oocytes allowing its activation (2). It is likely that KL starts oocyte growth, for instance, with the slow accumulation of factors required for meiosis resumption, such as p34cd2, cyclin B1, MAPK, cdc25. KL: Kit Ligand; c-Kit: receptor of Kit Ligand; Akt: signaling molecule; FKHRL1(Foxo3a): member of the FOXO subfamily and of forkhead transcription factors and is a substrate of Akt; PTEN: phosphatase and tensin homolog deleted on chromosome 10.

In addition to the PI3K–Akt pathway, several transcription factors are known to affect the regulation of oocyte-specific genes during early folliculogenesis. FIGLA (Factor in the Germline Alpha; Joshi *et al*., 2007), NOBOX (Newborn Ovary Homeobox gene; Choi *et al*., 2007), and Sohlh1 (Spermatogenesis and oogenesis helix-loop-helix 1; Pangas *et al*., 2006) are all critical to primordial follicle formation and maintenance. FIGLA is an oocyte-specific

basic helix-loop-helix (bHLH) transcription factor that regulates the expression of many genes in the ovary, including zona pellucida genes (Soyal *et al*., 2000). NOBOX is a transcription factor necessary for the expression of several key oocyte-specific genes, including GDF-9. Sohlh1 is another germ cell-specific gene that lies upstream of Lhx8 (LIM-homeobox protein 8), a gene involved in oogenesis. Sohlh1 is preferentially expressed in primordial oocytes (Pangas *et al*., 2006). Manipulations that delete the Sohlh1 gene lead to disturbances in the formation of primary follicles from primordial follicles (Pangas *et al*., 2006); these findings are similar to those observed in tests blocking the c-Kit gene (Yoshida *et al*., 1997). More recently, the novel transcription factor Sohlh2, which is preferentially expressed in germ cells of the embryonic ovary and oocytes of primordial and primary follicles, was discovered (Ballow *et al*., 2006). Both factors (Sohlh1 and Sohlh2) have a crucial role in oogenesis. Choi *et al*. (2008b) demonstrated that Sohlh2-knockout adult female mice are infertile due to a lack of ovarian follicles. Further, Sohlh2-deficient ovaries can form primordial follicles and, despite limited oocyte growth, do not differentiate surrounding granulosa cells into cuboidal and multilayered structures. In addition, Sohlh2 deficiency causes infertility in female mice and affects the expression of numerous oocyte-specific genes (e.g., GDF-9 and c-Kit) in the ovary. Similar results were observed by Toyoda *et al*. (2009) after inhibition of the Sohlh2 gene in mice. These authors concluded that the Sohlh2 gene might be a key gene in the transcriptional cascade responsible for germ-cell differentiation through the acquisition of KIT expression.

Another critical factor for the maintenance and differentiation of oocytes during early oogenesis is Lhx8. It is a member of the LIM-homeobox transcription factor family and is preferentially expressed in germ cells and primordial, primary, and antral follicles within the mouse ovary (Pangas *et al*., 2006). Choi *et al*. (2008a) verified that Lhx8-deficient ($Lhx8^{-/-}$) ovaries are similar to newborn wild-type ovaries. After $Lhx8^{-/-}$ inhibition, a large oocyte loss in mice ovaries that led to female infertility was observed. $Lhx8^{-/-}$ ovaries fail to maintain the primordial follicles, and the transition from primordial to growing follicles does not occur. $Lhx8^{-/-}$ ovaries misexpress oocyte-specific genes, such as GDF-9 and NOBOX. In addition, $Lhx8^{-/-}$ ovaries demonstrated a decrease in the expression of Bax and caspases 2 and 3, without loss of Bcl2 gene expression. A drastic reduction in the KL and c-Kit genes was also observed, and this reduction may explain the loss in oocyte number. On the contrary,

NOBOX$^{-/-}$ mice did not show decreased expression of KL, c-Kit, or apoptosis genes (Rajkovic *et al*., 2004).

The mechanism by which KL causes oocyte growth is unknown. Proteins involved in c-Kit signal transduction via PI3K, such as mitogen-activated protein kinase (MAPK) and Janus-Activated kinase 2 (JAK2), are possible candidates. It has been suggested that high levels of KL constantly activate PI3K signaling in oocytes, increasing their growth (Reddy *et al*., 2005). KL appears to trigger oocyte growth, for example with the slow accumulation of factors (p34cd2, cyclin B1, MAPK, cdc25) required for meiosis resumption (Reddy *et al*., 2005). It is not clear whether these intracellular factors are activated by other cytokines and growth factors to balance the KL signaling for oocyte growth and follicular development under KL-deficient conditions (Moniruzzaman *et al*., 2007). Furthermore, the activation of MAPK is a key event for many cellular processes, including proliferation, differentiation, and apoptosis (Davis, 1993). There are three main classes of MAPK: extacellular-regulated kinases (Erks; Hunter, 1995), c-Jun NH2-terminal protein kinases (JNKs; Derijard *et al*., 1994), and p38-MAPKs (Lee *et al*., 1994; Goedert *et al*., 1997). Erks are mainly activated in response to growth factors and cytokines, whereas JNKs and p38-MAPKs are activated in response to different cell stresses. In a study by Jin *et al*. (2005), it was demonstrated that KL activated only Erk types 1 and 2.

The cascade from granulosa cell KL to oocyte c-Kit PI3K-Akt-FKHRL1 is of great importance, not only to promote oocyte growth but also to promote the secretion of factors that influence granulosa cell proliferation and differentiation (Reddy *et al*., 2005). More recently, the action of glial-derived neurotrophic factor (GDNF) on the transition from primordial to primary follicles has been demonstrated (Dole *et al*., 2008). GDNF signaling occurs via a protein complex. The receptor GDNF and the ligand-receptor complex activate the ubiquitous tyrosine kinase receptor RET (Amoresano *et al*., 2005; Carmillo *et al*., 2005; Pozas and Ibanez, 2005; Vargas-Leal *et al*., 2005). Activation of RET by this complex leads to the activation of intracellular signaling pathways involved in cell proliferation and differentiation (Naughton *et al*., 2006). GDNF is localized to the oocyte cytoplasm in follicles from all developmental stages as well as to cumulus, theca, and granulosa cells in rat antral follicles. Its receptor, α1 (GFRα1), was localized to the oocyte cytoplasm of primordial and primary follicles, and reduced levels were noted in the oocytes of antral follicles, theca cells, and epithelium (Dole *et al*., 2008). In observations from organ cultures, Dole *et al*. (2008) noted that ovaries treated with GDNF for 10 days contained a significant increase in

developing follicles; this finding is similar to that observed with KL treatment, which was previously shown to promote follicle development. Moreover, it was verified that GDNF did not affect KL expression during primordial follicle development.

Effects of the KL/c-Kit system on PGC, oocyte, and follicle survival

It is known that KL is responsible for promoting the survival of different kinds of cells involved in the processes of hematopoiesis, melanogenesis, and gametogenesis, including PGCs, oogonia, and oocytes (Reynaud and Driancourt, 2000; Reynaud *et al*., 2001). *In vivo* studies have demonstrated that a mutation in the genes that encode KL and its receptor c-Kit induced sterility in mice, since PGCs did not survive during early oogenesis (Buehr *et al*., 1993). *In vitro* studies have demonstrated that the activation of the c-Kit receptor negatively regulates apoptosis of PGCs mediated by Fas antigen (a membrane-associated polypeptide that is a member of the tumoral necrosis factor receptor superfamily; Sakata *et al*., 2003). This inhibition occurs through PI3K-Akt signaling. Another study showed that the synergistic action of KL with insulin-like growth factor-1 (IGF-1) and leukemia inhibitory factor (LIF) improved the survival of PGCs of mice during *in vitro* culture (Lyrakou *et al*., 2002).

KL also seems to be involved in oocyte and preantral follicle survival (mice: Reynaud *et al*., 2000; rat: Jin *et al*., 2005). In these studies, *in vitro* culture of preantral follicles in a medium with KL either alone or with an anti-KL or anti-c-Kit antibody was performed. In mice, KL inhibited apoptosis of oocytes from primordial follicles after *in vitro* culture (Jin *et al*., 2005) through an increase in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL and a reduction in the expression of the pro-apoptotic factor Bax. These anti-apoptotic effects seem to be mediated by PI3K signaling (Jin *et al*., 2005). Other authors have demonstrated that inhibition of the KL/c-Kit interaction via an anti/c-Kit antibody promoted oocyte death *in vitro* (mouse: Reynaud *et al*., 2000). When this interaction was inhibited *in vivo*, however, no effect on primordial follicle survival was observed (mouse: Yoshida *et al*., 1997). Moreover, an increase in the number of atretic follicles was verified after blockade of the c-Kit receptor in humans (Carlsson *et al*., 2006). In a mouse model, Doneda *et al*. (2002) showed that the addition of anti-KL or anti-c-Kit antibodies (in the absence of exogenous KL) to the culture medium leads to a significant increase in oocyte apoptosis. Furthermore, *in vitro* culture of fetal ovaries in the absence of KL causes a significant reduction (up to 99%) in the number of mouse oocytes after 72 h (Morita *et al*., 1999). According to Yan *et al*. (2000), KL

production is much lower *in vitro* than *in vivo* due to the absence of FSH. Therefore, higher concentrations of this factor are necessary *in vitro*. In addition, some studies have reported that KL inhibits the expression of BMP-15, increasing the expression of FSH receptors (Thomas *et al*., 2005). This pathway is important for reducing follicular atresia in many species (human: Roy and Treacy, 1993; mouse: Baker and Spears, 1997; caprine: Matos *et al*., 2007).

The KL/c-Kit system regulates primordial follicle activation *in vitro*

To demonstrate the different signaling pathways involved in KL action, many studies have shown that KL promotes primordial follicle activation *in vitro*. Yoshida *et al*. (1997) demonstrated that blockade of c-Kit using a monoclonal antibody affects primordial follicle development. In addition, after *in vitro* culture of rat preantral follicles, Nilsson and Skinner (2004) have shown that KL (50 ng/ml) significantly increased the proportion of developing follicles in comparison to the control. This development did not occur after the addition of an anti-basic fibroblast growth factor antibody, which means that this antibody interferes with the ability of KL to promote follicular activation and development. An *in vitro* study showed that KL (100 ng/ml) increased the transition from rat primordial to primary follicles after 5 or 14 days of culture (Parrot and Skinner, 1999). According to these authors, low levels of KL may not be sufficient to promote follicular development. Therefore, it is necessary to use higher concentrations of this factor (Parrot and Skinner, 1997). Moreover, during culture of human preantral follicles, KL at 1, 10, or 100 ng/ml did not show any effect on the early stages of follicular development after 7 or 14 days (Carlsson *et al*., 2006). In a mouse model, however, 10 ng/ml of KL promoted the development of primordial follicles to the primary stage during *in vitro* culture for 9 days (Wang and Roy, 2004). Furthermore, after 8 days of culture, KL (50 or 150 ng/ml) led to the activation of mouse rather than rabbit primordial follicles (Hutt *et al*., 2006a). In another study, follicular activation was observed when higher concentrations of KL (i.e., 100, 200, or 400 ng/ml) were used; in contrast, the lowest concentration (25 ng/ml) did not cause activation (Fernandez *et al*., 2008). According to the literature, there are many conflicting results. In spite of differences in culturing conditions (e.g., the culture medium used), these controversial results may suggest the existence of differences between species (Carlsson *et al*., 2006).

In vitro effects of the KL/c-Kit system on oocyte and follicle growth

The expression of both KL and c-Kit is consistent with the role of this system in early oocyte growth (Manova *et al.*, 1993), which was already demonstrated *in vitro* (Packer *et al.*, 1994; Klinger and De Felici, 2002). It is suggested that KL synthesized by granulosa cells binds to c-Kit present in the oocyte, thereby promoting oocyte growth. Klinger and De Felici (2002) working with mouse oocytes reported a two-fold increase in oocyte diameter compared to controls after 4 days of culture in the presence of 50 or 100 ng/ml of KL. These same authors reported that mouse oocyte growth is characterized by three distinct phases: the initial stage of growth can be promoted by KL and does not require gap junctions; followed by a growth phase which depends on KL and gap junctions; and finally, growth phase is dependent only on gap junctions and is independent of KL (Klinger and De Felici, 2002). Moreover, the use of 50 ng/ml of KL stimulated theca cell growth, as estimated by DNA synthesis and the increase in androstenedione production in the absence of gonadotropins (Parrot and Skinner, 1997). Furthermore, oocytes of prepubertal animals seemed to show the ability to increase mRNA for KL in granulosa cells (Packer *et al.*, 1994); this finding was confirmed by Joyce *et al.* (1999). Therefore, it is suggested that the oocyte increases the expression of KL in the surrounding granulosa cells and this increase in KL stimulates oocyte growth (Driancourt *et al.*, 2000). Nevertheless, Cecconi and Colonna (1996) did not observe any effect of KL on the growth of 12-day-old mouse oocytes, suggesting that KL may have different actions in each stage of oocyte development. In addition, after culture of whole mouse ovaries for 9 days, KL (100 ng/ml) did not promote oocyte growth (Wang and Roy, 2004). These differences in results within the same species may be attributed to the stage of oocyte development, different experimental techniques used for the evaluation of oocyte growth, or differences in culture conditions cited previously.

Finally, the complex interaction between oocyte and granulosa cells is influenced by KL/c-Kit, hormones, and growth factors. Some studies suggested that low concentrations of FSH are necessary for the appropriate regulation of the paracrine factors that trigger oocyte development. In this way, the correct concentration of FSH may be important for the proper modulation of KL and BMP-15, increasing oocyte growth (Thomas *et al.*, 2005). BMP-15 produced by the oocyte increases KL expression by granulosa cells. Therefore, KL acts through c-Kit in the oocyte membrane to promote growth and negatively regulate BMP-15 expression, which produces a consequent increase in FSH receptors (Hutt *et al.*, 2006b;

Thomas and Vanderhyden, 2006), as shown in Fig. 4. In humans, Carlsson *et al*. (2006) have demonstrated that different concentrations of KL (1, 10, or 100 ng/ml) did not increase follicular diameter compared to controls after 7 or 14 days of culture. Nevertheless, after 8 days of mouse and rabbit preantral follicle culture, the addition of 50 or 150 ng/ml of KL with FSH caused a significant increase in oocyte diameter relative to controls (Hutt *et al*., 2006a).

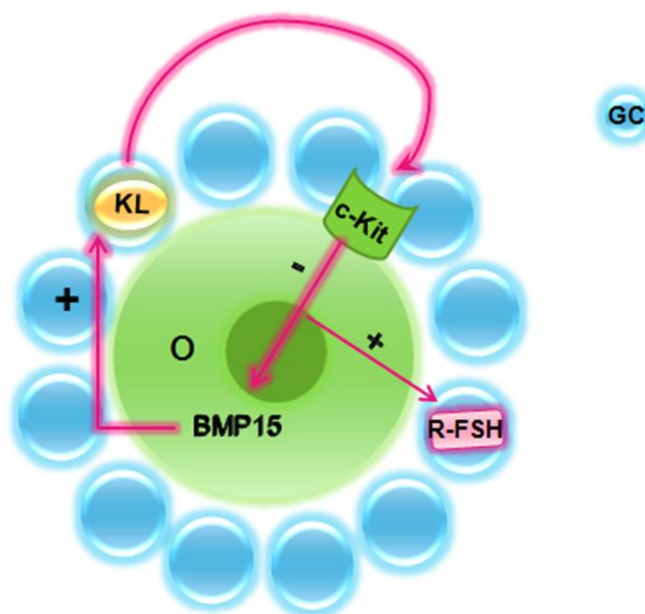

Figure 4. Kit Ligand/Bone Morphogenetic Protein negative feedback loop. BMP-15 produced by the oocyte increases KL expression by granulosa cells. Therefore, KL acts through c-Kit in the oocyte membrane to promote growth and negatively regulate BMP-15 expression, which produces a consequent increase in FSH receptors. KL: Kit Ligand; c-Kit: receptor of Kit Ligand; BMP-15: Bone Morphogenetic Protein-15; R-FSH: FSH receptor; O: oocyte; GC: granulosa cells. Adapted *et al.* (2006b).

Collectively, these results underline the crucial role of the KL/c-Kit system in the control of mammalian reproduction, especially oogenesis and folliculogenesis. The KL/c-Kit system, through different signaling proteins, regulates PGCs and follicular viability, the initiation of primordial follicle growth, and oocyte and follicle development. Further research in this field will greatly advance our understanding of ovarian physiology, which may help clinicians and reproductive biologists to find a role for the KL/c-Kit system in the diagnosis and treatment of reproductive disorders affecting human and animal fertility.

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8 CAPÍTULO 3

Implicações da proteína morfogenética óssea-15 na foliculogênese ovariana

Implications of bone morphogenetic protein-15 on the ovarian folliculogenesis

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Resumo

A proteína morfogenética óssea-15 (BMP-15) tem demonstrando importantes efeitos na reprodução e fertilidade de fêmeas mamíferas das mais diversas espécies. Estudos *in vivo* utilizando essa proteína têm contribuído para uma melhor compreensão da fisiologia ovariana, demonstrando que deleções no gene da BMP-15 são capazes de causar infertilidade, com parada no desenvolvimento folicular inicial. Além disso, estudos *in vitro* têm comprovado o papel relevante da BMP-15 no ovário mamífero, destacando sua atuação na proliferação de células da granulosa e no estímulo à expressão de alguns fatores de crescimento locais, os quais são necessários para um adequado crescimento folicular e oocitário. O objetivo desse artigo é revisar a importância da BMP-15 no desenvolvimento de folículos ovarianos, especialmente nos processos de sobrevivência, ativação e crescimento de folículos pré-antrais.

Palavras-chave: BMP-15. Folículo pré-antral. Fertilidade. Ovário.

Implications of bone morphogenetic protein-15 on the ovarian folliculogenesis

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Abstract

Bone morphogenetic protein-15 (BMP-15) has been implicated in important effects on female reproduction and fertility in several mammalian species. *In vivo* studies using this protein have contributed to a better comprehension of ovarian physiology, demonstrating that the deletion of BMP-15 gene is capable to cause infertility, with arrest in the early follicular development. Moreover, *in vitro* studies have shown the relevant role of BMP-15 in the mammalian ovary, highlighting its action in the granulosa cell proliferation and in the stimulation of some local growth factors expression, which are necessary for an adequate follicular and oocyte growth. The purpose of this article is to review the importance of BMP-15 in ovarian follicular development, especially in the processes of survival, activation and growth of preantral follicles.

Keywords: BMP-15, preantral follicle, fertility, ovary

1. Introduction

The bone morphogenetic protein (BMP) family corresponds to the largest group of growth factors members of TGF-β superfamily (Shimasaki et al., 2004a,b). Among the BMPs, the BMP-15 has received great distinction in the last decades, being its role widely studied in different species, including rodents, domestic animals and humans. Such protein, codified by a gene linked to the X chromosome and secreted by the oocyte, has been considered an important regulator of follicular development, acting positively in the maturation of oocytes enclosed in preovulatory follicles, in the determination of fertility and ovulation rate in mammals, in addition to be directly involved in premature luteinization (Galloway et al., 2000; Juengel et al., 2004a; Knight and Glister, 2006). Furthermore, BMP-15 has a significant mitogenic effect on the granulosa cells, contributing to the growth during the different phases of folliculogenesis, including the process of follicular activation (Otsuka et al., 2000; Juengel et al., 2004b).

In this context, the present review will highlight the aspects related to the expression of BMP-15 and its receptors in the ovary, cellular signaling pathways of BMP-15, and the effects of this protein on the survival, activation and follicular growth, as well in the ovulation and steroidogenesis.

2. Expression of BMP-15 and its receptors in the ovary

BMP-15, also known as growth differentiation factor-9B (GDF-9B), is one of the 35 members of the TGF-β superfamily (Knight and Glister, 2006). In this superfamily, the ligand interacts with two categories of signaling receptors, called type I and type II (Massagué and Chen, 2000). For the BMPs, the main type I receptors are BMPR-IA or activin receptor-like kinase-3 (ALK-3) and BMPR-IB or ALK-6, while the type II receptor is called BMPR-II (Ten Dijike et al., 2003; Abir et al., 2008). Specifically, BMP-15 plays its biological effects through the formation of heterodimeric complexes with a type I receptor (ALK-6) and a type II receptor (BMPR-II) on the surface of the cell (Lebrun et al., 1997; Miyazono et al., 1997).

In all studied species, the ovary is the local with the greatest production of BMP-15 (Peng et al., 2009), being this protein also found in the pituitary, *rete testis* and other tissues from some species (Fitzpatrick et al., 1998; Aaltonen et al., 1999; Galloway et al., 2000; Eckery et al., 2002; Otsuka and Shamasaki, 2002a). Regarding the ovary, in some species, such as marsupial (*Brushtail possums* – Eckery et al., 2002), ovine (Mery et al., 2007), bovine (Bodensteiner et al., 1999) and human (Shimasaki et al., 2004a), BMP-15 is expressed in oocytes from primordial follicles onward. In rodents, the expression of this protein is observed only in the oocyte from primary follicles onward (Laitinen et al., 1998; Jaatinen et al., 1999). For caprine species, BMP-15 has been found in the oocytes of all types of follicles and granulosa cells of primary, secondary and antral follicles but not in primordial stage. In this species, the mRNA for BMP-15 and their associated receptors were detected in primordial, primary and secondary follicles, as well as in the oocyte and granulosa cells of antral follicles (Silva et al., 2005). In sheep, the protein and mRNA for BMP-15 receptors were detected both in the granulosa cells and oocyte of preantral follicles (Wilson et al., 2001; Souza et al., 2002), thus suggesting an important role of BMP-15 in the regulation of the function of these two cell types during the follicular development.

3. Signaling pathways of BMP-15 in the regulation of folliculogenesis

Both BMP-15 receptors, ALK-6 (type I) and BMPR-II (type II), have an extracellular binding domain rich in cysteine, a membranary and a cytoplasmic domain of serine/treonine quinase. First, binding of BMP-15 to the extracellular domain of type I receptor leads to the recruitment of the type II receptor, which is constitutively phosphorylated and

transphosphorylates the quinase domain of the type I receptor (Zimmerman and Mathews, 1996; Miyazono et al., 1997). The activated type I receptor phosphorylates one or more cytoplasmic signaling intermediates, known as Smads receptors (R-Smads), specifically for BMP-15 the intracellular pathway Smad 1/5/8 (Moore et al., 2003; Ten Dijike et al., 2003; Shimasaki et al., 2004a). Once activated, these Smads associate with a further Smad, a co-Smad (Smad 4). The activated complex R-Smad-co-Smad is translocated to the nucleus, promoting the transcription of the target genes and determining the action of BMP-15 in different organs, including the ovary (Fig. 1) (Nishimura et al., 1998; Moore and Shimasaki, 2005).

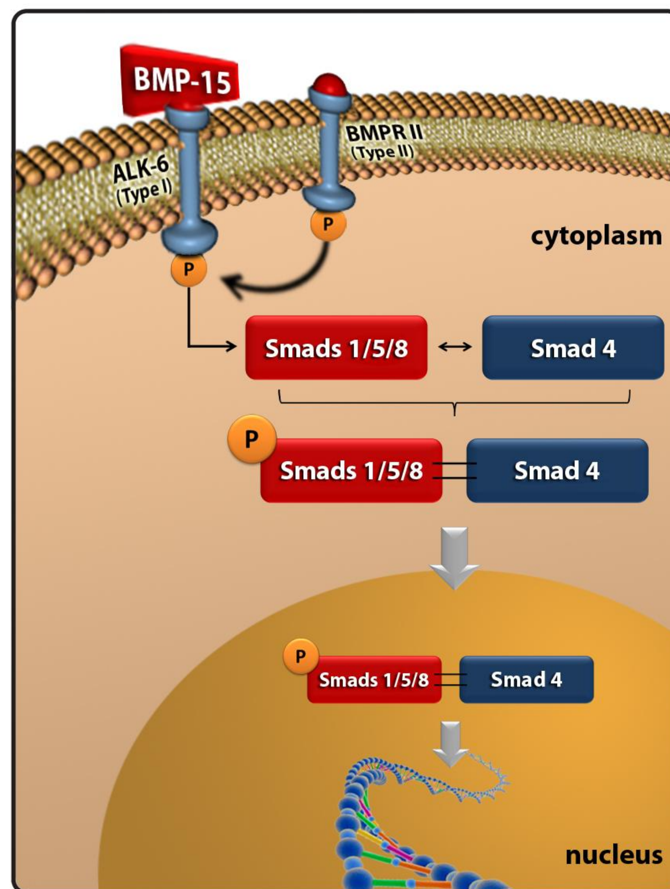

Figure 1. Signaling pathways of bone morphogenetic protein-15 (BMP-15). BMP-15 primarily binds to ALK-6 receptor, to which has more affinity, and thereafter recruits BMPR-II receptor, constitutively phosphorylated. BMPR-II, in turn, transphosphorylates ALK-6 previously bound to BMP-15, inducing the phosphorilation of sinalization proteins, called Smads 1, 5 and 8. Smads 1, 5 and 8 phosphorylated interact with Smad 4 and the complex is translocated to the nucleus, where interacts with transcriptional factors and regulates the expression of target genes, determining the action of BMP-15.

Additional regulation also occurs by inhibitory Smads (Smads 6 and 7), which can attenuate the pathway of signal transduction (Miyazono et al., 1997; Knight and Glister, 2003). In addition to the cell signaling way mediated by Smads, evidences indicate that other ways also may be involved in the BMP-15 and other BMPs action (Von Bubnoff and Cho, 2001; Nohe et al., 2002), for example, the signaling way mediated by the protein-quinase activated by mitogens (MAPK). Studies have demonstrated that MAPK can modulate the transduction of the BMP-15 signals through acting in common sites with the Smad way. In fact, it has been verified that activated MAPK molecules can positively transducer the BMP-15 signals (Su et al., 2002) or act as inhibitors of the Smad signaling (Von Bubnoff and Cho, 2001). According to Moore et al. (2003), the action of BMP-15 on the proliferation and differentiation of the granulosa cell in dependent on the ERK1/2 molecule, which is a member of MAPK family. This emphasizes the existence of an alternative way for the regulation of the BMP-15 action. In addition, other works indicate that follistatin, important binding protein expressed by the granulosa cells of growing follicles, can also bind to BMP-15, and thus block its bioactivity (Otsuka et al., 2001a).

4. Effects of BMP-15 on the follicular viability

BMP-15, as well as others BMPs (-2, -5 and -6), acts in the granulosa cells, promoting the survival of the follicles through the maintenance of cell proliferation and prevention of precocious luteinization and/or atresia (Knight and Glister, 2006). Studies also have demonstrated that BMP-15 levels in the follicular fluid seems to be a potential factor to determine the oocyte quality and further embryo development, being demonstrated that high BMP-15 levels in human follicular fluid warrant a higher cleavage rate and good embryo morphology (Wu et al., 2007). In addition, BMP-15 was capable to maintain a low incidence of apoptosis in the cumulus cells, suggesting that this may be involved in the follicular development (Hussein et al., 2005; Wu et al., 2007). In a study with mouse in which the specific gene for BMP-15 was deleted, the authors observed defects in the integrity of the cumulus oocyte complexes (Varani et al., 2002). Other study showed that after neutralization of a great portion of the gene for GDF-9 and BMP-15, there was a reduction in the oocyte quality and in the ability of the sheep to carry pregnancies to term (Juengel et al., 2004a).

Some studies have evidenced that BMP-15 did not act by itself but interacts with other substances, such as GDF-9, kit ligand (KL), follicle stimulating hormone (FSH) and follistatin

(Huang et al., 2001; Otsuka et al., 2001a,b; Yan et al., 2001; Juengel et al., 2002; Otsuka and Shimasaki, 2002a,b; Moore et al., 2003; Shimasaki et al., 2004a; Edwards et al., 2008; Sadeu et al., 2008), possibly in a species-specific way (Margulis et al., 2009). It is known that BMP-15 stimulates the expression of KL in the granulosa cells (Otsuka and Shimasaki, 2002b; Moore and Shimasaki, 2005), which is an essential factor for the early folliculogenesis (Otsuka and Shamasaki, 2002a; Peng et al., 2009). In humans (Carlsson et al., 2006) and caprine (Celestino et al., 2010), it has been demonstrated the importance of KL and its receptor c-kit for the survival of preantral follicles cultured *in vitro*. Other studies have demonstrated that KL inhibits apoptosis in oocytes from mouse primordial follicles through an increase in the mRNA levels of antiapoptotic proteins, Bcl-2 and Bcl-cL, and a reduction in the levels of the pro-apoptotic factor Bax. These antiapoptotic effects seem to be mediated by a fosfatidilinositol 3-quinase (PI3K) signaling, which acts promoting the inhibition of apoptosis mediated by Fas ligand, a polypeptide associated to the membrane (Jin et al., 2005).

5. Involvement of BMP-15 in the activation of primordial follicles

In the early folliculogenesis, one of the main activities of BMP-15 is to promote granulosa cell mitosis (Moore et al., 2003). In fact, studies performed in rodents (rat: Otsuka et al., 2000) and ruminants (sheep and bovine: McNatty et al., 2005) characterize BMP-15 as a potent stimulator of granulosa cell proliferation, demonstrating an intense capacity to increase the incorporation of tritiated thymidine and the number of follicular cells *in vitro*.

The presence of BMP-15 in primordial follicles from some species suggests that this protein, produced by the oocyte, has a role in the recruitment of the follicles, i.e., in the follicular activation (Otsuka et al., 2000). In caprine, not only the protein (expressed exclusively in the oocyte) but also the mRNA for the BMP-15 ligand, as well as the mRNA for its two receptors, were all expressed as early as from primordial follicle stage (Silva et al., 2005), thus suggesting the possible role of BMP-15 in the follicular activation in this species. We recently confirmed this hypothesis in a study showing that BMP-15 promotes the *in vitro* transition from caprine primordial to primary follicle stage (Celestino et al., unpublished data). The role of BMP-15 in the process of follicular activation, determined in a great part by its action in cell proliferation, occurs mainly through its BMPR-II receptor present in the surface of the granulosa cells (Shimasaki et al., 2004a,b).

In ovarian follicles, the BMP-15 action is directly related with the KL, including in the proliferation of the granulosa cells (Moore and Shimasaki, 2005). In fact, the ability of BMP-15 for regulating cell mitosis is controlled in part by KL. A negative feedback between these two factors seems to exist, with BMP-15 stimulating the expression of KL in the granulosa cells, and KL inhibiting the expression of BMP-15 in the oocyte (Juengel et al., 2004b). In sheep and women mutations for the BMP-15 gene, this negative feedback between BMP-15 and KL is lost, damaging the early follicular development (Moore and Shimasaki, 2005).

6. BMP-15 and follicular and oocyte growth

Evidences indicate that due to its effects on the cellular proliferation, BMP-15 has an essential role for the ovarian follicle development (Juengel et al., 2004b). In addition, it has been demonstrated that BMP-15 activity is independent of FSH, suggesting that BMP-15 can stimulate the mitosis of granulosa cells in early preantral follicles in the absence of this gonadotropin, and also inhibiting the expression of FSH receptor (FSH-R) (Otsuka et al., 2001b).

Otsuka et al. (2000) suggests that the signaling pathway of BMP-15 leads to the stimulation and inhibition of effector genes, as well as to biological specific responses, which are critical for follicular growth and development. In mouse, when the gene for BMP-15 was deleted, the animals show defects in the ovulation process and in the quality of the fertilizable oocytes, resulting in lowest sizes of these oocytes (Yan et al., 2001). In sheep, immunization against BMP-15 resulted in a blockage of follicular growth (Juengel et al., 2002).

Additional studies have related a high expression of the mRNA for BMP-15 and/or of the protein in growing or completely grown oocytes (Shimasaki et al., 2004a; Juengel and McNatty, 2005; Li et al., 2008). Moreover, the absence of BMP-15 receptor (ALK-6 and BMPR-II) production in ovine follicles can lead to a break in their growth (Mery et al., 2007). In mouse, it was reported an increase in the levels of mRNA expression for BMP-15 from day 7 to 10 of *in vitro* culture, being this increase associated to the presence of early stage follicles with growing oocytes (Sadeu et al., 2008). In another study, BMP-15 increased the competence for bovine oocyte development (Gilchrist et al., 2008). Similarly, it has been demonstrated that BMP-15 in human follicular fluid is positively correlated with the potential for the oocyte development (Wu et al., 2007). In this same species, the expression of the

mRNA for BMP-15 increased in a direct correlation with the follicular growth (Teixeira Filho et al., 2002). A summary of the main implications of bone morphogenetic protein-15 (BMP-15) in the reproduction of different species as shown in Table 1.

Table 1. Summary of the main implications of bone morphogenetic protein-15 (BMP-15) in the reproduction of different species.

Effects	Species	References
Improvement of embryo quality	Human	Wu et al., 2007
Reduction of apoptosis in cumulus cells	Human Mouse	Wu et al., 2007 Varani et al., 2002
Improvement of oocyte quality	Sheep	Juengel et al., 2004a
Stimulates the expression of KL	Rat	Otsuka and Shimasaki, 2002b
Granulosa cell proliferation	Rat Sheep and Bovine	Otsuka et al., 2000 McNatty et al., 2005
Follicular activation	Goat	Celestino et al., unpublished data
Inhibition of FSH-R expression	Rat	Otsuka et al., 2001b
Follicle and oocyte growth	Mouse Sheep Human	Yan et al., 2001; Sadeu et al., 2008 Juengel et al., 2002; Mery et al., 2007 Wu et al., 2007

KL: kit ligand; FSH-R: FSH receptor.

7. Final considerations

Several researches in the field of reproductive biotechnologies have been developed to establish *in vivo* and *in vitro* systems which reveal the functions of different proteins synthesized in the ovary, including BMP-15. This protein, highlighted in the present review, has a crucial role on mammalian folliculogenesis and fertility, showing together with other local factors, regulatory actions on the follicular somatic cells, which are essential for warrant an adequate development of ovarian follicles. However, despite many researches be related to the study of BMP-15, less is known about its specific role on the female reproduction, as well as on its interaction with other regulatory substances. Therefore, it is of great importance the complete elucidation of the mechanisms of BMP-15 action in the ovary because, in the future, it can provide a correct monitoring and manipulation of the ovarian function, with a consequent improvement of the fertility of domestic animals, endangered species and humans.

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9 CAPÍTULO 4

Fator de crescimento epidermal como mediador de sobrevivência e desenvolvimento folicular

Epidermal growth factor as mediator of survival and follicular development

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Resumo

Diversos fatores intra-ovarianos atuam no ovário dos mamíferos regulando o desenvolvimento folicular. Dentre eles destaca-se o fator de crescimento epidermal (EGF), considerado um potente fator mitogênico para células foliculares e luteais. Tendo em vista a importância deste fator no âmbito do desenvolvimento folicular, a presente revisão de literatura tem como objetivo descrever as principais implicações do EGF na foliculogênese, destacando seu padrão de expressão no ovário, suas principais vias de sinalização celular, bem como seu efeito como fator de sobrevivência e de desenvolvimento folicular.

Palavras-chave: EGF. Ovário. Folículo. Mamífero.

Fator de crescimento epidermal como mediador de sobrevivência e desenvolvimento folicular

(Epidermal growth factor as mediator of survival and follicular development)

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Resumo

Diversos fatores intra-ovarianos atuam no ovário dos mamíferos regulando o desenvolvimento folicular. Dentre eles destaca-se o fator de crescimento epidermal (EGF), considerado um potente fator mitogênico para células foliculares e luteais. Tendo em vista a importância deste fator no âmbito do desenvolvimento folicular, a presente revisão de literatura tem como objetivo descrever as principais implicações do EGF na foliculogênese, destacando seu padrão de expressão no ovário, suas principais vias de sinalização celular, bem como seu efeito como fator de sobrevivência e de desenvolvimento folicular.
Palavras-chave: EGF, ovário, folículo, mamífero.

Abstract

Several intra-ovarian factors act regulating the mammalian follicular development in the ovary. Among them highlights the epidermal growth factor (EGF), considered a potent mitogenic factor for follicular and luteal cells. Given the importance of this factor within the follicular development, this review describes the main implications of EGF in the folliculogenesis, focusing on its expression pattern in the ovary, the major pathways of cell signaling and its effect as a survival and follicular development factor.
Keywords: *EGF, ovary, follicle, mammal.*

Introdução

A foliculogênese é uma complexa orquestra de eventos que propicia o desenvolvimento folicular (Fair, 2003). Atualmente é bem estabelecido que o crescimento dos folículos presentes nos ovários dos mamíferos é regulado por gonadotrofinas e por fatores intra-ovarianos (Fortune, 2003). O papel das gonadotrofinas, Hormônio Folículo Estimulante (FSH) e Hormônio Luteinizante (LH) tem sido bastante investigado, principalmente sobre o desenvolvimento folicular terminal, tornando-se uma prioridade o estudo dos fatores de crescimento, sobretudo durante o crescimento folicular inicial (Bristol-Gould e Woodruff, 2006).

O início do desenvolvimento folicular é marcado pela ativação folicular, ou seja, a passagem de folículos primordiais do *pool* de reserva ou quiescentes (oócito circundado por

uma camada de células da pré-granulosa de morfologia plana) para o estádio de folículos de transição (oócito circundado por uma camada de células da granulosa de morfologia plana e/ou cúbica) (van den Hurk e Zhao, 2005). De acordo com Fortune (2003), este evento ocorre devido a estímulos de diferentes fatores de crescimento produzidos localmente no ovário. Além disso, Eppig (2001) demonstrou que vários fatores de crescimentos, como o fator de crescimento epidermal (EGF) produzido pelo oócito e células da granulosa, frequentemente atuam modulando a foliculogênese ovariana.

O EGF é considerado um fator de crescimento protéico pertencente à família EGF, a qual consiste de, no mínimo, oito membros (Riese e Stern, 1998). Sua atividade biológica é mediada por receptores de membrana EGF-R (ErbB1) do tipo tirosina-quinase, pertencentes à superfamília ErbB (Conti et al., 2006). Na década de 80, vários estudos já demonstravam que o EGF induzia o crescimento de folículos ovarianos e modulava a função das células da granulosa (Schomberg et al., 1983). Atualmente ele é considerado um potente fator mitogênico que estimula a proliferação de diferentes tipos celulares (Toyoda et al., 2007). No ovário, é tido como um importante regulador da fisiologia, estando envolvido na regulação de diversos processos, incluindo a ativação folicular (Celestino et al., 2009), proliferação e diferenciação das células da granulosa, (Saha et al., 2000; Wang et al., 2007) esteroidogênese e maturação oócitária (Gall et al., 2004). Além disso, tem recebido notável atenção por inibir a apoptose, garantindo assim uma maior sobrevivência folicular em condições *in vitro* (Markström et al., 2002).

Tendo em vista a grande relevância do EGF para o desenvolvimento folicular, a presente revisão de literatura tem como objetivo descrever as principais implicações deste fator sobre a foliculogênese ovariana, enfatizando seu padrão de expressão no ovário, as principais vias de sinalização celular, bem como seu efeito como fator de sobrevivência e de desenvolvimento folicular.

Expressão do EGF e EGF-R no ovário

O RNAm para proteína e receptor de EGF (EGF-R) têm sido identificados no oócito e células da granulosa de folículos iniciais e em estádios mais avançados de desenvolvimento, em diferentes espécies (ratas: Chabot et al., 1986; Feng et al., 1987, mulheres: Maruo et al., 1993; Bennett et al., 1996; Qu et al., 2000, porcas: Singh et al., 1995a, vacas: Lonergan et al., 1996, camundongas: Hill et al., 1999, hamsters: Garnett et al., 2002 e cabras: Gall et al., 2004;

Silva et al., 2006). Além disso, foi encontrado em células luteais de porcas (Kennedy et al., 1993; Singh et al., 1995b), ratas (Tekpetey et al., 1995) e cabras (Silva et al., 2006).

Após análise por imunohistoquímica para localização da proteína EGF, em ovários de hamsters, foi observada uma intensa marcação nas células da granulosa de folículos pré-antrais (1 a 6 camadas de células da granulosa) (Roy e Greenwald, 1990). Por outro lado, Wu e Tian (2007) verificaram que a produção e a ação do EGF foram mais significativas em células da granulosa de folículos pré-antrais em desenvolvimento do que em folículos quiescentes (primordiais). Em caprinos, após quantificação do RNAm para o EGF foi observada uma expressão significativamente superior em folículos secundários quando comparada com folículos primordiais, não diferindo dos folículos primários (Celestino et al., dados não publicados). No entanto, esses achados não têm sido totalmente reproduzidos por outros estudos (Skinner e Coffey, 1988; Tamura et al., 1995; Reeka et al., 1998).

Reeka et al. (1998) e Qu et al. (2000) relataram em humanos que a expressão de RNAm para EGF foi somente detectada em oócitos de folículos primordiais e primários, mas não nos estádios subsequentes. Maruo et al. (1993), também em humanos, relataram que a expressão do EGF foi encontrada em oócitos a partir do estádio primário até a fase antral. Roy e Greenwald (1990) verificaram por imunohistoquímica, que em hamsters ocorre uma expressão flutuante nos níveis de EGF em função das diferentes fases do ciclo estral. Skinner e Coffey (1988) e Tamura et al. (1995), utilizando a técnica de hibridização *in situ* e marcação por imunohistoquímica, não detectaram imunoreatividade para EGF em células foliculares, luteais e células do estroma em ovários de bovinos e humanos, respectivamente. De acordo com Lafky et al. (2008) estas discrepâncias no padrão de expressão do EGF durante o desenvolvimento folicular podem ser atribuídas ao uso de diferentes técnicas, bem como a espécie estudada.

Em folículos antrais, a presença de RNAm para o receptor e proteína do EGF foi descrita em oócitos de suínos (Singh et al., 1995a), humanos (Reeka et al., 1998) e caprinos (Silva et al., 2006), bem como nas células da granulosa de folículos suínos (Singh et al., 1995a). Na espécie humana, uma fraca marcação através da técnica de imunohistoquímica para a proteína EGF foi verificada em células da teca (Qu et al., 2000). Na espécie caprina, Celestino et al. (dados não publicados) demonstraram que a expressão de RNAm para o EGF foi significativamente superior nas células do cumulus do que nas suas respectivas células da granulosa/teca. Após a formação da cavidade antral, a presença de receptores funcionais para EGF no ovário de primatas não humanos parece ser regulada pelas gonadotrofinas (Fru et al.,

2007). Em hamsters, o receptor de EGF localizado nas células da granulosa e da teca completamente diferenciadas, bem como no oócito de folículos antrais avançados, teve sua expressão regulada positivamente pelo FSH e estrógeno (Garnett et al., 2002).

Caracterização estrutural e sinalização celular via EGF/EGF-R

O receptor do fator de crescimento epidermal (EGF-R) e seus múltiplos ligantes são considerados os maiores reguladores de diversos processos reprodutivos (Schneider e Wolf, 2008). O sistema EGF compreende oito ligantes e quatro receptores. Como ligantes, pode-se citar: o próprio EGF, fator de crescimento transformante alfa (TGF-α), fator de crescimento semelhante ao EGF ligado à heparina (HB-EGF), anfiregulina (AR), betacelulina (BTC), epiregulina (EPR), neuregulinas (NRG 1-4) e epigen (Strachan et al., 2001; Schneider e Wolf, 2008). Todos esses membros são derivados de precursores ligados à membrana que são proteoliticamente clivados da membrana plasmática (Plowman et al., 1990; Holmes et al., 1992; Abraham et al., 1993). Cada ligante possui um domínio extracelular com uma sequência altamente conservada caracterizada por seis cisteínas formando três pontes dissulfetos intramoleculares. Essa sequência confere especificidade de ligação ao EGF-R (Schneider e Wolf, 2008).

O receptor EGF-R é uma glicoproteína transmembranária com um domínio ligante extracelular, um domínio lipofílico transmembrana e um domínio intracelular com atividade tirosina-quinase intrínseca (Ullrich e Schlessinger, 1990). O EGF-R (ErbB1; HER1) é um dos membros da família dos quatro receptores tirosina-quinase, na qual também inclui ErbB2 (neu; HER2), ErbB3 (HER3) e ErbB4 (HER4) (Schneider e Wolf, 2008). O domínio extracelular amino-terminal de EGF-R tem duas regiões ricas em cisteína que formam o domínio de ligação com o ligante. A região transmembrana é uma hélice alfa simples que ancora o receptor à célula (Wells, 1999). O domínio citoplasmático contém uma região tirosina-quinase e uma cauda carboxi-terminal que contém pelo menos seis sítios de autofosforilação de tirosina: Y845, Y992, Y1045, Y1068, Y1148 e Y1173 (Fig. 1). Vários ligantes da superfamília EGF podem interagir com o EGF-R (ErbB1), ErbB3 e ErbB4, com diferentes especificidades para cada receptor, resultando em distintos efeitos celulares (Riese e Stern, 1998; Jones et al., 1999; Normanno et al., 2003). Ao EGF-R podem se ligar o EGF, TGF-α, EPR, AR, HB-EGF, BTC e epigen (Riese et al., 1996; Strachen et al., 2001). O

ErbB2 não possui ligantes conhecidos (Klapper et al., 1999), enquanto o ErbB3 não possui atividades quinase intrínseca, parecendo atuar apenas como co-receptores (Guy et al., 1994).

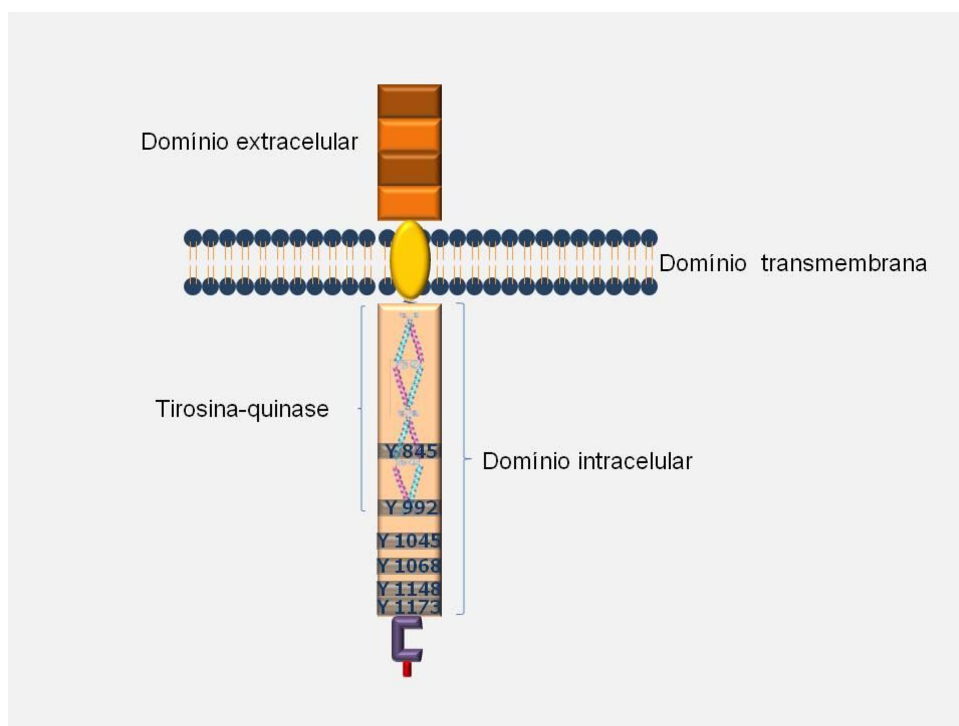

Figura 1. Estrutura do receptor do fator de crescimento epidermal (EGF-R) composto por um domínio ligante extracelular, um domínio lipofílico transmembrana e um domínio intracelular com atividade tirosina-quinase intrínseca. O domínio citoplasmático contém uma região tirosina-quinase e uma cauda carboxi-terminal que contém pelo menos seis sítios de autofosforilaçãso de tirosina: Y845, Y992, Y1045, Y1068, Y1148 e Y1173.

Pesquisas têm demonstrado sistematicamente que a ligação do ligante induz nove das dez possíveis combinações de homo e heterodímeros ErbB, valendo destacar aqui que não tem sido observado homodímeros ErbB2, provavelmente pelo fato deste receptor não possuir os domínios I e III da porção extracelular, não havendo portanto interação com o ligante (Riese et al., 1996; Tzahar et al., 1996). O EGF-R existe como um monômero inativo que se ativa por intermédio de um ligante externo. Isto leva o receptor a dimerizar-se com outro monômero de EGF-R (homodimerização) ou com outro membro da família de receptores (heterodimerização) (Sako et al., 2000; Hynes et al., 2001; Yarden e Sliwkowski, 2001). Esta dimerização do EGF-R induz a atividade catalítica tirosina-quinase, o que leva a autofosforilação em vários sítios de tirosina dentro da cauda carboxil-terminal do receptor. As

fosfotirosinas resultantes Y845, Y992, Y1045, Y1068, Y1148 e Y1173 agem como locais de ancoragem para enzimas transdutoras de sinais e proteínas de adaptação que desencadearão os efeitos celulares (Wells, 1999). Combinados, os quatro receptores contêm 89 tirosinas citosólicas, 40 das quais poderão interagir com uma ou mais proteínas adaptadoras para ativar vias de sinalização quando eles tornam-se fosforilados (Schulze et al., 2005). Essas proteínas adaptadoras podem ter múltiplos sítios de ligação para um único receptor ErbB e/ou múltiplos sítios de ligação para os membros da família de receptor ErbB, as quais permitem a ativação de uma diversa e complexa rede de padrões de sinalização, ou seja, vias de transdução (Lafky et al., 2008).

Uma vez ativados, os receptores desencadeiam o recrutamento e fosforilação de vários substratos intracelulares, levando à sinalização mitogênica e outras atividades celulares (Alroy e Yarden, 1997). A principal via de sinalização da família de receptores ErbB parece ser a via Ras/Raf/MEK/ proteínas-quinases ativadas por mitógenos (MAPK); fosfatidilinositol 3-quinase (PI3K)/Akt (também conhecida como proteína quinase B, PKB) e transdutor de sinal e ativador de transcrição (STAT) que culmina com a sinalização celular, através de ativadores de transcrição gênica (Prenzel et al., 2001; Jorissen et al., 2003; Citri e Yarden, 2006). A Fig. 2 ilustra de forma resumida a via de sinalização do EGF-R. O padrão de sinalização Ras/MAPK é bastante utilizado pelos receptores do tipo tirosina-quinase para promover diferentes respostas celulares, incluindo crescimento celular, diferenciação e apoptose (Chin et al., 1996; Garrington e Johnson, 1999; Wu e Tian, 2007). Já o padrão PI3K é um padrão de sinalização “clássico” consistindo de várias moléculas sinalizadoras incluindo quinases, fosfatases e fatores trancricionais que estabelecem cascatas de sinalização intracelular que são fundamentais para regulação da proliferação celular, sobrevivência, migração e metabolismo (Blume-Jensen e Hunter, 2001; Cantley, 2002).

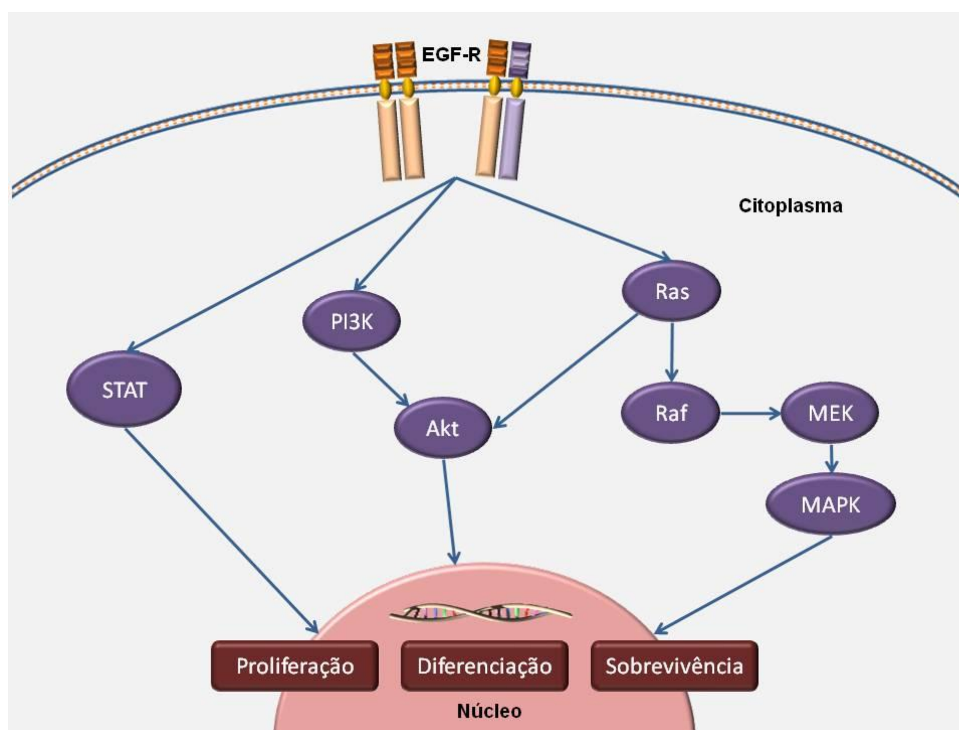

Figura 2. Proliferação, diferenciação e sobrevivência por diferentes vias de sinalização, decorrente da ligação do ligante fator de crescimento epidermal (EGF) ao seu receptor (EGF-R).

EGF como fator de sobrevivência folicular

O EGF além de ser considerado um potente fator que estimula a proliferação de diferentes tipos celulares (Toyoda et al., 2007), é conhecido como um fator de sobrevivência tanto *in vivo* como *in vitro* (van den Hurk et al., 2000). *In vitro*, quando utilizado na concentração de 10 ng/mL, inibiu a apoptose das células da granulosa e levou a um aumentou da formação de antro após o cultivo de folículos isolados suínos (Mao et al., 2004). Porém, em folículos pré-antrais de camundongas, a utilização de EGF não mostrou nenhum efeito na supressão da apoptose (McGee et al., 1999; Demeestere et al., 2005).

Em bovinos, quando o EGF foi utilizado em uma concentração de 0,5 ng/mL, reduziu os níveis de atresia em folículos pré-antrais cultivados *in vitro* (Gutierrez et al., 2000). Por outro lado, nesta mesma espécie, utilizando a concentração de 10 ng/mL, o EGF não alterou a sobrevivência de folículos primários e secundários cultivados *in vitro* (Derrar et al., 2000). Posteriormente, estudos de Zhou e Zhang (2005a,b) demonstraram que o EGF na concentração de 50 ng/mL estimulou a sobrevivência de oócitos caprinos após o cultivo *in*

vitro. Celestino et al. (2009) verificaram que a adição de baixas concentrações de EGF (1 ou 10 ng/mL) no cultivo *in vitro* de folículos pré-antrais caprinos manteve a morfologia e ultraestrutura folicular após 7 dias de cultivo. No entanto, Silva et al. (2004) não observaram efeito significativo do EGF sobre a sobrevivência de folículos pré-antrais caprinos após 5 dias de cultivo, provavelmente devido a elevada concentração (100 ng/mL) de EGF utilizada. Talmimani et al. (2005) ao testar o efeito de diferentes concentrações de EGF (0, 25, 50, 75 ou 100 ng/mL) sobre folículos pré-antrais ovinos, mostrou que as maiores concentrações, ou seja, 75 ou 100 ng/mL induziu à degeneração de todos folículos pré-antrais após cultivo de 6 dias. Achados semelhantes também foram descritos por Celestino et al. (2009) ao verificar que folículos pré-antrais caprinos cultivados *in vitro* por 7 dias em altas concentrações de EGF (100 ou 200 ng/mL) não sofreram ativação e apresentaram altas taxas de degeneração.

Papel do EGF no desenvolvimento folicular

Estudos *in vitro* mostraram que o EGF promove a ativação folicular (ovinos: Andrade et al., 2005), estimula a proliferação das células da granulosa (suínos: Morbeck et al., 1993), evidenciado pela incorporação de timidina em folículos pré-antrais (hamster: Roy, 1993). Além disso, aumenta o diâmetro folicular (suínos: Mao et al., 2004) e promove o crescimento de oócitos em folículos primários (caprinos: Silva et al., 2004). Ademais, o EGF tem mostrado regular a expressão da conexina 43 (suíno: Bolamba et al., 2002; coelho: Kennedy et al., 2003) que é uma proteína essencial para a formação das junções *gaps*, sendo portanto importante para o desenvolvimento de folículos pré-antrais.

A ação do EGF sobre a ativação, diferenciação, proliferação e esteroidogênese das células da granulosa tem se mostrado controversa na literatura e dependente do estádio de desenvolvimento folicular. Alguns estudos têm mostrado que embora o EGF não seja essencial para ativação de folículos primordiais (Braw-Tal e Yossefi, 1997; Fortune et al., 1998; Wright et al., 1999), ele é importante para os estádios mais avançados de desenvolvimento folicular (Gutierrez et al., 2000; Nayudu et al., 2002; Peng et al., 2010). Em caprinos, Silva et al. (2004) demonstraram que o EGF (100 ng/mL) no cultivo *in situ*, não promoveu a ativação de folículos primordiais, mas teve um efeito benéfico no crescimento de oócitos de folículos primários (Silva et al., 2004). Em ovinos, sua utilização nesta mesma concentração, promoveu a ativação de folículos primordiais *in situ* e manutenção da viabilidade por até 6 dias de cultivo (Andrade et al., 2005). Já em pequenos e médios folículos

pré-antrais bovinos (60-179 µm), a utilização de 50 ng/ml de EGF garantiu a sobrevivência e promoveu o crescimento e produção de progesterona (Wandji et al., 1996), bem como estimulou a formação de antro em grandes folículos pré-antrais (166 µm), mas não o crescimento do oócito (Gutierrez et al., 2000). Contrariamente em suínos, o EGF em baixas concentrações (0,75; 1,5 ou 3 ng/mL) associado ao FSH melhorou a qualidade dos oócitos levando a uma maior taxa de desenvolvimento embrionário (Wu e Tian, 2007), concordando com o estudo de Park et al (2004) que demonstraram que o EGF melhora a qualidade dos oócitos oriundos de folículos cultivados *in vitro*.

Na fase antral, o EGF estimula a retomada da meiose de oócitos em várias espécies (camundonga: De La Fuente et al., 1999; ovelha: Guler et al., 2000; vaca: Lonergan et al., 1996; mulher: Goud et al., 1998; porca: Li et al., 2002; Prochazka et al., 2003), induz a expansão das células do cumulus (camundonga: O'Donnell et al., 2004), estimula a proliferação das células da granulosa (porca: May et al., 1992), bem como a síntese de hormônio esteroides (mulher: Misajon et al., 1999). Além disso, o EGF parece agir localmente no ovário controlando a expressão de receptores para FSH e LH (Luciano et al., 1994; Hattori et al., 1995).

Considerações finais

A partir das inúmeras informações disponíveis na literatura, torna-se evidente a grande importância do EGF para a foliculogênese ovariana. No entanto, mais estudos são necessários acerca de sua atuação, sobretudo no que se refere ao seu mecanismo de ação e interação com outros fatores de crescimento e hormônios. Esses conhecimentos poderiam ser de grande valia para implantação de bancos de germoplasmas de animais de alto valor genético, bem como para aquelas espécies ameaçadas de extinção.

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10 CAPÍTULO 5

Níveis de RNAm para o Kit Ligand em Ovários Caprinos e o Papel do Kit Ligand na Sobrevivência e Crescimento In Vitr*o* de Folículos Pré-antrais

Steady-State Level of Kit Ligand mRNA in Goat Ovaries and the Role of Kit Ligand in Preantral Follicle Survival and Growth In Vitro

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Resumo

Os objetivos desse estudo foram investigar os níveis de RNAm para o Kit Ligand (KL) e os efeitos dele na sobrevivência e crescimento de folículos pré-antrais caprinos. A RT-PCR foi utilizada para analisar os níveis de RNAm para o KL em folículos primordiais, primários e secundários, e em pequenos (1-3 mm) e grandes (3-6 mm) folículos antrais. Além disso, fragmentos ovarianos foram cultivados por 1 ou 7 dias em Meio Essencial Mínimo (MEM^+) suplementado com KL (0, 1, 10, 50, 100 ou 200 ng/ml). Fragmentos não-cultivados (controle) e cultivados foram processados para histologia e microscopia eletrônica de transmissão (MET). A RT-PCR demonstrou um aumento nos níveis de RNAm para o KL durante a transição de folículos primários para secundários. Pequenos folículos antrais tiveram níveis superiores de RNAm para o KL em células da granulosa e tecais do que grandes folículos. Após 7 dias, somente 50 ng/ml de KL manteve a percentagem de folículos normais similar ao controle. Após 1 dia, todas as concentrações de KL reduziram a percentagem de folículos primordiais e aumentaram a percentagem de folículos em crescimento. Após 7 dias, KL 10, 50, 100 ou 200 ng/ml aumentou o percentual de folículos primários quando comparados ao MEM^+. Um aumento no diâmetro folicular e oocitário foram observados com KL 50 ng/ml. A MET confirmou a integridade ultraestrutural dos folículos após 7 dias de cultivo em KL 50 ng/ml. Em conclusão, os RNAm para o KL foram detectados em todas as categorias foliculares. Além disso, 50 ng/ml de KL manteve a integridade de folículos pré-antrais caprinos cultivados por 7 dias e estimulou a ativação de folículos primordiais e o crescimento folicular.

Palavras-chave: Folículo ovariano. Ativação. Desenvolvimento folicular. KL. Caprino.

Steady-State Level of Kit Ligand mRNA in Goat Ovaries and the Role of Kit Ligand in Preantral Follicle Survival and Growth In Vitro

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SUMMARY

The aims of this study were to investigate steady-state level of Kit Ligand (KL) mRNA and its effects on in vitro survival and growth of caprine preantral follicles. RT-PCR was used to analyze caprine steady-state level of KL mRNA in primordial, primary, and secondary follicles, and in small (1–3 mm) and large (3–6 mm) antral follicles. Furthermore, ovarian fragments were cultured for 1 or 7 days in Minimal Essential Medium (MEM^+) supplemented with KL (0, 1, 10, 50, 100, or 200 ng/ml). Noncultured (control) and cultured fragments were processed for histology and transmission electron microscopy (TEM). RT-PCR demonstrated an increase in steady-state level of KL mRNA during the transition from primary to secondary follicles. Small antral follicles had higher steady-state levels of KL mRNA in granulosa and theca cells than large follicles. After 7 days, only 50 ng/ml of KL had maintained the percentage of normal follicles similar to control. After 1 day, all KL concentrations reduced the percentage of primordial follicles and increased the percentage of growing follicles. KL at 10, 50, 100, or 200 ng/ml increased primary follicles, compared to MEM^+ after 7 days. An increase in oocyte and follicular diameter was observed at 50 ng/ml of KL. TEM confirmed ultrastructural integrity of follicles after 7 days at 50 ng/ml of KL. In conclusion, the KL mRNAs were detected in all follicular categories. Furthermore, 50 ng/ml of KL maintained

the integrity of caprine preantral follicle cultured for 7 days and stimulated primordial follicle activation and follicle growth.

INTRODUCTION

Female gametes are stocked in the ovary as primordial follicles, which are composed of small, immature, quiescent oocytes surrounded by a single layer of flattened granulosa cells. During the female reproductive life, primordial follicles are stimulated to grow each day, a process known as follicular activation (Fortune, 2003; Choi et al., 2008a,b). After growth begins, the oocytes enter into a pre-programmed course of maturation and development that is necessary for the success of ovulation and fertilization. Primordial follicles from the quiescent pool are lost by atresia without these processes (Knight and Glister, 2006; Dole et al., 2008). The size of the ovarian follicular reserve associated with the rate of follicular activation is a critical determinant of the female reproductive lifespan, since the number of gametes in the ovary is supposedly finite (Choi et al., 2008a; Dole et al., 2008). However, the mechanisms responsible for activation of primordial follicles remain unknown. Kit Ligand (KL) is one of the first discovered of a few known factors with a recognized role in primordial follicle activation (Hutt et al., 2006a; Thomas and Vanderhyden, 2006).

KL, also known as stem cell factor (SCF), steel factor (SF), or mast cell growth factor (MCGF), is a locally produced growth factor with many roles in ovarian function from the embryonic stage onwards (Yoshida et al., 1997; Driancourt et al., 2000). The presence of KL mRNA in follicles has been detected in the granulosa cells of several species (rat: Ismail et al., 1996; sheep: Tisdall et al., 1999; mouse: Doneda et al., 2002; human: Hoyer et al., 2005; and goat: Silva et al., 2006). KL can be expressed as a soluble protein (KL-1) or a membrane associated protein (KL-2) depending on how the mRNA is spliced (Huang et al., 1992). KL-2 is the main isoform required for oocyte growth, survival, and formation of germ cells (Thomas et al., 2008). However, soluble KL-1 can promote the primordial to primary transition (Parrot and Skinner, 1999). Both forms are present in rodent (Manova et al., 1990; Ismail et al., 1997) and goat ovaries (Silva et al., 2006). In goats, the protein and mRNA for KL were expressed in granulosa cells during all stages of follicular development (Silva et al., 2006). However, quantification of the steady-state level of KL mRNA during different stages of follicular development has not been performed yet. KL influences the target cells through the c-kit receptor, a member of the tyrosine kinase receptor family. During postnatal ovarian development, both the mRNA and protein for c-kit are expressed in the oocytes during all

stages of follicular development. Moreover, c-kit mRNA has been detected in the interstitial and thecal cells of antral follicles (rodents: Motro and Bernstein, 1993; sheep: Clark et al., 1996; goat: Silva et al., 2006).

In vitro studies of rodents and sheep have implicated the KL/c-kit system in the migration, proliferation, and survival of primordial germ cells (CGP) (Zama et al., 2005), activation of primordial follicles (Parrot and Skinner, 1999), oocyte growth and survival (Jin et al., 2005), granulosa cell proliferation (Otsuka and Shimasaki, 2002), maintenance of meiotic competence (Ismail et al., 1997), recruitment of theca cells, and regulation of ovarian steroidogenesis (Hutt et al., 2006a). In addition, the KL/c-kit system is essential for human preantral follicle survival in vitro (Carlsson et al., 2006). When tested in mouse and rabbit ovaries at two different concentrations (50 and 150 ng/ml), KL promoted an increase in the diameter of oocytes in preantral follicles from both species and stimulated mouse primordial follicle activation in vitro (Hutt et al., 2006b). Although several studies have been performed, particularly in murine and ovine models, little is known regarding the KL/c-Kit interaction during early folliculogenesis in goats.

The present study was designed with the following aims: (1) to determine the steady-state level of KL mRNA during different follicular stages in goat ovaries, and (2) to investigate a possible influence of KL at different concentrations (0, 1, 10, 50, 100, or 200 ng/ml) on the survival, activation, and growth of preantral follicles enclosed in caprine ovarian tissue cultured for 1 or 7 days.

RESULTS

Steady-State Level of KL mRNA in Goat Ovarian Follicles

Quantification of the steady-state level of mRNA demonstrated a significant increase in the steady-state level of KL mRNA during the transition from primary to secondary follicle stage ($P < 0.05$), but no significant difference was observed between primordial and primary follicles ($P > 0.05$; Fig. 1A). When the steady-state level of KL mRNA was compared between cumulus–oocyte complexes (COCs) collected from small and large antral follicles, no significant difference was observed ($P > 0.05$; Fig. 1B). A significantly higher steady-state level of KL mRNA was observed in granulosa/theca cells from small antral follicles in comparison to large antral follicles ($P < 0.05$; Fig. 1C). Additionally, real-time PCR demonstrated that granulosa/theca cells and COCs from small antral follicles exhibited a similar steady-state level of KL mRNA ($P > 0.05$; Fig. 1D). However, COCs from large antral

follicles produced significantly more KL than respective granulosa/theca cells ($P < 0.05$; Fig. 1E).

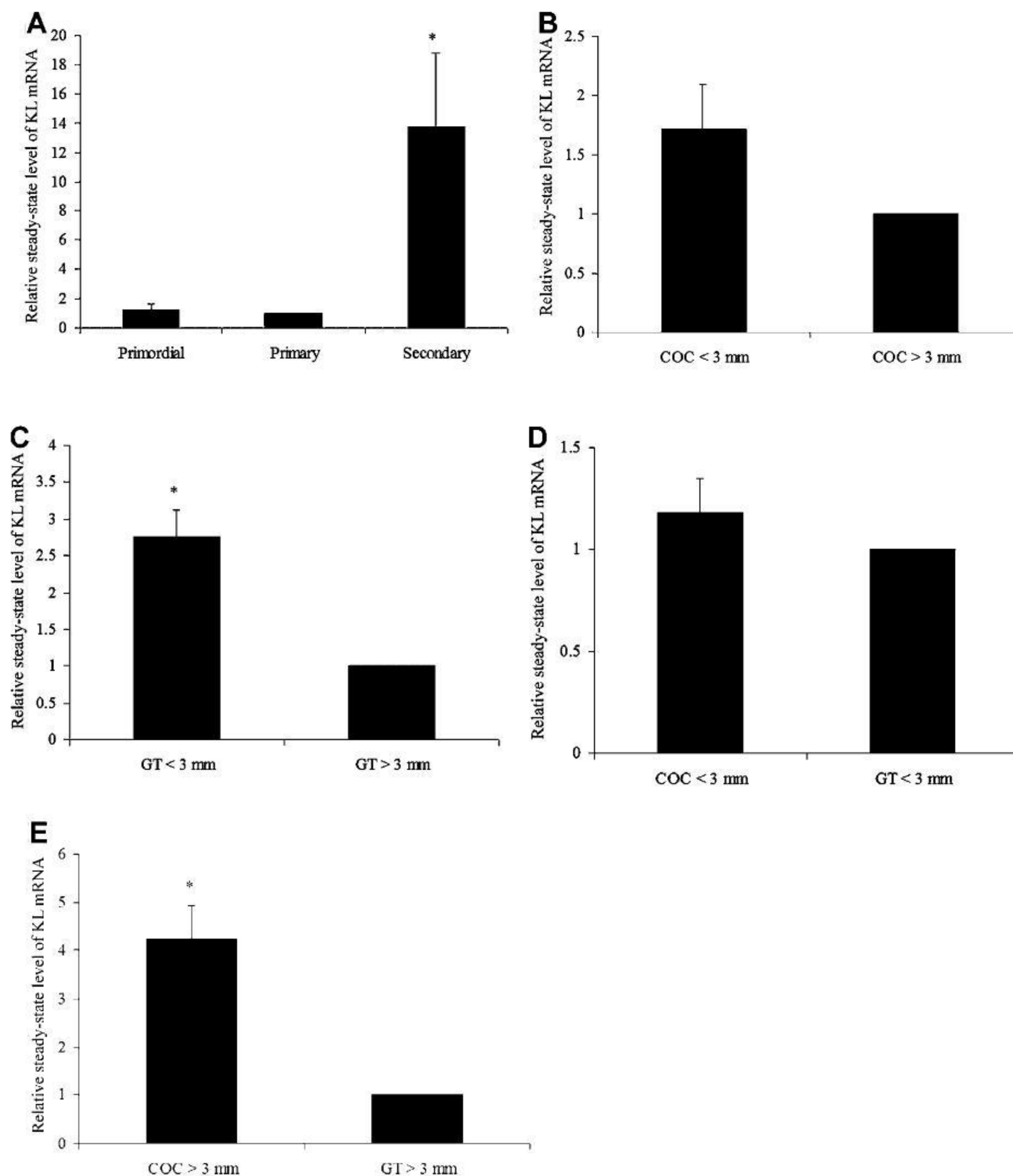

Figure 1. Steady-state level of KL mRNA in goat ovarian follicles (mean ± SD). **A**: Primordial, primary, and secondary follicles, (**B**) COCs from small and large antral follicles, (**C**) granulosa/theca cells from small and large antral follicles, (**D**) COCs and granulosa/theca

cells from small antral follicles, and (**E**) COCs and granulosa/theca cells from large antral follicles. Thirty follicles per category or structure follicular * ($P < 0.05$).

Caprine Preantral Follicle Survival Before and After In Vitro Culture

A total of 1,950 preantral follicles were analyzed. Figure 2A shows a normal primary follicle after culture in KL at 50 ng/ml, while Figure 2B illustrates a degenerated follicle after culture in KL at 1 ng/ml. Both are after 7 days of culture. Degenerated follicles had a retracted oocyte, pyknotic nucleus, and disorganized granulosa cells (Fig. 2B).

The percentages of morphologically normal preantral follicles in the control (fresh tissue) and 1- or 7-day cultures in different treatments are shown in Figure 3. After 7 days of culture, there was a significant reduction ($P < 0.05$) in the percentage of normal follicles in all treatments compared to the fresh control (89%) except when the tissues were cultured in 50 ng/ml of KL (82.5%) ($P > 0.05$). In addition, all concentrations of KL significantly ($P < 0.05$) increased the percentage of normal follicles after 1 week in comparison to MEM^+ alone except for 1 ng/ml of KL ($P > 0.05$). Moreover, there was no significant difference in the percentages of normal follicles between 10 (77%), 50 (82.5%), and 100 ng/ ml (78%) of KL ($P > 0.05$), which were higher than those observed with 1 and 200 ng/ml of KL ($P < 0.05$). With the progression of the culture period from 1 to 7 days, a significant decrease ($P < 0.05$) in the percentage of normal follicles in MEM^+ alone or supplemented with 1 or 200 ng/ml of KL was observed.

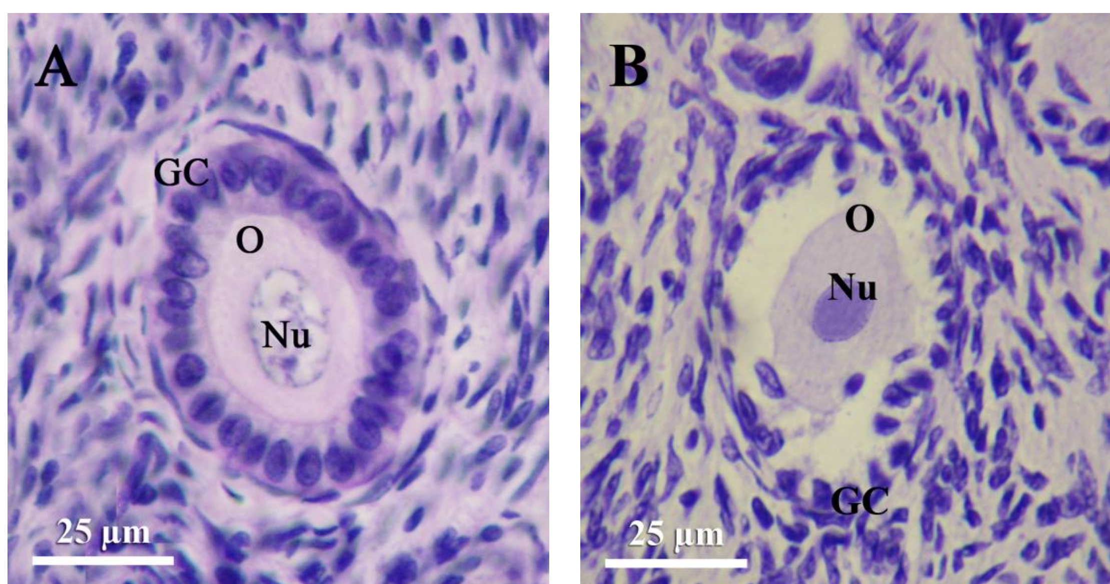

Figure 2. Histological section after staining with periodic acid Schiff-hematoxylin, showing (**A**) a normal follicle after culture in KL at 50 ng/ml and (B) a degenerated follicle after

culture in KL at 1 ng/ml. Both were after 7 days of culture. Note the cuboidal granulosa cell (GC) layer in the normal primary follicle (A) and the retracted oocyte with a pyknotic nucleus (**B**). O: Oocyte; Nu: oocyte nucleus (400x, bar = 25 µm).

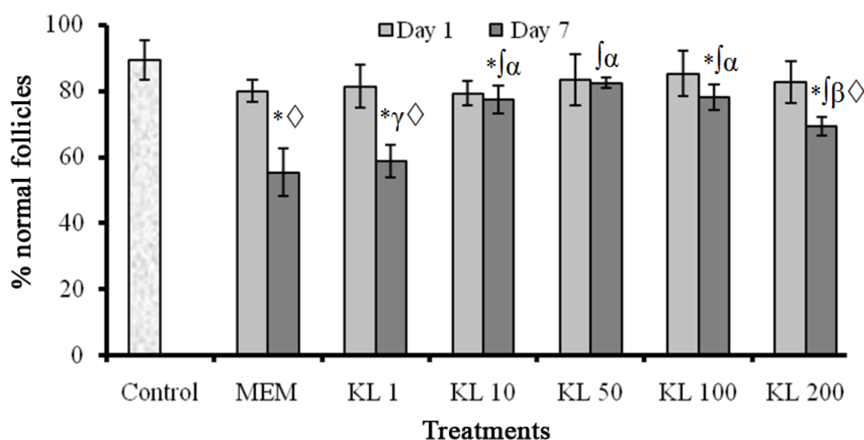

Figure 3. Percentage (mean ± SD) of morphologically normal preantral follicles in fresh control (non-cultured) and after culture for 1 or 7 days in the absence or presence of Kit Ligand. * Differs significantly from control follicles ($P < 0.05$); $^{\int}$ differs significantly from MEM^{+} in each day of culture ($P < 0.05$); $^{\alpha,\beta,\gamma}$ differs significantly among concentrations in each day of culture ($P < 0.05$); $^{\Diamond}$ differs significantly with the progression of the culture period from day 1 to 7 in the same treatment ($P < 0.05$).

Follicular Activation and Growth After In Vitro Culture

As early as day 1 of culture, a significant reduction in the percentage of primordial follicles was observed in all treatments containing KL (Fig. 4A, $P < 0.05$), which was associated with an increase in the percentage of intermediate follicles (Fig. 4B, $P < 0.05$) compared to the fresh control. Similar results were observed after culture with 50, 100, and 200 ng/ml of KL compared to MEM^{+} ($P < 0.05$). When the culture period progressed from 1 to 7 days, there was a significant decrease in the percentage of primordial follicles for all treatments ($P < 0.05$), while a significant increase in the percentage of intermediate follicles was only observed in MEM^{+} alone or with 1 ng/ml of KL ($P < 0.05$). All concentrations of KL (except 1 ng/ml) significantly increased the percentage of primary follicles on 7 days of culture compared to the fresh control and MEM^{+} (Fig. 4C, $P < 0.05$). The percentage of primary follicles significantly increased from days 1 to 7 of culture in medium supplemented with 10, 50, 100, or 200 ng/ml of KL. Furthermore, fresh and cultured caprine ovarian tissue

contained a low (often zero) and variable number of secondary follicles (data not shown) that did not permit the performance of statistical analysis.

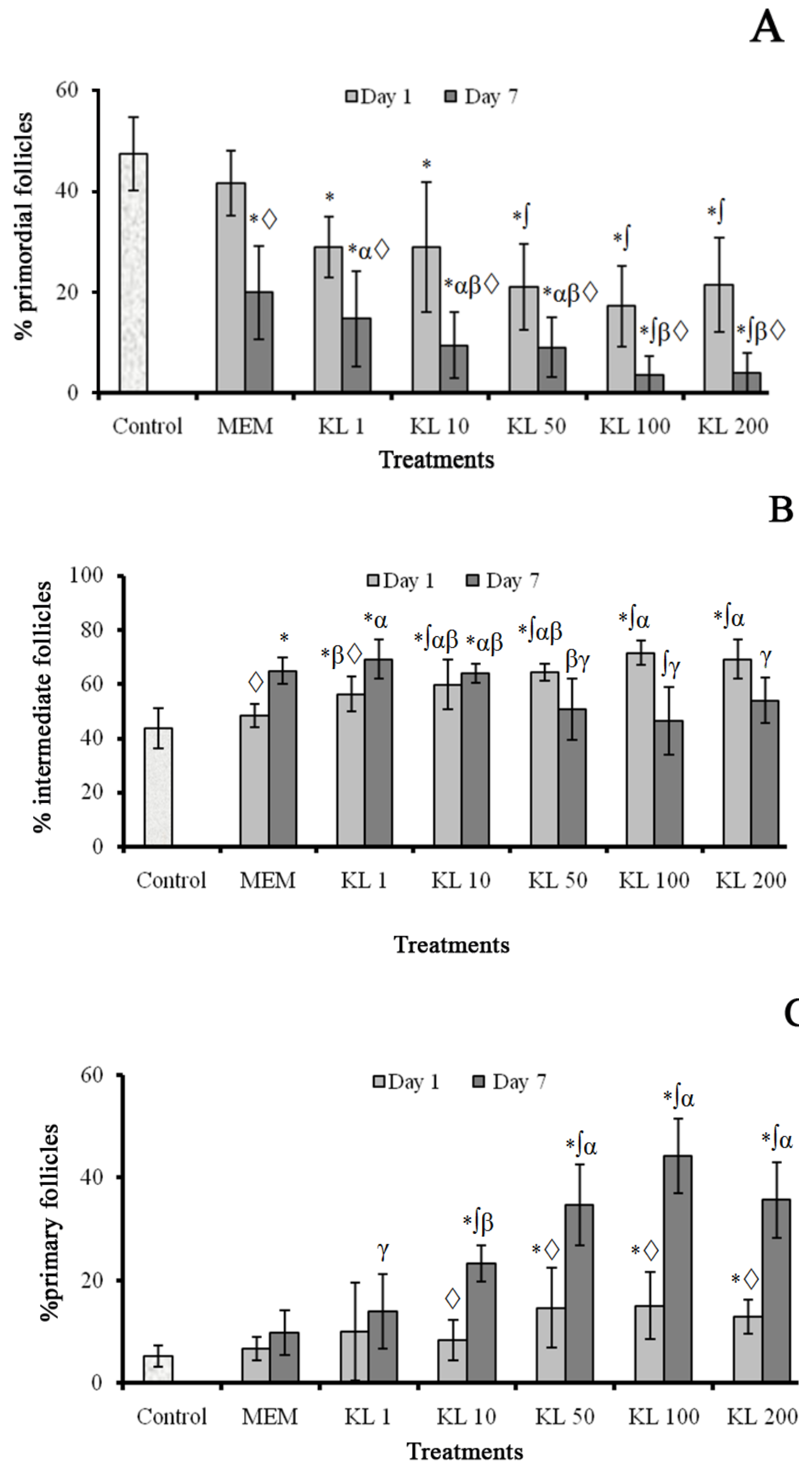

Figure 4. Percentage (mean ± SD) of primordial (**A**), intermediate (**B**), and primary (**C**) follicles in fresh control (non-cultured) and after culture for 1 or 7 days in the absence or presence of Kit Ligand. * Differs significantly from control follicles ($P < 0.05$); $^{\int}$ differs significantly from MEM^{+} in each day of culture ($P < 0.05$); $^{\alpha,\beta,\gamma}$ differs significantly among concentrations in each day culture ($P < 0.05$); $^{\lozenge}$ differs significantly with the progression of the culture period from day 1 to 7 under the same treatment ($P < 0.05$).

According to Table 1, there was an increase in oocyte and follicular diameters after 7 days of culture in medium containing 50 ng/ml of KL compared to the fresh control ($P < 0.05$). This same concentration of KL for the same period had a higher follicular diameter than MEM^+ after 7 days ($P < 0.05$). Similar results were also observed with the progression of the culture period from 1 to 7 days.

TABLE 1. Oocyte and Follicle Diameters (Mean ± SD) in Noncultured Tissues and in Tissues Cultured for 1 or 7 days in MEM (Control Medium) and MEM Supplemented With Various Concentrations of Kit Ligand.

Treatments	Oocyte diameter (μm)		Follicle diameter (μm)	
Non-cultured (Day 0)	38.47 ± 6.98		54.85 ± 6.64	
Cultured	Day 1	Day 7	Day 1	Day 7
MEM^+	36.62 ± 4.40	39.71 ± 7.39	56.70 ± 6.20	59.02 ± 7.36
KL 1	40.32 ± 6.61	41.87 ± 6.69	58.25 ± 8.70	60.60 ± 6.63 $^{\alpha\beta}$
KL 10	43.41 ± 8.71 ∫	40.63 ± 7.32	61.18 ± 9.70	61.34 ± 9.31 $^{\alpha\beta}$
KL 50	42.02 ± 6.21 ∫	45.73 ± 8.77 *	60.72 ± 8.58 ◊	67.36 ± 9.11 *∫$^{\alpha}$
KL 100	42.40 ± 5.69 ∫	38.93 ± 5.79	59.64 ± 7.37	59.64 ± 6.50 $^{\beta}$
KL 200	42.18 ± 6.75 ∫	43.26 ± 10.70	60.56 ± 7.79	61.25 ± 10.03 $^{\alpha\beta}$

* differs significantly from non-cultured tissues ($P < 0.05$)

∫ differs significantly from MEM alone within each day of culture ($P < 0.05$)

$^{\alpha,\beta}$ differs significantly among concentrations within each day of culture ($P < 0.05$)

◊ differs significantly with the progression of the culture period from day 1 to 7 in the same treatment ($P < 0.05$)

Ultrastructural Features of Caprine Preantral Follicles

To better evaluate the follicular quality, ultrastructural analysis was performed using morphologically normal preantral follicles from the fresh control, cultured with MEM^+ alone for 7 days and cultured in 50 ng/ml of KL, which was the treatment that demonstrated the best results according to previous histological analysis. Ultrastructural features of follicles from the control (Fig. 5A) and 50 ng/ml of KL (Fig. 5B) were similar in some aspects, such as intact basement and nuclear membranes as well as a large oocyte nucleus. In addition, there were organelles uniformly distributed in the ooplasm, especially the mitochondria and endoplasmic reticulum. Granulosa cells were ultrastructurally normal and well organized

around the oocyte, revealing an elongated and large nucleus. However, when the ovarian tissue was cultured for 7 days in MEM^+ with KL 50 ng/ml, their oocytes had irregular nuclear membranes as well as a detachment of the granulosa cells from the oocyte. When follicles were cultured for 7 days with MEM^+ alone, there was an absence of nuclear and basement membrane integrity, a highly vacuolated oocyte cytoplasm, low density of organelles and disorganized and degenerated granulosa cells (Fig. 5C).

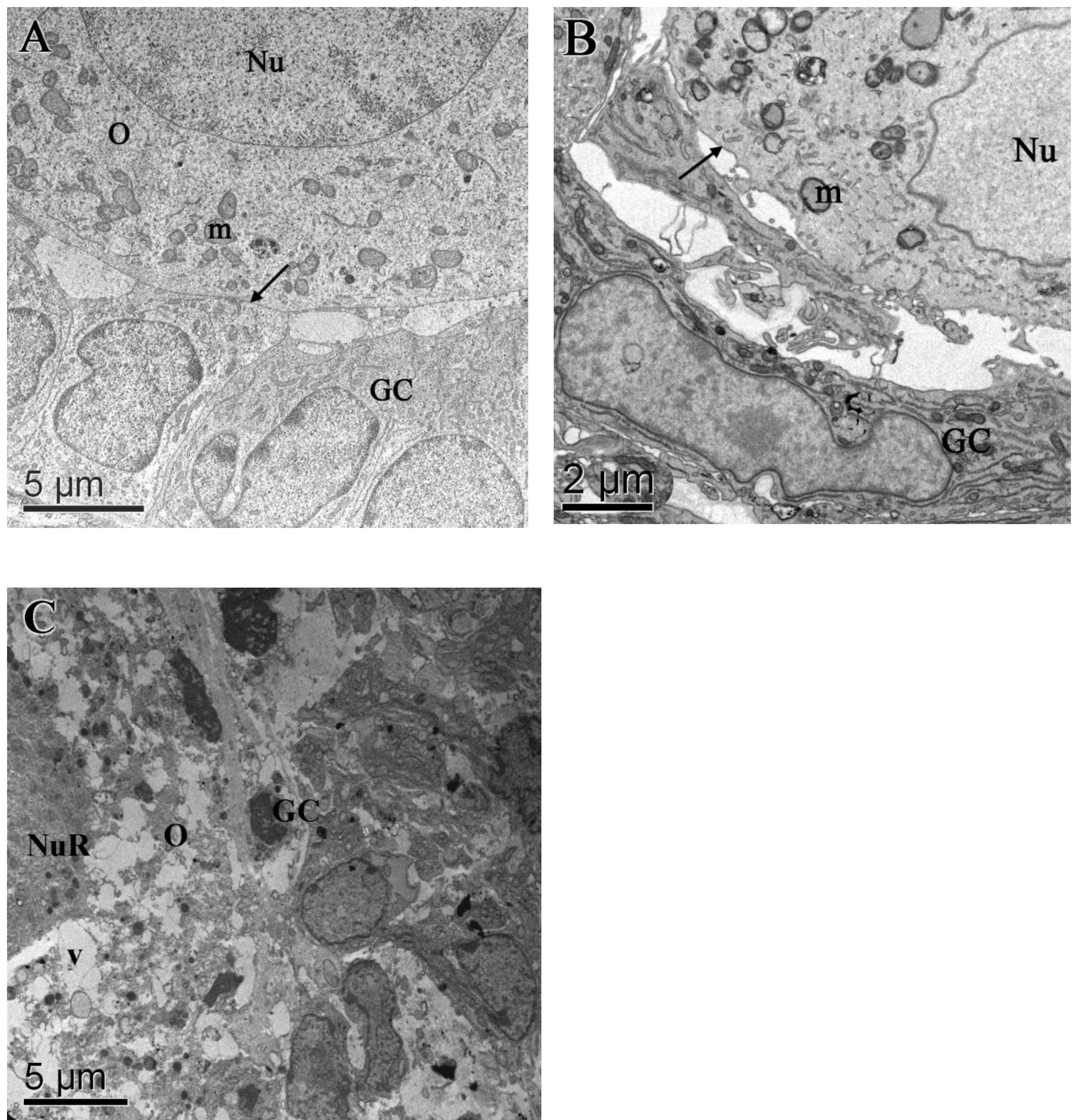

Figure 5. Ultrastructural analysis of non-cultured preantral follicle (**A**) and follicle cultured for 7 days in medium containing 50 ng/ml Kit Ligand (**B**) or MEM^+ alone (**C**). Note the large nucleus of the granulosa cells in the control follicles and follicles cultured in 50 ng/ml of Kit Ligand and absence of nuclear and basement membrane in follicles cultured with MEM^+ alone. O: oocyte; Nu: oocyte nucleus; NuR: nuclear region; GC: granulosa cells; m: mitochondria; v: vacuole; arrow- oocyte membrane. (A: 6000x, bar = 5µm; B: 10000x, bar = 2 µm; C: 5000x, bar = 5µm).

DISCUSSION

Analysis of the steady-state level of mRNA encoding KL demonstrated an increase in the steady-state level of KL mRNA during the transition from primary to secondary follicle stages. KL is involved in the control of early follicle development in goats, since a stimulation of primordial follicle activation and growth of activated follicles was observed after culturing ovarian cortical tissue in medium containing KL. In a mouse model, a higher level of KL mRNA was demonstrated in one and two-layered growing follicles 8 days after birth, while minimal expression was found in primordial follicles cells until this time (Doneda et al., 2002). In addition, the steady-state level of KL mRNA was very low in primordial and primary follicles (Manova et al., 1993; Motro and Bernstein, 1993). In humans, mRNA encoding KL was detected in primary follicles; however, there was no effect on the stages of follicular development after in vitro culture with KL for 7 days, which was independent of the concentration used (Carlsson et al., 2006). In rabbit ovaries, low steady-state level of KL mRNA in primordial follicles, high steady-state level of mRNA in growing follicles, and variable levels in antral follicles were observed (Hutt et al., 2006b). In this study on goat antral follicles, regarding the levels of mRNA in granulosa/theca cells, a higher steady-state level was observed in small antral follicles in comparison to large antral follicles. In large antral follicles, a higher steady-state level of mRNA was detected in COCs as compared with granulosa/theca cells. In contrast, Joyce et al. (1999) demonstrated that the development of mouse preovulatory follicles was associated with a large increase in the steady-state level of KL mRNA in mural granulosa cells but not in cumulus cells. These differences could be due to the use of goat follicles ranging from 3 to 6 mm in this study instead of preovulatory follicles. In rabbit antral follicles, low levels of KL mRNA were expressed in mural granulosa cells, and moderate to insignificant steady-state levels of mRNA were observed in the cumulus cells of antral follicles (Hutt et al., 2006b).

This study is the first to report the positive effect of KL on the in vitro development of caprine preantral follicles, which indicated that KL at a concentration of 50 ng/ml not only maintained the morphological integrity of 7-day cultures of caprine preantral follicles, but also stimulated the activation of the primordial follicles and the growth of the activated follicles. After 7 days of culture, the follicular survival was similar to the fresh control only with the addition of 50 ng/ml of KL to the culture medium. These results demonstrate the importance of the KL/c-kit interaction for preantral follicle survival. According to Yoshida et al. (1997), there was a significant reduction in normal mouse preantral follicles after in vivo

injection of antibodies that block the interaction between these two components. Moreover, the inhibition of the KL/c-kit interaction by anti-c-kit antibody promoted the death of oocytes in vitro (Reynaud et al., 2000). In vitro studies have demonstrated that KL inhibits apoptosis in oocytes of mouse primordial follicles by increasing the steady-state level of mRNA of the anti-apoptotic proteins, Bcl-2 and Bcl-cL, and reducing the steady-state level of mRNA of the pro-apoptotic factor, Bax. These anti-apoptotic effects appear to be mediated through a phosphoinositide-3 kinase pattern, which promotes the inhibition of Fas-mediated apoptosis (Jin et al., 2005). Other studies have demonstrated that the oocytes had no visible signs of degeneration after addition of 50 or 100 ng/ml of KL even after 20 days of culture (Klinger and De Felici, 2002). However, KL did not have any effect on the survival of rabbit and murine preantral follicles at 50 or 150 ng/ml (Hutt et al., 2006b). Similar to the results observed in our study at 1 ng/ml of KL, Yan et al. (2000) concluded that low levels of KL are not sufficient for the survival of rat male germ cells. According to this previous study, the steady-state level of KL mRNA is lower in vitro than in vivo due to the absence of FSH, as high concentrations of this factor are necessary in vitro. Regarding the unsatisfactory result at 200 ng/ml, this KL concentration may have interfered with the steady-state level of mRNA of other important factors for follicular survival, such as FSH receptors (Thomas et al., 2005; Matos et al., 2007).

After 1 day of culture, all treatments containing KL promoted follicular activation compared to the fresh control. Additionally, all KL concentrations (except 1 ng/ml) increased the percentage of primary follicles after 7 days of culture compared to the fresh control and MEM^+ alone. Studies have demonstrated that blockage of KL affects primordial follicle development (Yoshida et al., 1997) and that KL increases the transition from primordial to primary follicles in rats (Parrot and Skinner, 1999; Dole et al., 2008) and hamsters (Wang and Roy, 2004) after in vitro culture. However, KL did not have any effect on human preantral follicle development after 7 or 14 days of culture at 1, 10, or 100 ng/ml (Carlsson et al., 2006). KL has been hypothesized to play a role in follicular activation and further development through activation of Akt (a signaling molecule known to increase cellular proliferation and survival as well as glycogen and protein synthesis) and inhibition of FKHRL1 (the Akt substrate and transcriptional factor that leads to apoptosis and cell cycle arrest) in the oocyte (Reddy et al., 2005). Although an elevated steady-state level of KL mRNA in secondary follicles was verified in this study, which is similar to previous results (human: Carlsson et al., 2006, rabbit: Hutt et al., 2006b), few follicles were observed at this

stage after 7 days of culture with KL. This lack of follicles most likely occurs due to the short culture period or the absence of some hormones or other growth factors in the culture medium that could be important for the transition from primary to secondary follicles, such as activin (Zhao et al., 2001), bone morphogenetic protein-15 (BMP-15) (Galloway et al., 2000), or growth and differentiation factor-9 (GDF-9) (Hayashi et al., 1999).

In relation to growth, an increase in the follicular and oocyte diameter was observed after 7 days of culture with 50 ng/ml of KL compared to the control. The role of KL in the promotion of early oocyte growth has already been demonstrated in vitro (Packer et al., 1994), and the steady-state level of mRNA of both KL and c-kit is consistent with this role (Manova et al., 1993). Previous studies have indicated that KL stimulates oocyte growth during earlier stages of follicular development (mouse: Klinger and De Felici, 2002; rabbit: Hutt et al., 2006b). However, other investigations did not observe any effect of KL on oocyte (mouse: Cecconi and Colonna, 1996; hamster: Wang and Roy, 2004) or follicle (human: Carlsson et al., 2006) growth, suggesting that KL's actions differ according to the stage of follicular development. In addition, Parrot and Skinner (1997) demonstrated that 50 ng/ml of KL stimulated theca cell growth. The mechanism by which KL results in oocyte growth is unknown. However, proteins involved in the translation of the c-kit signal, such as PI3-kinases, MAP kinases, and Janus-activated kinase 2 (JAK2), are possible candidates. KL is likely to initiate oocyte growth with the slow accumulation of required factors for meiosis resumption (e.g., p34cd2, cyclin B1, MAPK, cdc25) (Reddy et al., 2005). Moreover, based on the ability of KL to induce oocyte growth, which requires both the presence of KL and contact with granulosa cells (Thomas and Vanderhyden, 2006), KL actions are suggested to be modulated by the presence of gap junctions (Klinger and De Felici, 2002) according to the oocyte stage.

Our results on follicular viability after classical histology were confirmed by ultrastructural analysis. This technique, which is able to detect damage to cellular membranes and organelles that is not visible at the level of light microscopy, is considered an important tool to discern the quality of the follicle and oocyte (Lucci et al., 2001). Important structures, such as the mitochondria, endoplasmic reticulum, and granulosa, were preserved after transmission electron microscopy (TEM) in addition to the basement and nuclear membranes after 7 days of culture in the presence of 50 ng/ml of KL. However, cortical tissues cultured with 50 ng/ml KL for 7 days demonstrated some detachment of granulosa cells from the oocyte. This result was also observed in caprine preantral follicles cultured in vitro (Lima-

Verde, unpublished work), and does not necessarily correlate with follicular degeneration. Normal follicles exhibited a similar ultrastructure to the previously described caprine follicles (Lucci et al., 2001) as well as the bovine and ovine follicles (Cran et al., 1980; van Wezel and Rodgers, 1996).

In conclusion, the mRNAs for KL were detected for all investigated follicular categories and cellular types. Furthermore, this study demonstrated that the 50 ng/ml concentration of KL promoted follicular survival and growth as well as the transition from primordial to primary follicles after 7 days of culture in vitro. The results on the steady-state level of KL mRNA as well as the culture system established in this work contribute to future investigations of the mechanisms and factors involved in early follicular development and therefore promote the development of new strategies in pro-fertility and contraceptive biotechnologies.

MATERIALS AND METHODS

Steady-State Level of KL mRNA in Goat Ovarian Follicles

To evaluate steady-state level of mRNA, 30 ovaries from 15 goats (Capra hircus) were collected at a local slaughterhouse and rinsed in saline (0.9% NaCl) containing antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). After this preparation, 10 ovaries from 5 goats were utilized for isolation of primordial, primary, and secondary follicles. Fifteen ovaries were used for collection of COCs, mural granulosa cells, and thecal cells from small and large antral follicles. Primordial, primary, and secondary follicles were isolated using a mechanical procedure, as previously described (Lucci et al., 1999). After isolation, these follicles were washed several times to completely remove the stromal cells and were then placed by category into separate Eppendorf tubes in groups of 10. This procedure was completed within 2 hr, and all samples were stored at -80°C until the RNA was extracted. From a second group of ovaries (n = 20), COCs aspirated from small (1–3 mm) and large (3–6 mm) antral follicles were recovered. Compact COCs were selected from the follicle content as described by van Tol and Bevers (1998). Thereafter, groups of 10 COCs were stored at -80°C until RNA extraction. To collect mural granulosa and theca cell complex, small (n = 10) and large antral follicles (n = 10) were isolated from ovaries (n = 5) and dissected free from stromal tissue with forceps as previously described (van Tol and Bevers, 1998). The follicles were then bisected and granulosa and theca cell complexes were collected and stored at -80°C.

Isolation of total RNA was performed using Trizol plus purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 ml of Trizol solution was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000*g* for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz units/ml) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 µl RNAse-free water.

Prior to reverse transcription, the eluted RNA samples were incubated for 5 min at 70°C, and chilled on ice. Reverse transcription was then performed in a total volume of 20 µl, which was comprised of 10 µl of sample RNA, 4 µl 5X reverse transcriptase buffer (Invitrogen), 8 U RNAseout, 150 U Superscript III reverse transcriptase, 0.036 U random primers (Invitrogen), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 hr at 42°C, for 5 min at 80°C, and then stored at -20°C. Negative controls were prepared under the same conditions but without the inclusion of the reverse transcriptase.

Quantification of the mRNA for KL was performed using SYBR Green. PCR reactions were composed of 1 µl cDNA as a template in 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µl of ultra-pure water, and 0.5 µM of each primer. The primers were designed to perform amplification of mRNA for KL. The GAPDH and b-actin (Table 2) were used as endogenous controls for normalization of steady-state level of mRNA of gene. The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 min at 94°C, followed by 40 cycles of 15 sec at 94°C, 30 sec at 60°C, and 45 sec at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany). The delta-delta-CT method was used to transform CT values into normalized relative steady-state level of mRNA.

TABLE 2. Oligonucleotide Primers Used for PCR Analysis of Goat Cells and Tissues.

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
GAPDH	TGTTTGTGATGGGCGTGAACCA	s	287-309	GI: 27525390
	ATGGCGTGGACAGTGGTCATAA	as	440-462	(2005)*Capra hircus* GAPDH
Beta-Actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI: 28628620
	TCCTTGATGTCACGGACGATTTCC	as	386-410	(2003)*Capra hircus* Beta-Actin
KL	AGCGAGATGGTGGAACAACTGTCA	s	211-235	GI: 16580734
	GTTCTTCCATGCACTCCACAAGGT	as	35-59	(2001)*Capra hircus* KL

s,sense; as, antisense

In Vitro Culture of Ovarian Tissue and Morphological Evaluation

Ovarian cortical tissues were collected at a local slaughterhouse from five adult (1–3 years old), mixed-breed goats (two ovaries from each goat). Immediately postmortem, the ovaries were washed in 70% alcohol for 10 sec, followed by two washes in Minimum Essential Medium (MEM) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin. The pairs of ovaries were transported within 1 hr to the laboratory in MEM at 4°C.

The organ culture system utilized herein was previously described in detail (Matos et al., 2007; Celestino et al., 2009). In the laboratory, the ovaries from each animal were stripped of surrounding fat tissue and ligaments. Moreover, the medulla, visible growing follicles and corpora lutea were removed. Ovarian cortex tissue samples from each ovarian pair were cut into 13 slices (approximate size 3 x 3 mm^2, with 1 mm thickness) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultrastructural analysis (fresh control) or placed in culture for a total of 1 or 7 days. Caprine tissues were transferred to 24-well culture dishes containing 1 ml of culture media. The culture was performed at 39°C in 5% CO_2 in a humidified incubator and all the media were incubated for 1 hr prior to use. The basic culture medium (culture control) consisted of 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) (Sigma, St. Louis, MO), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxantine, and 1.25 mg/ml bovine serum albumin (BSA), and was called MEM^+. For experimental conditions, the medium was supplemented with recombinant human KL (rhKL) at different concentrations (0, 1, 10, 50, 100, or 200 ng/ml) (Cell Sciences, Canton, MA). Each treatment was repeated five times, that is, five different goats were used, and the culture media were replenished every other day.

Before culture (fresh control) and after 1 or 7 days in culture, all tissues were fixed in Carnoy's solution for 12 hr and then dehydrated with increasing concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), 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 on a microscope (Nikon, Tokyo, Japan) under 400x magnification. The follicles were carefully counted once, as performed in our earlier studies (Matos et al., 2007; Celestino et al., 2009). Each follicle was examined in every section that the follicle appeared in and matched with the same follicle on adjacent sections to avoid double counting and therefore ensure that each follicle was only counted once regardless of size.

The developmental stages of follicles have been previously defined (Silva et al., 2004) as primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened and 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 individually classified as histologically normal when an intact oocyte was present and surrounded by granulosa cells, which were well organized into one or more layers and no pyknotic nucleus was present. Atretic follicles were defined as follicles 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 in one repetition x 5 repetitions = 150 follicles).

To evaluate follicular activation, the percentage of healthy primordial and growing follicles were calculated before (fresh control) and after culture in each medium. Follicle and oocyte diameters were only measured in unilaminar (primordial, intermediate, and primary follicles) and healthy follicles with the aid of an ocular micrometer. Both diameters, from the basement membrane, at right angles to each other in the largest cross-section of each growing oocyte and follicle, were measured and averaged.

Ultrastructural Analysis

To better evaluate follicular morphology, ultrastructural analysis was performed on preantral follicles from noncultured tissue, follicles cultured in MEM^+ alone, as well as on the treatment which demonstrated the best results for morphology, activation, and growth. Small pieces (1 mm^3) of caprine ovarian tissues from treatments mentioned above were fixed in 2%

paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 hr 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 hr. Subsequently, the samples were dehydrated through a gradient of acetone solutions and the tissues were embedded in Spurr's resin. Semi-thin sections (3 µm) were cut on an ultramicrotome (Reichert Supernova, Heidelberg, Germany) for light microscopy studies and stained with toluidine blue. The ultra-thin sections (60–70 µm) were contrasted with uranyl acetate and lead citrate, and examined under a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope. Parameters were further evaluated, such as the density and integrity of ooplasmic and granulosa cell organelles, vacuolization, and basement membrane integrity. For ultrastructural analysis, 3–5 follicles were examined per group.

Statistical Analysis

The percentages of morphologically normal, primordial, and developing follicles after 1 or 7 days of culture were initially subjected to Smirnov–Kolmogorov and Bartlett tests to confirm normal distribution and homogeneity of variance, respectively. Analysis of variance (ANOVA) was then performed using the GLM procedure of SAS (1997) and the Dunnett test was applied for comparison of each treatment tested against the control groups (Control and MEM^{+}) (Steel et al., 1997). The Student–Newman–Keuls (SNK) test was utilized to evaluate the effects of different concentrations of KL and days of culture. Due to heterogeneity, steady-state levels of mRNA in primordial, primary, secondary, and in small and large antral follicles were analyzed by the Kruskal–Wallis nonparametric test. Differences were considered to be significant when $P < 0.05$, and results were expressed as the mean ± standard deviation (SD).

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11 CAPÍTULO 6

Níveis da proteína morfogenética óssea-15 em ovários caprinos e sua influência no desenvolvimento *in vitro* e sobrevivência de folículos pré-antrais

Steady-state level of bone morphogenetic protein-15 in goat ovaries and its influence on *in vitro* development and survival of preantral follicles

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Resumo

Este estudo investiga os níveis de RNAm para a proteína morfogenética óssea-15 (BMP-15) em ovários caprinos, e os efeitos da BMP-15 no desenvolvimento *in vitro* de folículos pré-antrais. Fragmentos ovarianos foram cultivados por um ou sete dias em Meio Essencial Mínimo (MEM^+) com BMP-15 (0, 1, 10, 50, 100 ou 200 ng/mL), e posteriormente foram analisados por histologia, microscopia eletrônica de transmissão e de fluorescência. O RNAm para BMP-15 em folículos secundários foi superior que em folículos primordiais e primários. Após sete dias, 10, 50 ou 100 ng/mL de BMP-15 mantiveram a percentagem de folículos normais similar ao controle (não-cultivado), e aumentou os diâmetros oócitário e folicular quando comparado ao controle e ao MEM^+. BMP-15 100 ng/mL aumentou a percentagem de folículos secundários e manteve a integridade ultraestrutural. Em conclusão, os RNAm para BMP-15 foram detectados em todas as categorias foliculares. BMP-15 (100 ng/mL) manteve a integridade e promoveu o crescimento de folículos pré-antrais caprinos cultivados por sete dias.

Palavras-chave: Caprino. Folículos pré-antrais. BMP-15. Ativação. Crescimento.

Steady-state level of bone morphogenetic protein-15 in goat ovaries and its influence on *in vitro* development and survival of preantral follicles

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Abstract

This study investigates steady-state level of bone morphogenetic protein-15 (BMP-15) mRNA in caprine follicles, and the effects of BMP-15 on *in vitro* development of preantral follicles. Ovarian fragments were cultured for one or seven days in Minimal Essential Medium (MEM^+) with BMP-15 (0, 1, 10, 50, 100 or 200 ng/mL), and further analysed by histology, transmission electron and fluorescent microscopy. BMP-15 mRNA in secondary follicles was higher than in primordial and primary follicles. After seven days, 10, 50 or 100 ng/mL of BMP-15 maintained the percentage of normal follicles similar to the control (noncultured), and increased the oocyte and follicle diameters when compared to the control and MEM^+. BMP-15 at 100 ng/mL increased the secondary follicles and maintained their ultrastructural integrity. In conclusion, the BMP-15 mRNAs were detected in all follicular categories. BMP-15 (100 ng/mL) maintained the integrity and promoted the growth of caprine preantral follicles cultured for seven days.

Keywords: Caprine; Preantral Follicles; BMP-15; Activation; Growth.

1. Introduction

The mechanisms that control the initiation of follicular development, i.e., the activation of primordial follicles, and the time necessary for their growth and differentiation remains unknown (Fortune, 2003). Therefore, the development of an efficient culture system that allows the activation and development of early stage follicles (preantral) up to later stages is essential. This system would provide oocytes that can be further used for the *in vitro* embryo production. In addition, this system would provide a better understanding of the factors involved in folliculogenesis. Among these factors, bone morphogenetic protein-15 (BMP-15) can be highlighted.

BMP-15, also known as growth differentiation factor-9B (GDF-9B), is a member of the transforming growth factor-β (TGF-β) superfamily. The ligands of this superfamily regulate cell proliferation, differentiation and apoptosis, and they are essential for embryo development, organogenesis, bone formation, reproduction and other biologic processes (Chang et al., 2002; Mazerbourg and Hsueh, 2006). BMP-15 binds through a type I receptor, i.e., BMPR-IA (activin receptor-like kinase [ALK]3) and BMPR-IB (ALK-6), and a type II receptor on the surface of the cell (Miyazono et al., 1997; Lebrun et al., 1997). The activated type I receptor phosphorylates one or more cytoplasmic signaling intermediates, known as Smads receptors (R-Smads) (Moore et al., 2003; Shimasaki et al., 2004a).

In addition to being found in other tissues (Galloway et al., 2000; Eckery et al., 2002; Otsuka and Shimasaki, 2002a), BMP-15 is produced in the largest quantities in the ovary (Peng et al., 2009). In marsupial (Eckery et al., 2002), ovine (Mery et al., 2007), bovine (Bodensteiner et al., 1999) and human (Aaltonen et al., 1999), BMP-15 is expressed in oocytes from primordial follicles onward. In rodents, the expression of this protein is observed only in the oocyte from primary follicles onward (Laitinen et al., 1998; Jaatinen et al., 1999). For caprine species, BMP-15 has been found in the oocytes and granulosa cells of all types of follicles but not in granulosa cells of primordial follicles. The mRNAs for BMP-15 and their associated receptors were detected in primordial, primary and secondary follicles and in the oocyte and granulosa cells of antral follicles (Silva et al., 2005). However, quantification of the steady-state level of BMP-15 mRNA during different stages of goat follicular development has not yet been performed.

In vitro studies have demonstrated that BMP-15 promotes the proliferation of the granulosa cells and stimulates the development of primordial and primary follicles in rodents

(Otsuka et al., 2000; Vitt et al., 2000a; Fortune, 2003). *In vivo* studies have shown that BMP-15-mutant sheep, i.e., without BMP-15, are infertile because they do not continue their development beyond the primary follicle stage (Dong et al., 1996; Galloway et al., 2000). In rats, recombinant BMP-15 stimulated the proliferation of granulosa cells independent of follicle stimulating hormone (FSH) (Otsuka et al., 2000). Furthermore, BMP-15 is able to inhibit the expression of the FSH receptor (Otsuka et al., 2001) and stimulate the expression of the kit ligand (KL) and epidermal growth factor (EGF) in rat granulosa cells (Otsuka and Shimasaki, 2002b) and mouse cumulus cells (Yoshino et al., 2006), respectively. In a study performed with sheep, BMP-15 was suggested to be required for the follicular development up to the ovulatory stage (Juengel et al., 2002). However, the influence of BMP-15 during caprine folliculogenesis is unknown. Results for this species are of great economic importance due to its production of milk, meat and fibers, and its potential to provide the main source of proteins for the population of the tropics. Therefore, the present study was designed with the following aims: (1) to determine the steady-state level of BMP-15 mRNA during different follicular stages in goat ovaries and (2) to investigate a possible influence of BMP-15 at different concentrations (0, 1, 10, 50, 100 or 200 ng/mL) on the survival, activation and further growth of preantral follicles enclosed in caprine ovarian tissue that was cultured for one or seven days.

2. Materials and Methods

2.1. Steady-state level of BMP-15 mRNA in goat ovarian follicles

To evaluate the steady-state level of mRNA, 30 ovaries from 15 goats (*Capra hircus*) were collected at a local slaughterhouse and rinsed in Minimum Essential Medium (MEM) containing antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). After this preparation, 10 ovaries from five goats were used for the isolation of primordial, primary, and secondary follicles. The remaining 20 ovaries were used for collection of the cumulus oocyte complex (COCs), mural granulosa and thecal cells from small and large antral follicles. Primordial and primary follicles were isolated using a mechanical procedure, as previously described (Lucci et al., 1999). Using a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) that was adjusted for a sectioning interval of 75 µm, samples were cut into small pieces, placed in MEM, resuspended 40 times using a large Pasteur pipette

(approximate diameter of 1600 μm) and resuspended again 40 times with a smaller Pasteur pipette (approximate diameter of 600 μm) to dissociate preantral follicles from stroma. The material obtained was passed through 100-μm nylon mesh filters and resulted in a suspension containing preantral follicles with less than a 100-μm diameter. This procedure was carried out at room temperature (RT, approximately 25°C) within a 10-min time frame.

To isolate the secondary follicles, ovarian cortical slices (1 - 2 mm thickness) were cut from the ovarian surface using a surgical blade under sterile conditions. Then, the ovarian cortex was placed in fragmentation medium, consisting of MEM plus HEPES. Secondary follicles of approximately 150-μm diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the strips of ovarian cortex using 26-gauge (26-G) needles. After isolation, all follicles were washed several times to completely remove the stromal cells and were then placed by category into separate Eppendorf tubes in groups of 10. This procedure was completed within 2 h, and all samples were stored at -80°C until the RNA was extracted. From a second group of ovaries (n=15), COCs aspirated from small (1–3 mm) and large (3–6 mm) antral follicles were recovered. Compact COCs were selected from the follicle content as described by van Tol and Bevers (1998). Thereafter, groups of 10 COCs were stored at -80°C until RNA extraction. To collect mural granulosa and theca cell complexes, small (n=10) and large antral follicles (n=10) were isolated from ovaries (n=5) and dissected from stromal tissue with forceps as previously described (van Tol and Bevers, 1998). The follicles were then bisected and granulosa and theca cell complexes were collected and stored at -80°C.

Isolation of total RNA was performed using Trizol and a purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 mL of Trizol solution was added to each frozen sample and the lysate was aspirated through a 20-G needle before centrifugation at 10,000 g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After the binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz units/mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 μL RNAse-free water.

Prior to reverse transcription, the eluted RNA samples were incubated for 5 min at 70°C and chilled on ice. Reverse transcription was then performed in a total volume of 20 μL, which was comprised of 10 μL sample RNA, 4 μL 5X reverse transcriptase buffer (Invitrogen), 8 U RNAse-out, 150 U Superscript III reverse transcriptase, 0.036 U random

primers (Invitrogen), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C, for 5 min at 80°C, and then stored at -20°C. Negative controls were prepared under the same conditions but without the inclusion of the reverse transcriptase.

Quantification of the mRNA for BMP-15 was performed using SYBR Green. PCR reactions were composed of 1 µL cDNA as a template in 7.5 µL SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µL ultra-pure water, and 0.5 µM each primer. The primers were designed to perform amplification of mRNA for BMP-15. GAPDH and b-actin (Table 1) were used as endogenous controls for normalization of the steady-state level of mRNA of the genes. The thermal cycling profile for the first round of PCR was as follows: initial denaturation and activation of the polymerase for 15 min at 94°C, followed by 40 cycles of 15 sec at 94°C, 30 sec at 60°C, and 45 sec at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany). The delta-delta-CT method was used to transform CT values into a normalized relative steady-state level of mRNA.

Table 1. Oligonucleotide primers used for PCR analysis of goat cells and tissues.

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
GAPDH	TGTTTGTGATGGGCGTGAACCA	s	287-309	GI:27525390 (2005)*Capra hircus*
	ATGGCGTGGACAGTGGTCATAA	as	440-462	GAPDH
Beta-Actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI:28628620 (2003)*Capra hircus*
	TCCTTGATGTCACGGACGATTTCC	as	386-410	Beta-Actin
BMP-15	AAGTGGACACCCTAGGGAAA	s	237-257	GI: 8925958 (2000)*Ovis aries*
	TTGGTATGCTACCCGGTTTGGT	as	362-384	BMP15

s,sense; as, antisense

2.2. *In vitro* culture of ovarian tissue and morphological evaluation

Ovarian cortical tissues (n=8 ovaries) were collected at a local slaughterhouse from four adult (1 – 3 years old) mixed-breed goats (*Capra hircus*). Immediately postmortem, the ovaries were washed in 70% alcohol for 10 seconds followed by two washes in MEM supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The ovary pairs were

transported within 1 hour to the laboratory in MEM at 4°C (Chaves et al., 2008). All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

Our organ culture system was described in detail previously (Celestino et al., 2010). Ovarian tissue samples from each ovarian pair were cut in 13 slices (3 mm x 3 mm x 1 mm) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultrastructural analysis (fresh control tissue) or placed in culture for one or seven 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 of the media was incubated for 1 h prior to use. The basic culture medium (cultured control tissue) consisted of MEM (pH 7.2 – 7.4) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5.0 ng/mL selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxantine and 1.25 mg/mL bovine serum albumin (BSA), which combined was called MEM^+. For the experimental conditions, the medium was supplemented with recombinant human BMP-15 (rhBMP-15, R&D Systems, Minneapolis, MN, USA) at different concentrations (1, 10, 50, 100 or 200 ng/mL). Each treatment was repeated four times, and the culture media was replenished every other day.

Before culture (fresh control tissue) and after one or seven days in culture, all of the pieces were fixed in Carnoy's solution for 12 h and then dehydrated in increasing concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the caprine tissue 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, anonymous slides were examined on a microscope (Nikon, Japan) under 400X magnification.

The developmental stages of follicles have been defined previously (Silva et al., 2004) as either primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened or 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 and surrounded by granulosa cells, which are well organized in one or more layers and have no pyknotic nucleus. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells

detached from the basement membrane. Overall, 120 follicles were evaluated for each treatment (30 follicles per treatment for one repetition x four repetitions = 120 follicles).

To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before (fresh control tissue) and after culture in each medium. Follicle and oocyte diameters were only measured in healthy follicles with the aid of an ocular micrometer. Both basement membrane diameters were measured at right angles to each other in the largest cross-section of each growing oocyte and follicle were then averaged. Care was taken to count each follicle only once as previously described (Celestino et al., 2010). Each follicle was examined in every section 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.

2.3. Ultrastructural analysis of caprine preantral follicles

For a more in-depth evaluation of follicular morphology after histological analysis, ultrastructural studies were performed on fragments of fresh control tissue and treatment groups that maintained follicular survival and promoted growth after seven days of culture. A portion with a maximum dimension of 1 mm^3 was cut from each fragment of ovarian tissue and fixed in Karnovsky solution (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2) for 3 h at RT. After three washes in sodium cacodylate buffer, specimens 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 at RT. The samples were then dehydrated through a gradient of acetone solutions, and thereafter, they were embedded in SPIN-PON resin (Sigma Company, St Louis, MO). Afterwards, semi-thin sections (3 µm) were cut, stained with toluidine blue and analyzed by light microscopy at 400× magnification. Ultra-thin sections (60–70 nm) were obtained from preantral follicles, which were classified as morphologically normal in semi-thin sections according to the criteria adopted in histology. Subsequently, ultra-thin sections were contrasted with uranyl acetate and lead citrate, and they were examined under a Morgani-FEI transmission electron microscope (TEM) operating at 80 kV. The density and integrity of ooplasmic and granulosa cell organelles as well as vacuolization and membrane integrity were evaluated.

2.4. Assessment of preantral follicle viability by fluorescence microscopy

Based on the results of the morphological and ultrastructural analyses, the viability of follicles from control preparations and preparations cultured with the concentration of BMP-15 that maintained follicular morphology and ultrastructural integrity was further analyzed using fluorescent probes.

Additional pairs of goat ovaries (n = 2) were collected from a slaughterhouse, and cut into fragments at the laboratory. One of these fragments was immediately processed for follicle isolation (fresh control tissue) and the remaining fragments were cultured for seven days with BMP-15 (100 ng/mL) as described above. After the culture period, fragments were processed for mechanical isolation using the method described by Lucci et al. (1999). Preantral follicles were analyzed using a two-color fluorescence cell viability assay based on the simultaneous detection of live and dead cells with calcein-AM and ethidium homodimer-1, respectively. While the first probe detected intracellular esterase activity in viable cells, the latter labeled nucleic acids in 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, Eugene, OR, USA) to a suspension of isolated follicles and incubating them at 37°C for 15 min. After being labeled, follicles were washed once by centrifugation at 100× g for 5 min and resuspended in MEM. Cells were then mounted on glass microscope slides in 5 μl anti-fading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching, and they were examined using a fluorescence microscope (Nikon Eclipse 80*i*, Tokyo, Japan). The emitted fluorescence signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. Oocytes and granulosa cells were considered viable if their cytoplasm stained positively with calcein-AM (green) and their chromatin was not labeled with ethidium homodimer-1 (red).

2.5. Statistical analysis

Data of mRNA expression in primordial, primary and secondary follicles were analyzed by Kruskal-Wallis test, while t test was used for paired comparisons of mRNA expression in small and large antral follicles ($P<0.05$). Percentages of morphologically normal, primordial and growing follicles after one or seven days of culture were initially submitted to Smirnov-Kolmogorov and Batlett tests to confirm a normal distribution and

homogeneity of variance, respectively. Analysis of variance (ANOVA) was then carried out using GLM procedure of SAS (1997), and a Dunnett test was applied for the comparison of each treatment tested against control groups (Control and MEM^+). Student Newman Keuls (SNK) test was used to evaluate the effects of different concentrations of BMP-15 and days of culture with the results expressed as mean ± standard deviation (SD). The number of secondary follicles varied widely amongst ovarian fragments obtained from the four animals used in the assay. Thus, these structures were analyzed as a pool for each treatment, and comparisons were done by a Chi-square test with the results expressed as a percentage of morphologically normal secondary follicles. Data obtained from fluorescence microscopy for validation of histological and ultrastructural findings were also taken as a pool and analyzed by Chi-square test. In all cases, differences were considered to be statistically significant when $P<0.05$.

3. Results

3.1. Steady-state level of BMP-15 mRNA in goat ovarian follicles

Quantification of mRNA expression demonstrated that secondary follicles had significantly higher levels of mRNA for BMP-15 than primordial to primary follicle stages ($P<0.05$ – Figure 1A). When the levels of mRNA for BMP-15 in primordial and primary follicles were compared, no significant differences were observed ($P>0.05$ – Figure 1B). In addition, no significant difference was observed between COCs collected from small and large antral follicles ($P>0.05$ – Figure 1B). Similar results were observed for granulosa / theca cells from small and large antral follicles ($P>0.05$ – Figure 1C). On the other hand, real-time PCR showed that COCs from either small or large antral follicles had significantly higher levels of mRNA for BMP-15 than their respective granulosa / theca cells ($P<0.05$ – Figure 1 D, E).

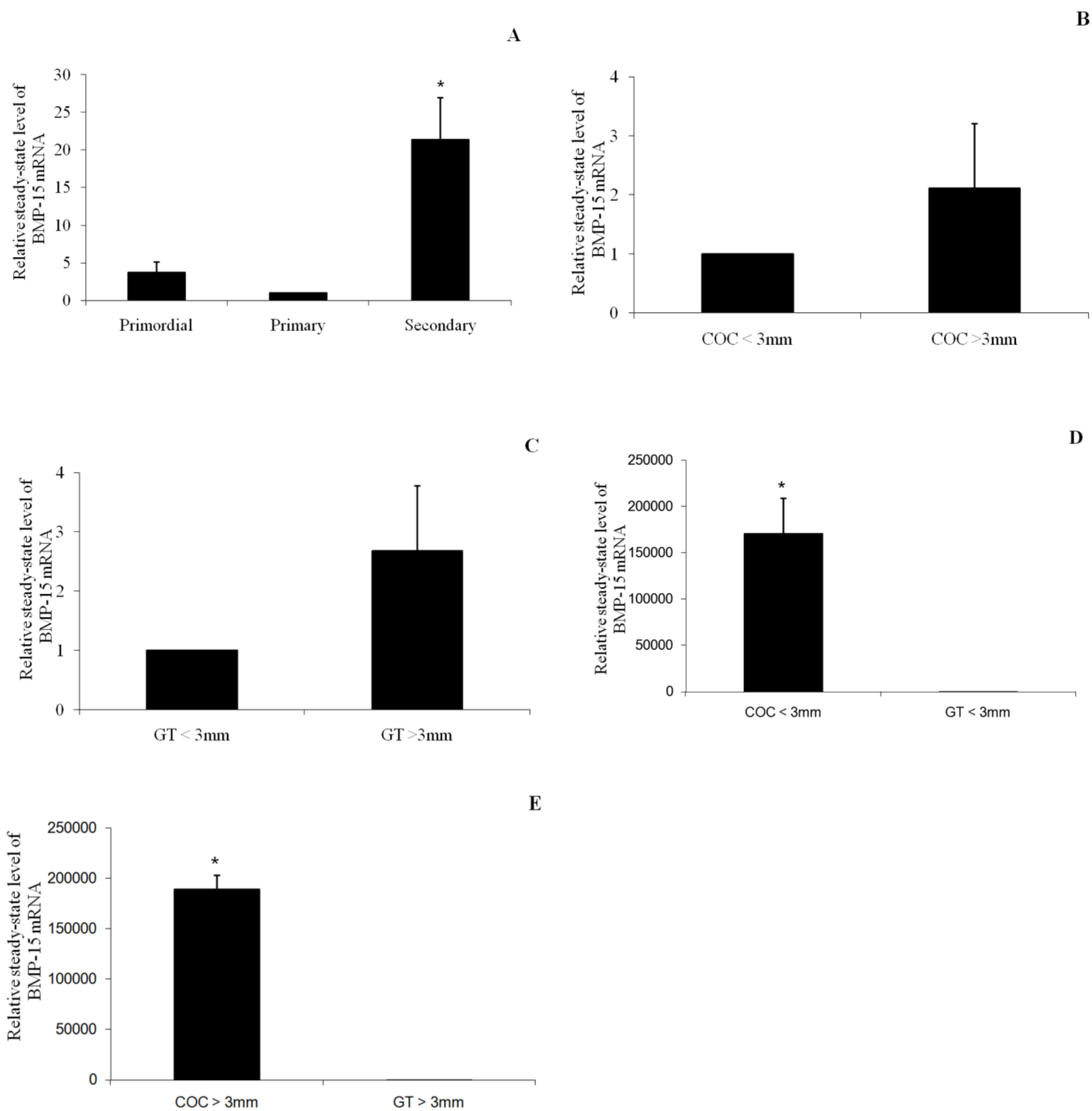

Figure 1. Steady-state level of BMP-15 mRNA in goat ovarian follicles (mean ± SD). A) Primordial, primary, and secondary follicles, B) COCs from small and large antral follicles, C) granulosa/theca cells from small and large antral follicles, D) COCs and granulosa/theca cells from small antral follicles, and E) COCs and granulosa/theca cells from large antral follicles.

* ($P<0.05$).

3.2. Caprine preantral follicles survival before and after *in vitro* culture

A total of 1,560 preantral follicles were analyzed. Figure 2A shows normal secondary follicle after culture in BMP-15 at 100 ng/mL, whereas Figure 2B illustrates degenerated follicle after culture in MEM^+ alone, both after seven days of culture. In the degenerated follicle (Figure 2B), we observed retracted oocyte, pycnotic nucleus and disorganized granulosa cells.

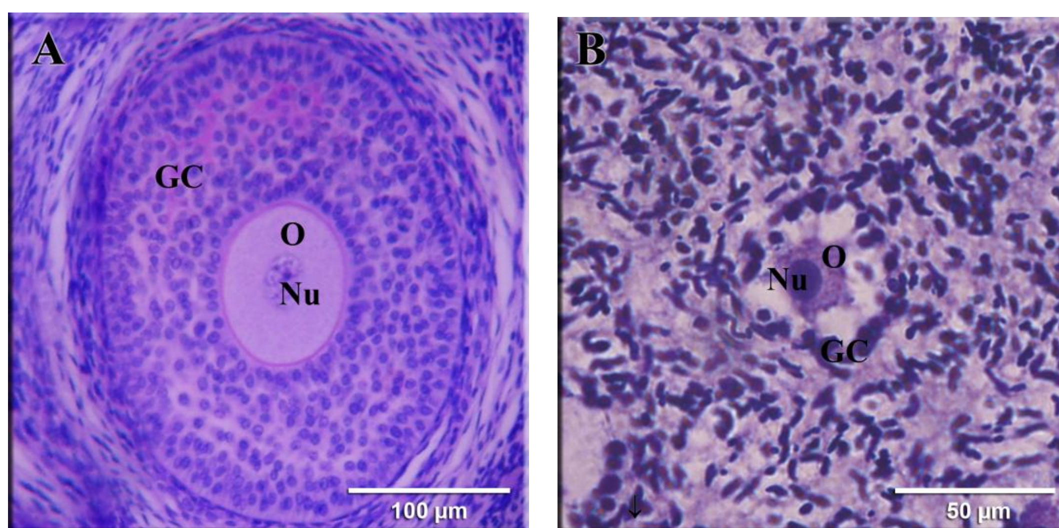

Figure 2. Histological section after staining with periodic acid Schiff-hematoxylin, showing (A) a normal follicle after culture in BMP-15 at 100 ng/mL and (B) a degenerated follicle after culture in MEM^+ alone. Both were after seven days of culture. Note the cuboidal granulosa cells (GC) layers in the normal secondary follicle (A) and the retracted oocyte with a pyknotic nucleus (B). O: oocyte; Nu: oocyte nucleus. (A: bar = 100 µm; B: bar = 50 µm).

The percentages of morphologically normal preantral follicles in control (fresh) tissue and in tissue cultured in different treatments for one or seven days are shown in Figure 3. After seven days of culture, the percentage of normal follicles was similar ($P>0.05$) to fresh control tissue (83%) when the fragments were cultured in BMP-15 at 10 (79%), 50 (76%) or 100 ng/mL (75%), and no differences ($P>0.05$) existed among these concentrations and 1 ng/mL (72%). However, culture in medium supplemented with 200 ng/mL BMP-15 significantly reduced ($P<0.05$) the percentage of normal follicles compared to fresh control tissue and other concentrations of BMP-15, except 1 ng/mL. In addition, after seven days, in all BMP-15 concentration tested, higher percentages ($P<0.05$) of normal follicles were found

compared to MEM$^+$ alone. Examining the progression of the culture period from one to seven days, a significant decrease (P<0.05) in the percentage of normal follicles in MEM$^+$ alone or supplemented with 200 ng/mL BMP-15 was observed.

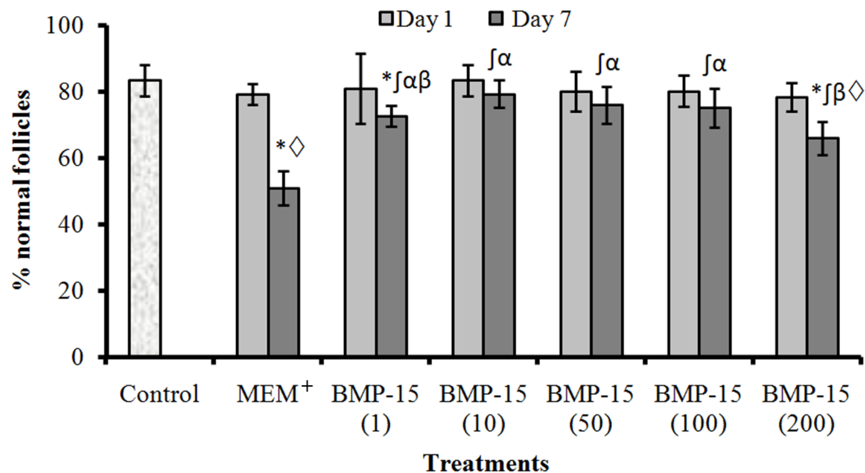

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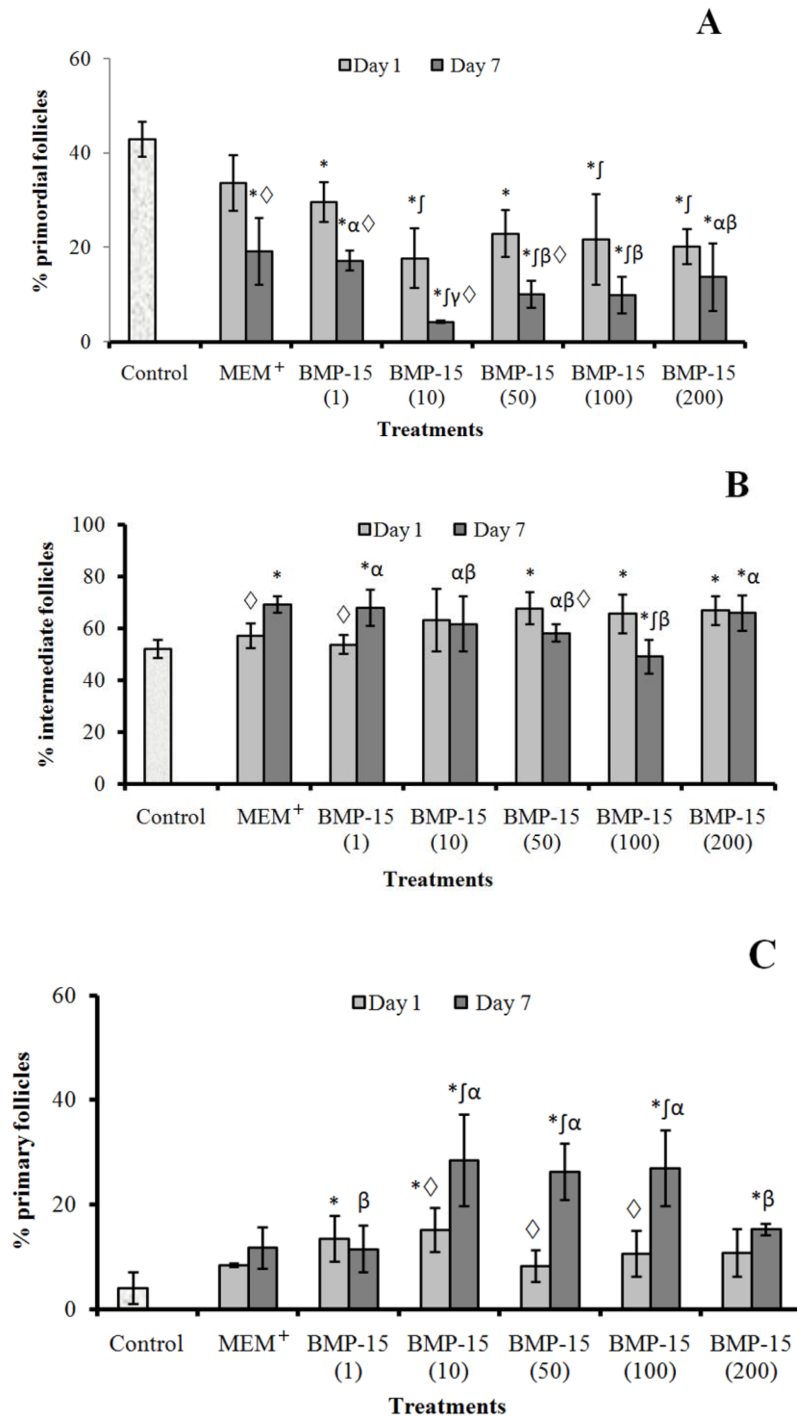
Figure 3. Percentage (mean ± S.E.M.) of morphologically normal preantral follicles in control (non-cultured) and after *in vitro* culture for one or seven days in the absence or presence of bone morphogenetic protein-15. * differs significantly from control follicles (P<0.05); ʃ differs significantly from MEM$^+$ in each day of culture (P<0.05); α,β differs significantly among concentrations in each day of culture (P<0.05); ◊ differs significantly with the progression of the culture period from one to seven days in the same treatment (P<0.05).

3.3. Follicular activation and growth after *in vitro* culture

Even after one day of culture, BMP-15 at higher concentrations (50, 100 or 200 ng/mL) promoted a significant reduction in the percentage of primordial follicles (Figure 4A, P<0.05) and was associated with an increase in the percentage of intermediate follicles (Figure 4B, P<0.05), compared to fresh control tissue. When the culture period progresses from one to seven days, a significant (P<0.05) increase in the percentage of intermediate follicles in MEM$^+$ alone or supplemented with 1 ng/mL BMP-15 was observed. After seven days of culture, all BMP-15 concentrations (except 1 ng/mL) significantly increased the percentage of primary follicles compared to fresh control tissue. The same result was observed in the concentrations of 10, 50 or 100 ng/mL, compared to MEM$^+$ alone (Figure 4C, P < 0.05). With relation to the secondary follicles, there was a significant increase (P<0.05)

after culturing in 100 ng/mL of BMP-15 in comparison to fresh control and MEM^{+}. Furthermore, at the same concentration, a significant increase in the percentage of secondary follicles with the progression of the culture from one to seven days was observed (Figure 4D, $P < 0.05$).



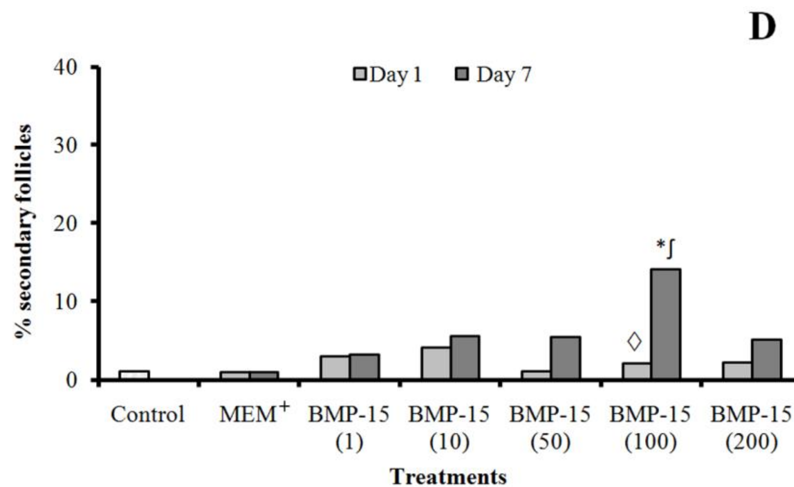


Figure 4. Percentage (mean ± S.E.M.) of primordial (A), intermediate (B), primary (C) and secondary (D) follicles in control (non-cultured) and after *in vitro* culture for one or seven days in the absence or presence of bone morphogenetic protein-15. * differs significantly from control follicles ($P<0.05$); ∫ differs significantly from MEM^+ in each day of culture ($P<0.05$); α,β,γ differs significantly among concentrations in each day culture ($P<0.05$); ◊ differs significantly with the progression of the culture period from one to seven days in the same treatment ($P<0.05$).

According to Table 2, after seven days, a significant increase in oocyte diameter compared to fresh control and MEM^+ groups was observed, which was independent of BMP-15 concentration (except 200 ng/mL) ($P<0.05$). The same result was observed for the follicular diameter for all BMP-15 concentrations ($P<0.05$). With the progression of the culture period from one to seven days, all concentrations of BMP-15 significantly increased oocyte and follicular diameters.

Table 2. Oocyte and follicle diameters (mean ± SD) in non-cultured tissues and in tissues cultured for one or seven days in MEM$^+$ (control medium) and MEM$^+$ supplemented with various concentrations of bone morphogenetic protein-15.

Treatments	Oocyte diameter (μm)		Follicle diameter (μm)	
Non-cultured (Day 0)	41.87 ± 6.46		56.86 ± 5.96	
Cultured	Day 1	Day 7	Day 1	Day 7
MEM$^+$	41.86 ± 5.25	42.18 ± 7.32	59.79 ± 4.04	62.42 ± 6.06
BMP-15 (1)	41.41 ± 6.75 ◊	48.20 ± 8.41 *ʃ	61.03 ± 6.86 ◊	68.91 ± 6.50 *ʃ
BMP-15 (10)	40.01 ± 5.16 ◊	48.30 ± 6.28 *ʃ	60.87 ± 4.82 ◊	70.30 ± 8.14 *ʃ
BMP-15 (50)	38.93 ± 5.15 ◊	51.29 ± 6.60 *ʃ	59.17 ± 5.04 ◊	74.16 ± 8.97 *ʃ
BMP-15 (100)	40.48 ± 6.72 ◊	49.13 ± 6.17 *ʃ	59.02 ± 5.48 ◊	73.08 ± 8.58 *ʃ
BMP-15 (200)	39.24 ± 6.19 ◊	47.59 ± 7.53	59.64 ± 6.11 ◊	68.75 ± 7.69 *ʃ

* differs significantly from control follicles (P<0.05)

ʃ differs significantly from MEM$^+$ alone in each day of culture (P<0.05)

◊ differs significantly with the progression of the culture period from one to seven days in the same treatment (P<0.05)

3.4. Ultrastructural features of cultured follicles

For a better evaluation of follicular integrity, ultrastructural analysis was performed in fresh control tissue and tissue cultured for seven days with MEM$^+$ supplemented with 100 ng/mL BMP-15, which was the treatment that demonstrated the best results according to the previous histological analysis (survival, growth, aside from the activation of follicles with the transition from primary to secondary follicles). Ultrastructural features of the follicles from the control tissue (Figure 5A) and 100 ng/mL BMP-15 cultured tissue (Figure 5B) were similar. These follicles showed intact oocyte membranes and organelles that were uniformly distributed in the ooplasm; notably, mitochondria with visible cristae were observed. Granulosa cells were ultrastructurally normal and well organized around the oocyte, showing an elongated and large nucleus with an irregular membrane.

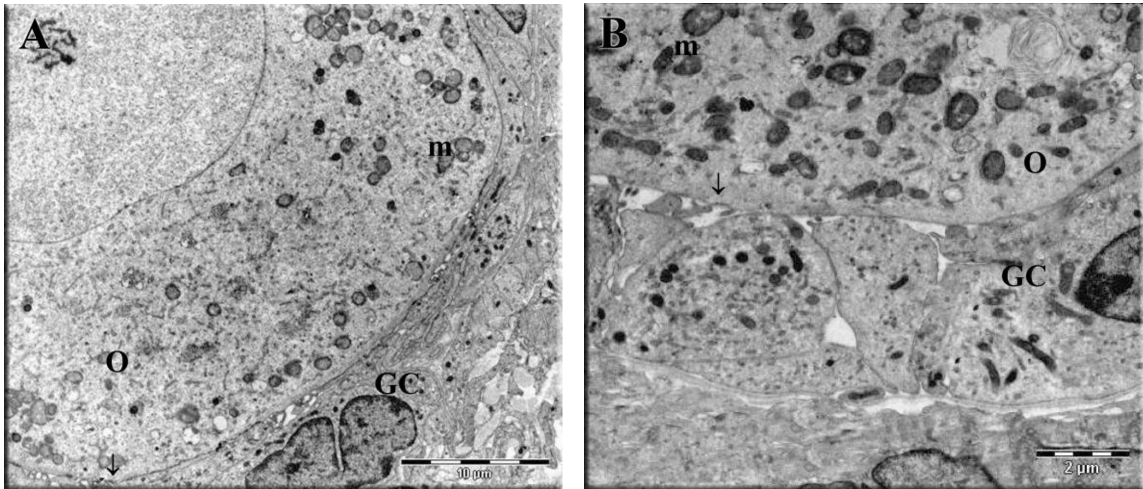


Figure 5. Ultrastructural analysis of noncultured preantral follicle (A) and follicle cultured for seven days in medium containing 100 ng/mL bone morphogenetic protein-15 (B). O: oocyte; GC: granulosa cells; m: mitochondria; arrow-oocyte membrane. (A: bar = 10 µm; B: bar = 2 µm). Three to five follicles per group were examined and the photomicrographs are representative examples.

3.5. Viability assessment of follicles cultured with BMP-15

The results from the morphological and ultrastructural evaluation demonstrated that culturing follicles with 100 ng/mL BMP-15 maintained the percentages of normal follicles, which was similar to levels seen in fresh control tissue, and it preserved the ultrastructure of the sample. Thus, a viability trial using this treatment was performed. In the present study, 30 caprine preantral follicles were analyzed after seven days of culture with MEM^+ supplemented with 100 ng/mL BMP-15. After this quantitative analysis, 90.0% of follicles remained viable, assessed by calcein-AM–ethidium homodimer assays (Figure 6), and this percentage of viable follicles was similar to the percentage observed in the fresh control group (96.7%).

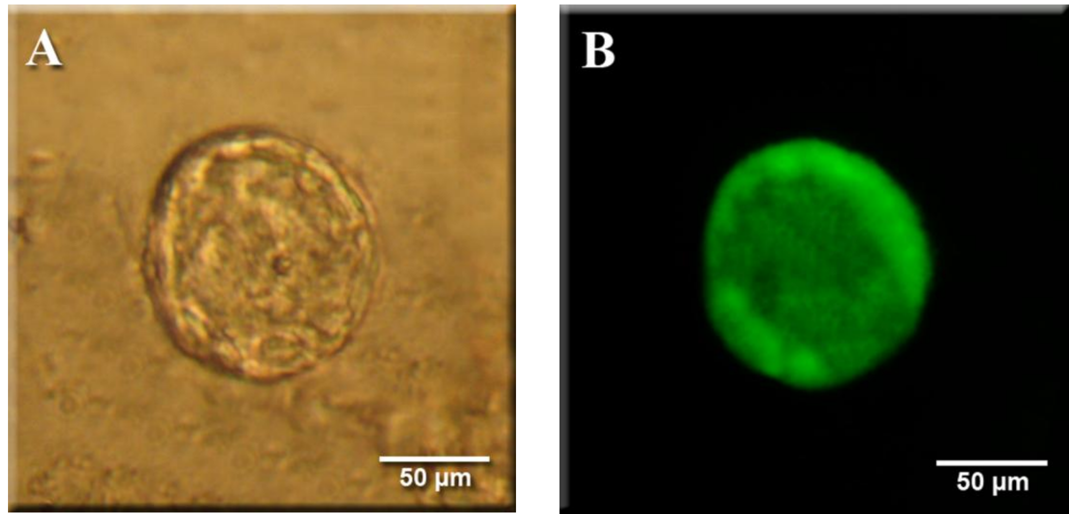


Figure 6. Viability assessment of caprine preantral follicles using fluorescent probes. (A) An isolated preantral follicle after culture with 100 ng/mL BMP-15 that was classified as viable, (B) because cells were labeled by calcein-AM (green fluorescence). Scale bars = 50 µm.

4. Discussion

In the current study, analysis of the steady-state level of mRNA that encodes BMP-15 in goat's early follicles demonstrated an increase in this level during the transition from primary to secondary follicle stages. BMP-15 has a key role in promoting follicular growth beyond the primary stage (Knight and Glister, 2003). Transgenic mice with high levels of BMP-15 expression showed a fast follicular growth with a decrease in the primary follicles and an increase in the secondary follicles (McMahon et al., 2008). In addition, in mouse oocytes, several studies have shown that BMP-15 is expressed only from the primary stage of development onward (Burns et al., 2003; Wu et al., 2003a,b; Tong et al., 2004), which differs from the present results in caprine species that demonstrated the importance of BMP-15 in the beginning of follicular growth. Similarly to the caprine species, the mRNA for BMP-15 is expressed in human primordial follicles, and its increase is directly related to follicular growth (Teixeira Filho et al., 2002). These conflicting results regarding expression may be due to differences among the species and/or differences in the methodology used, as cited by several authors (Juengel et al., 2004a; McNatty et al., 2005a,b; Chen et al., 2009).

In the current study, COCs from either small or large antral follicles had higher levels of mRNA for BMP-15 than their respective granulosa / theca cells. These data demonstrated that although BMP-15 was found in the follicular wall, the COC was mainly responsible for its production. BMP-15 has been identified as an important factor in the later stages of folliculogenesis and is related to both the inhibition of premature luteinization and cumulus cells expansion (Gilchrist et al., 2004; Shimasaki et al., 2004b, Su et al., 2008). In mice, mutations in the BMP-15 gene are harmful to the normal development of the COC (Yan et al., 2001). In this same species, when the specific gene for BMP-15 was eliminated, some defects in the process of ovulation and in the oocyte quality were observed, which resulted in a smaller size of these oocytes (Yan et al., 2001). In swine COC obtained from antral follicles (3-7 mm), the mRNA and protein for BMP-15 were expressed in low levels in immature oocytes (Li et al., 2008; Zhu et al., 2008) and increased their levels within 18 h of *in vitro* maturation, which was coincident with cumulus cells expansion (Li et al., 2008).

This study is the first to report the positive effect of BMP-15 on the *in vitro* development of caprine preantral follicles. After seven days of culture, the follicular survival was similar to the fresh control tissue with the addition of 10, 50 or 100 ng/mL BMP-15 to the culture medium. In addition, all BMP-15 concentrations maintained a higher percentage of normal follicles than MEM^+ alone. Similar to our results, studies using BMP-15 have verified its important role in preventing atresia in follicular somatic cells (Hussein et al., 2005; Yoshino et al., 2006). Previous studies have shown that after neutralization of the gene for BMP-15, a decrease in the integrity of ovine oocytes (Juengel et al., 2004b) and mouse COC (Varani et al., 2002) was observed. BMP-15 can promote follicular survival through the maintenance of granulosa cell proliferation and prevention of premature luteinization and/or atresia (Knight and Glister, 2006). Furthermore, BMP-15 is known to stimulate KL expression, which is an important factor for the *in vitro* survival of murine (Jin et al., 2005) and caprine (Celestino et al., 2010) preantral follicles. However, as suggested by Mery et al. (2007), the expression of both BMP-15 and its receptors may be reduced in *in vitro* cultured ovaries compared with those *in vivo*. Thus, larger concentrations of BMP-15 may be needed to be administered exogenously. This fact may explain the unsatisfying results obtained in our study with the concentration of 1 ng/ml BMP-15 regarding the maintenance of follicular survival. In contrast, the concentration of 200 ng/ml may have induced the expression of inhibitory Smads (I-Smads), which can act as part of an autocrine negative feedback (Miyazono, 2000), and attenuate the pattern of transduction of BMP-15 signals (Miyazono et

al., 1997). A recent study demonstrated that transgenic adult mice with increased BMP-15 expression showed a higher amount of atretic antral follicles compared to the non-transgenic control mice (McMahon et al., 2008). Another explanation for our findings is that the high concentration of BMP-15, such as 200 ng/mL, may change the expression of the follistatin growth factor, which is expressed in granulosa cells of small growing follicles in most species, including caprine (Silva et al., 2006). The follistatin may bind to BMP-15 and inhibit its bioactivity (Otsuka et al., 2001).

All BMP-15 concentrations (except for 1 ng/mL) increased the percentage of primary follicles after seven days of culture compared to the fresh control tissue and at 10, 50 or 100 ng/mL when compared to MEM^+ alone. The presence of the BMP-15 protein in some oocytes of primordial follicles (Otsuka et al., 2000) suggest that it has a role in the follicular activation, i.e., the transition from the primordial to primary follicle stage (Shimasaki et al., 2004a,b; Moore and Shimasaki, 2005). In this case, the BMP-15 would act through the BMPR-II receptor (Shimasaki et al., 2004a), found on the surface of granulosa cells, to directly stimulate their mitosis (Vitt et al., 2000b; Di Pasquale et al., 2004; McNatty et al., 2005a,b). In rodents, BMP-15 stimulated the *in vitro* development of primordial and primary follicles (Otsuka et al., 2000; Fortune, 2003). In caprine species, the protein and mRNA for BMP-15 ligands and receptors (BMPR-IA, BMPR-IB and BMPR-II) were expressed even from the primordial follicle stage (Silva et al., 2005), thus providing evidence for the role of BMP-15 in follicular activation observed in the current study.

The elevated steady-state level of BMP-15 mRNA in secondary follicles found in this study agreed with previous results (rat: Otsuka et al., 2000, human: Teixeira Filho et al., 2002). Thus, BMP-15, at 100 ng/mL, increased the percentage of secondary follicles. Genes that encode for BMP-15 are essential for the early stages of follicular growth, especially for the transition from primary to secondary follicles (Mery et al., 2007). In ovine, after 7.5 months of immunization against BMP-15, the ovaries did not show normal development beyond the primary stage (Juengel et al., 2004b). An *in vitro* study showed that the addition of 100 ng/mL BMP-15 to a culture of granulosa cells increased the expression of phosphorylated Smads 1/5/8, i.e., active Smads. Furthermore, the treatment of rat granulosa cells with 30 or 200 ng/mL human rBMP-15 stimulated cell proliferation and DNA synthesis (Otsuka et al., 2000).

In relation to growth, an increase in the follicular diameter was observed after seven days of culture for all BMP-15 concentrations compared to the fresh control group or MEM^+

alone. The same result was observed for the oocyte diameter, except when 200 ng/mL BMP-15 was added to the culture medium. BMP-15 is essential for ovarian follicular growth (Juengel et al., 2004a) because granulosa cells are the target cells for the BMP-15 ligand (Otsuka et al., 2000). Some authors have described a high expression of the mRNA and/or BMP-15 protein in oocytes of growing or completely grown follicles (Shimasaki et al., 2004b; Juengel and McNatty, 2005; Li et al., 2008), which can suggest that BMP-15 plays a role in the increase of follicle diameter. Other studies reported that a lack in the production of BMP-15 receptors, such as BMPR-II and ALK-6 (BMPR-IB), may lead to a break in the growth (Mery et al., 2007). In mice, an increase in the levels of BMP-15 mRNA expression from day one to seven was associated with the presence of early follicles that contained growing oocytes (Sadeu et al., 2008).

Our results regarding to the percentage of normal follicular morphology were confirmed by ultrastructural analysis. TEM is considered a powerful technique for the evaluation of cellular organelles and ultrastructural changes (Salehnia et al., 2002) that occur during follicular atresia and is an important tool for analyzing preantral follicles after *in vitro* culture (Matos et al., 2007). Using TEM, important cellular structures, such as mitochondria, the endoplasmic reticulum, granulosa cells, and the basement and nuclear membranes, were preserved after seven days of culture in the presence of 100 ng/mL BMP-15. Normal follicles had ultrastructure similar to those described in caprine (Lucci et al., 2001; Celestino et al., 2009, 2010), bovine (Cran et al., 1980) and ovine (van Wezel and Rodgers, 1996) studies. In addition to the ultrastructural analysis, preantral follicles cultured for seven days in a medium containing 100 ng/mL BMP-15 were further analyzed using a more accurate method based on fluorescent probes; this method confirmed the previous results obtained with a light microscope and TEM regarding to follicular survival. Thus, this viability assessment appears to be a reliable, practical and fast method to analyze follicular viability (Cortvrindt and Smitz, 2001). Recently, this method has also been used successfully to evaluate preantral follicle viability in goats (Bruno et al., 2009; Silva et al., 2010).

In conclusion, the BMP-15 mRNAs were detected for all investigated follicular categories and cellular types. In addition, this study demonstrated that 100 ng/mL BMP-15 not only maintained the morphological integrity of caprine preantral follicles cultured for seven days but also promoted growth and transition to the secondary follicle stage. BMP-15 seems to be an essential factor for female fertility. However, new studies are still necessary for a better understanding of the ovarian regulatory mechanisms. These mechanisms could

ease the monitoring and manipulation of the ovarian function, further improving the fertility of domestic animals, endangered species and/or humans.

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12 CAPÍTULO 7

Fator de Crescimento Epidermal Recombinante Mantém a Ultraestrutura Folicular e Promove a Transição para Folículos Primários em Tecido Ovariano Caprino Cultivado In Vitro

Recombinant Epidermal Growth Factor Maintains Follicular Ultrastructure and Promotes the Transition to Primary Follicles in Caprine Ovarian Tissue Cultured In Vitro

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Resumo

Neste estudo foram investigados os efeitos do fator de crescimento epidermal sobre a sobrevivência e crescimento de folículos pré-antrais caprinos. Fragmentos ovarianos foram cultivados por 1 e 7 dias em meio essencial mínimo enriquecido com fator de crescimento epidermal (0, 1, 10, 50, 100 ou 200 ng/mL). Tecidos não-cultivados e cultivados foram processados para estudos histológicos e ultraestruturais. Os resultados mostraram que após 7 dias, o fator de crescimento epidermal (1 e 10 ng/mL) mantiveram a percentagem de folículos normais similar ao controle. Um aumento na percentagem de folículos primários foi observado com 1, 10 e 50 ng/mL de fator de crescimento epidermal comparado ao meio essencial mínimo enriquecido. Estudos ultraestruturais confirmaram a integridade folicular após 7 dias em fator de crescimento epidermal (1 e 10 ng/mL). Concluindo, baixas concentrações do fator de crescimento epidermal mantêm a viabilidade folicular caprina e promove a transição de folículos primordiais para primários.

Palavras-chave: EGF. Folículos pré-antrais. Ativação. Ruminante.

Recombinant Epidermal Growth Factor Maintains Follicular Ultrastructure and Promotes the Transition to Primary Follicles in Caprine Ovarian Tissue Cultured In Vitro

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We investigated the effects of epidermal growth factor on the survival and growth of caprine preantral follicles. Ovarian fragments were cultured for 1 and 7 days in enriched minimal essential medium with epidermal growth factor (0, 1, 10, 50, 100, or 200 ng/mL). Non-cultured and cultured tissues were processed for histological and ultrastructural studies. Results showed that after 7 days, the epidermal growth factor (1 and 10 ng/mL) maintained the percentage of normal follicles similar to control. An increase in the percentage of primary follicles was observed with 1, 10, and 50 ng/mL of epidermal growth factor compared to enriched minimal essential medium. Ultrastructural studies confirmed follicular integrity after 7 days in epidermal growth factor (1 and 10 ng/mL). In conclusion, the low concentrations of epidermal growth factor maintain caprine follicular viability and promote the transition from primordial to primary follicles.

KEY WORDS: EGF, preantral follicles, activation, ruminant.

INTRODUCTION

Mammalian folliculogenesis is a complex process that involves changes in follicular morphology such as oocytes growth and differentiation of the granulosa cells around it.[1] The mechanisms responsible for the beginning of follicular growth that is primordial follicle activation as well as the time required for follicle growth and differentiation are not yet elucidated. It is known that the growth of follicles in the mammalian ovary is regulated by gonadotrophins and intraovarian factors.[2] Several growth factors produced by the oocyte and granulosa cells frequently act modulating hormone effects, and folliculogenesis can be dysregulated when a factor is absent.[3]

One of the factors is the epidermal growth factor (EGF, a mitogenic factor), which stimulates the proliferation of different kinds of cells.[4] In the ovary, the EGF is involved in the regulation of several processes,[5] including proliferation and cellular differentiation, in addition to steroidogenesis.[6,7] Molecular biologic studies have demonstrated the expression of the protein and mRNA for EGF and its receptor in the oocyte and granulosa cells of preantral and antral follicles from rat, bovine, human, hamster, swine, mouse, and monkey[8-14] and also in luteal cells from rat, swine, and monkey.[14-16] In caprine, the EGF and its receptor are expressed in the developmental stages of all ovarian follicles, in corpus luteum, and in the ovarian epithelium surface.[5]

Studies about in vitro culture of preantral follicles showed that EGF promotes the proliferation of granulosa cells from swine, rodent, and human;[17,18] increases in follicular diameter from swine, bovine, rodent, and human,[19-22] reduces in atresia levels from bovine, swine, and caprine; [19,20,23] and promotes ovine primordial follicle activation and maintenance of viability for up to 6 days of culture.[24] Moreover, the culture of caprine ovarian tissue in 100 ng/mL of EGF had a benefic effect in the growth of oocyte from primary follicles but did not affect follicular viability.[25] However, in most of these studies performed with preantral follicle cultures, the EGF was tested at only one concentration, as well as the data obtained were based only on the histological evaluation of follicles. The aim of this study is to investigate a possible influence of different concentrations of EGF (0, 1, 10, 50, 100, or 200 ng/mL) on the survival, activation, and further growth of preantral follicles enclosed in caprine ovarian tissue cultured for 1 and 7 days.

MATERIALS AND METHODS

Source of Ovaries

Ovaries (n = 10) from 5 adult nonpregnant mixed-breed goats were collected at a local slaughterhouse. All animals were cyclic and in good body condition. The ovaries were removed, washed, and transported in minimum essential medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin (Vetec, Rio de Janeiro, Brazil), within 1 hour, to the laboratory in thermo flasks containing water at 33ºC.

Experimental Protocol

In the laboratory, goat ovaries were collected, stripped of all fat tissue and ligaments, and cut in half, after which the medulla, large antral follicles, and corpora lutea were removed. Following this, the ovarian cortex was divided into 13 fragments of approximately 3 x 3 mm (1 mm thick). The period from the collection of the ovaries up to the obtention of ovarian fragments lasts about 1 hour and a half. One fragment was immediately fixed for classic histological studies, while a smaller fragment (1 mm^3) was randomly collected and subsequently fixed for ultrastructural examination (non-cultured control). The other fragments of ovarian cortex were individually cultured in vitro in 1 mL of basic culture medium, which was MEM supplemented with ITS (insulin 6.25 g/mL, transferring 6.25 g/mL, and selenium 6.25 ng/mL), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, and 1.25 mg/mL bovine serum albumin (BSA), which was called MEM^+. This control medium (MEM^+) was tested alone or supplemented with different concentrations of recombinant human EGF (1, 10, 50, 100, or 200 ng/ mL [rhEGF; Cell Sciences, Canton, Mass]). All chemicals used in the current study were purchased from Sigma Chemical Co (St Louis, Mo), unless otherwise indicated. The tissues were cultured for 1 and 7 days at 39ºC with 5% CO_2 in air using a 24-well culture dish. Every 2 days, the culture medium was replaced with fresh medium. Each treatment was repeated 5 times using the ovaries of 5 different animals.

Histological Analysis and Assessment of In Vitro Follicle Growth

To evaluate caprine follicular morphology after 1 and 7 days of culture, a small part (1 mm^3) from each cultured fragment was randomly removed for transmission electron microscopy (TEM) studies, while the remainder was fixed overnight at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for histological studies. After fixation, the tissue fragments were dehydrated in a graded series of ethanol, clarified with xylene, and embedded in paraffin wax. For each piece of ovarian cortex, 7 µm sections were mounted on slides and stained with periodic acid-Schiff and hematoxylin (PAS-H staining system; Sigma, Inc). Coded anonymized slides were examined under a microscope (Zeiss, Jena, Germany) at x100 and x400 magnification by a single observer.

The follicles were classified as primordial or developing follicles (intermediate, primary, or secondary).[26] These follicles were further classified individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells that are well organized in one or more layers, and has no pyknotic nucleus. Degenerated follicles were

defined as those with a retracted oocyte that has a pyknotic nucleus and/or are surrounded by disorganized granulosa cells that are detached from the basement membrane. From each medium and culture period, approximately 150 follicles were randomly evaluated.

To evaluate follicular activation and growth, only intact follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and developing follicles were calculated at day 0 (control) and after 1 and 7 days of culture in the various media tested. In addition, major and minor axes of each oocyte and follicle were measured under a microscope with an ocular micrometer. The average of the minor and major axes was reported as oocyte and follicle diameters, respectively. These values were used to assess the effect of the EGF on follicular growth.

Ultrastructural Analysis

For ultrastructural analysis, small pieces of ovarian cortex were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer, pH 7.2. After washing the ovarian pieces with sodium cacodylate buffer, they were postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently, the samples were dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. First, semi-thin sections (3 µm) of the samples were cut on an ultramicrotome (Reichert Supernova, Heidelberg, Germany) for light microscopy studies and stained with toluidine blue. Subsequently, the follicles classified as histologically normal in semi-thin toluidin blue-stained sections were processed for ultrastructural analysis. For that purpose, thin sections (70 nm) were cut and then contrasted with uranyl acetate and lead citrate, and examined using a Jeol 1011 (Jeol, Tokyo, Japan) TEM, operating at 80 kV.

Statistical Analysis

Data expressed as mean ± SD (SEMs). The means of surviving follicles at all stages, primordial and developing (intermediate, primary, or secondary), obtained after 1 and 7 days in the various treatments were subjected to analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS (1999) and the Dunnett's test was implemented to compare the control and the MEM^+ groups against each treatment.[27] Duncan test was used to compare the differences among treatments. The Student *t* test was used to compare means between 1 and 7 days of culture. Differences among groups were considered significant when $P < .05$.

RESULTS

Caprine Preantral Follicles Survival Before and After In Vitro Culture

A total of 1950 preantral follicles were analyzed. Figure 1A shows normal follicles after culture in EGF at 1 ng/mL, while Figure 1B illustrates the degenerated follicles after culture in EGF at 200 ng/mL, both after 7 days of culture. In the degenerated follicles (Figure 1B), we observed retracted oocyte, pyknotic nucleus, and disorganized granulosa cells.

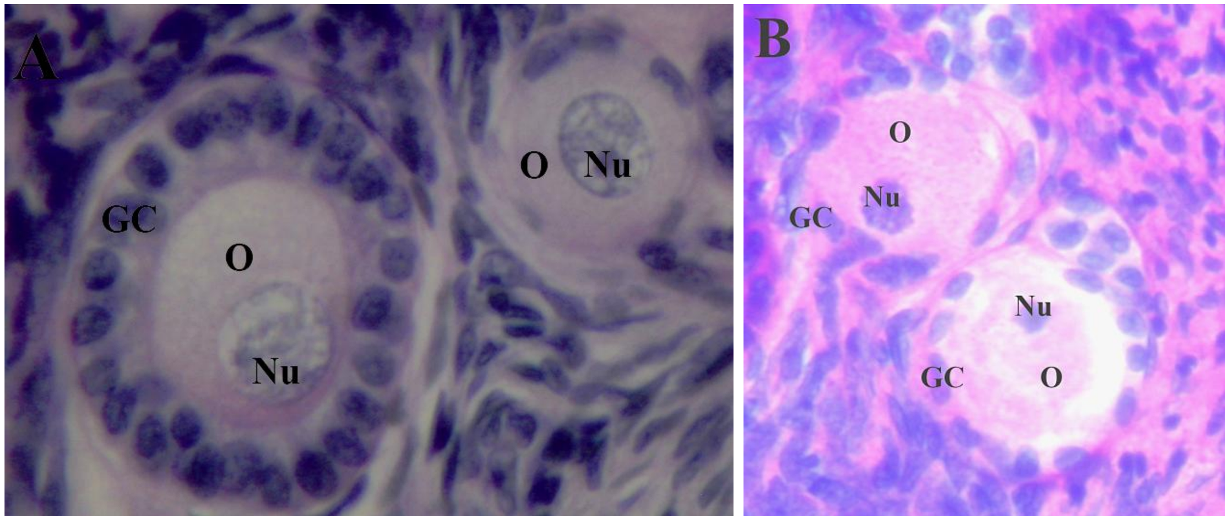


Figure 1. Histological section after staining with periodic acid-Schiff and hematoxylin showing (A) normal follicles after culture in epidermal growth factor (EGF) at 1 ng/mL and (B) degenerated follicles after culture in EGF at 200 ng/mL, both after 7 days of culture. Note the cuboidal granulosa cells (GC) layer in the normal primary follicle (Figure 1A). O = oocyte; Nu = oocyte nucleus (x 400).

The percentage of morphologically normal preantral follicles in control (non-cultured tissue) and after 1 and 7 days of culture in different treatments are shown in Figure 2. It was observed that after 7 days of culture, the percentage of normal follicles was similar ($P > .05$) to noncultured control (86%) only when the fragments were cultured in EGF at 1 (79%) or 10 (78%) ng/mL, and there is no difference ($P > .05$) in this percentage between both 1 or 10 ng/mL and 50 ng/mL (72%) concentrations. However, tissue culture in medium supplemented with 100 or 200 ng/mL of EGF significantly reduced ($P < .05$) the percentage of normal follicles when compared to control and other concentrations of EGF. In addition, after 7 days, higher ($P < .05$) percentage of normal follicles in tissues cultured with 1, 10, or 50 ng/mL of

EGF were observed when compared to MEM$^+$. With the progression of the culture from day 1 to 7, there was a significant reduction ($P < .05$) in the percentage of normal follicles in MEM$^+$ or supplemented with 100 or 200 ng/mL of EGF.

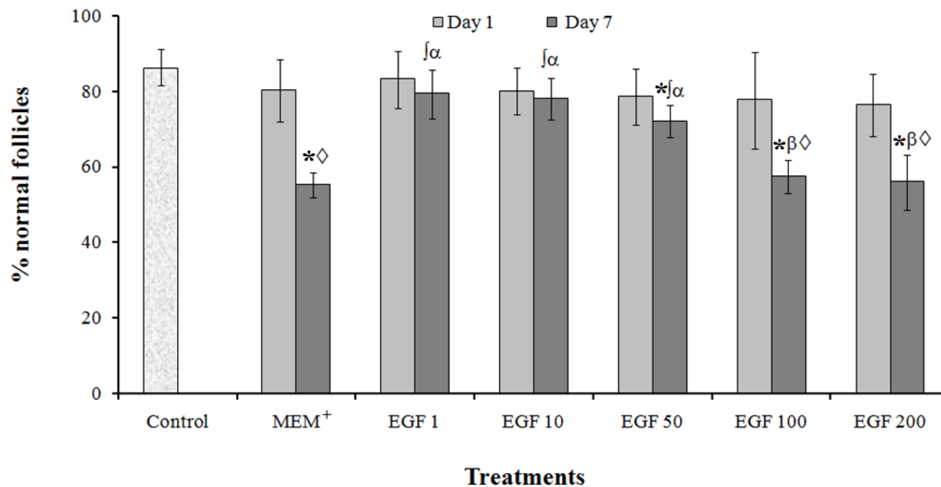


Figure 2. Percentage (mean ± S.E.M.) of morphologically normal preantral follicles in control (non-cultured) and after in vitro culture for 1 or 7 days in the absence or presence of epidermal growth factor. * differs significantly from control follicles ($P < .05$); ∫ differs significantly from MEM$^+$ in each day culture ($P < .05$); α,β differs significantly among concentrations in each day of culture ($P < .05$); ◊ differs significantly with the progression of the culture period from day 1 to 7 in the same treatment ($P < .05$).

Follicular Activation After In Vitro Culture

Even at day 1 of culture, in all treatments, a significant reduction ($P < .05$) in the percentage of primordial follicles (Figure 3A) associated with a concomitant increase ($P < .05$) in the percentage of intermediate follicles (Figure 3B) when compared to control was observed. Moreover, at day 1, addition of EGF to the culture medium significantly increased follicular activation compared with MEM$^+$ ($P < .05$). Furthermore, with the progression of the culture period from day 1 to 7 in the same treatment, we observed a significant increase ($P < .05$) in the percentage of intermediate follicles only in MEM$^+$ or with 100 ng/mL of EGF.

Regarding primary follicles, after 7 days of culture it was observed that all EGF treatments, except at 200 ng/mL, significantly increased ($P < .05$) the percentage of follicles from this stage compared to non-cultured control (Figure 3C). In addition, with increasing the culture period from 1 to 7 days, there was a significant reduction ($P < .05$) in the percentage

of primordial follicles and a significant increase ($P < .05$) in the percentage of primary follicles after culture in MEM^{+} or at 1, 10, or 50 ng/mL of EGF. However, only the low EGF concentrations (1, 10, and 50 ng/mL) significantly increased ($P < .05$) the rates of primary follicles in relation to MEM^{+}. Non-cultured and cultured caprine ovarian tissue contained a low (often zero) and variable number of secondary follicles (data not shown), which did not permit statistical analysis.

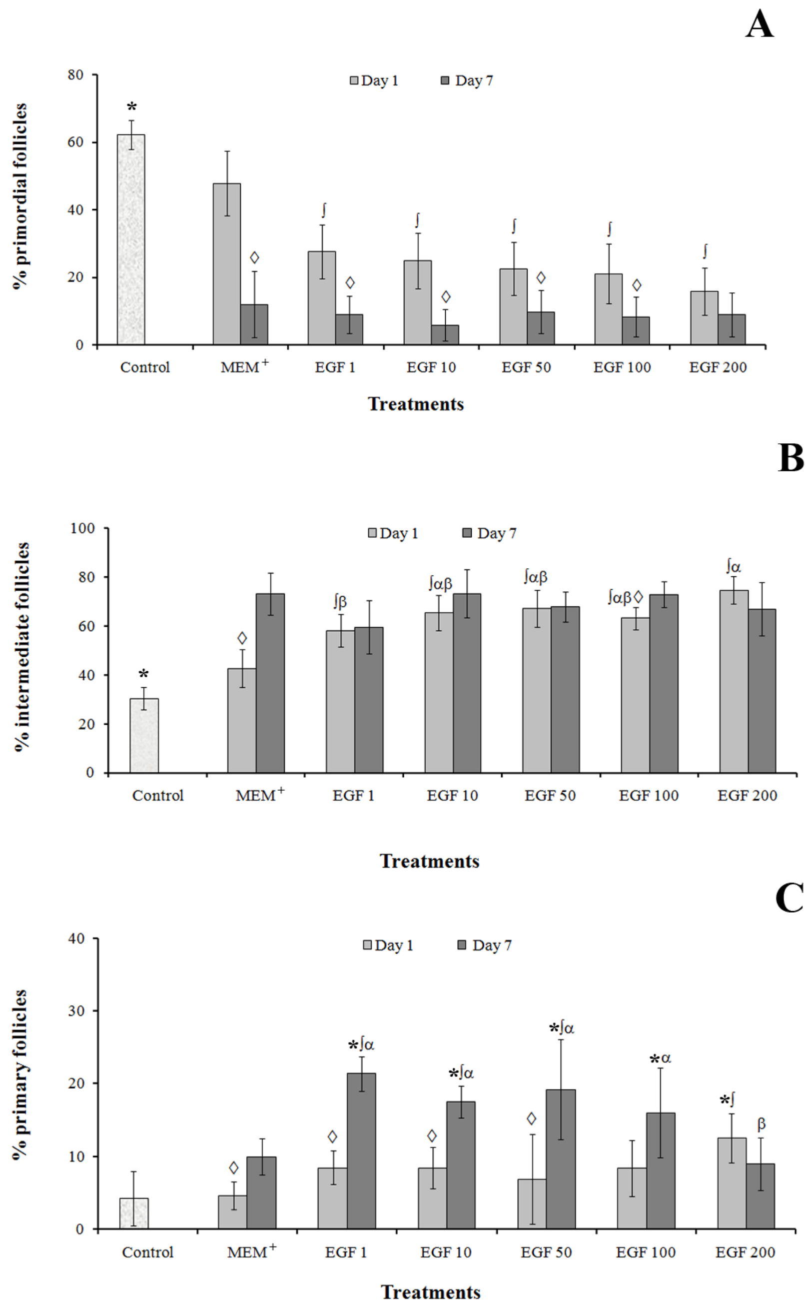


Figure 3. Percentage (mean ± S.E.M.) of primordial (A), intermediate (B) and primary (C) follicles in control (non-cultured) and after in vitro culture for 1 or 7 days in the absence or

presence of epidermal growth factor. * differs significantly from control follicles ($P < .05$); ∫ differs significantly from MEM^+ in each day culture ($P < .05$); α,β differs significantly among concentrations in each day culture ($P < .05$); ◊ differs significantly with the progression of the culture period from day 1 to 7 in the same treatment ($P < .05$).

According to Table 1, we did not observe any influence of EGF in the increase of follicular and oocytes diameters after 1 and 7 days of culture.

Table 1. Oocyte and follicle diameters (mean ± S.E.M.) in non-cultured tissues and in tissues cultured for 1 or 7 days in MEM^+ (control medium) and MEM^+ supplemented with various concentrations of epidermal growth factor. Per treatment 150 follicles were evaluated. *

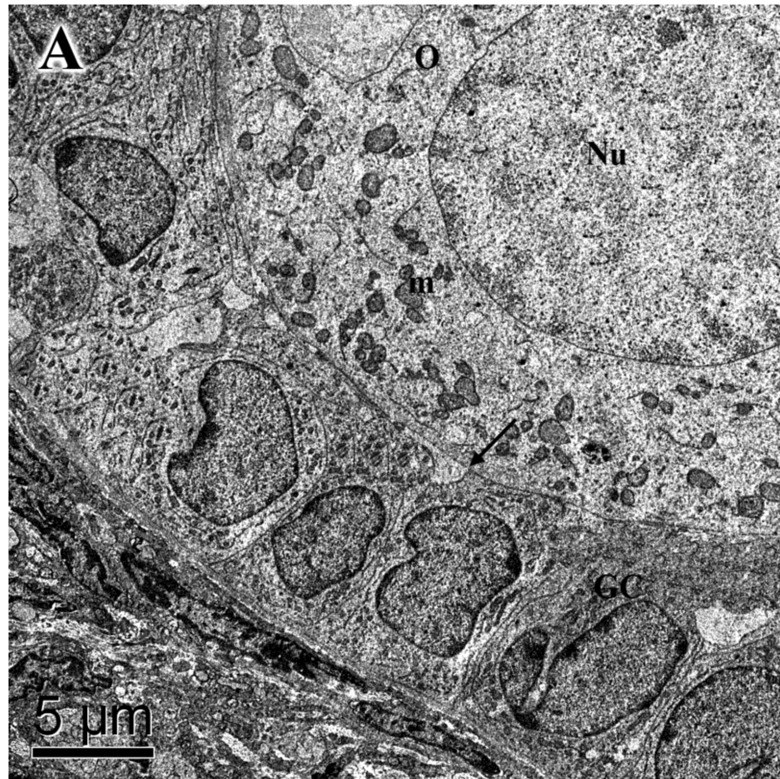
Treatments	Oocyte diameter (μm)		Follicle diameter (μm)	
Non-cultured (Day 0)	40.32 ± 6.22		54.38 ± 7.19	
Cultured	Day 1	Day 7	Day 1	Day 7
MEM^+	39.09 ± 7.45	39.40 ± 5.57	55.93 ± 11.38	55.46 ± 7.67
EGF 1	40.17 ± 7.43	40.01 ± 9.05	54.85 ± 10.17	54.07 ± 9.59
EGF 10	39.24 ± 8.33	39.09 ± 4.94	55.00 ± 10.96	53.77 ± 5.25
EGF 50	39.40 ± 6.56	39.71 ± 7.85	53.92 ± 8.23	54.23 ± 8.30
EGF 100	38.47 ± 4.07	37.08 ± 4.37	54.38 ± 6.21	53.30 ± 5.92
EGF 200	38.62 ± 6.91	39.71 ± 8.87	56.24 ± 9.44	54.54 ± 10.24

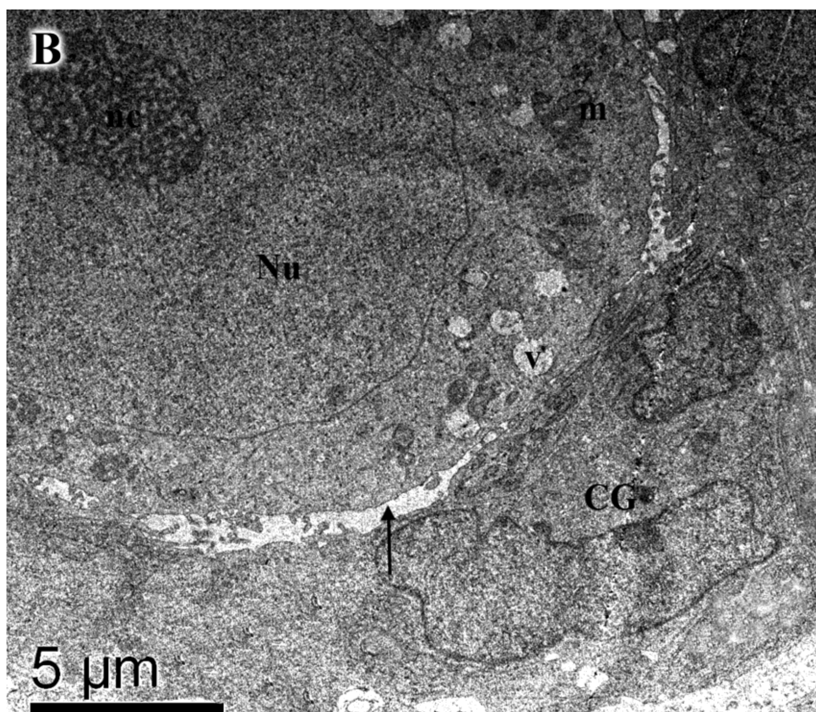
* No statistical difference was observed ($P > 0.05$).

Ultrastructural Features of Caprine Preantral Follicles Cultured With EGF

For better evaluation of follicular quality, ultrastructural analysis was performed using morphologically normal preantral follicles from control as well as from treatments that did not differ from control after 7 days of culture according to previous histological analysis (ovarian fragments cultured in EGF 1 or 10 ng/mL). Ultrastructural features of follicles from control (Figure 4A) and from 1 and 10 ng/mL of EGF (Figure 4B and 4C) were similar in some aspects such as intact basal and nuclear membranes, a large oocyte nucleus, and decondensed nuclear chromatin. In addition, there were organelles uniformly distributed in the ooplasm,

especially mitochondria. Granulosa cells were ultrastructurally normal and well organized around the oocyte, showing an elongated and large nucleus with irregular membrane. When the ovarian tissue was cultured for 7 days in MEM^{+} with EGF 1 or 10 ng/mL (Figures 4B and C, respectively), the preantral follicles appeared well preserved. However, their oocytes had irregular nuclear membrane as well as more microvilli, that is projections of the oocyte through the granulosa cells.





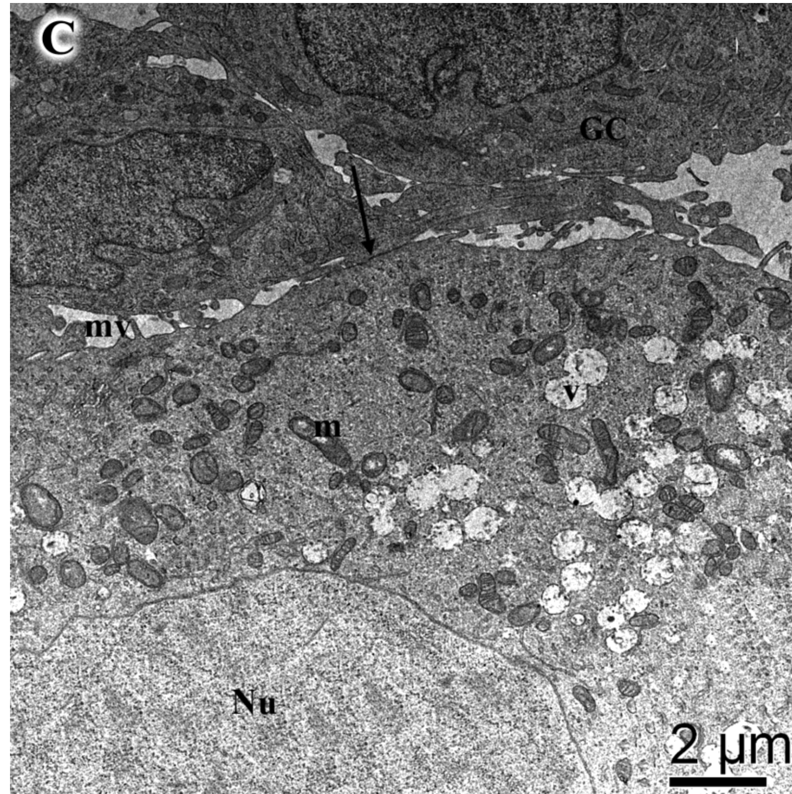


Figure 4. Ultrastructural analysis of non-cultured preantral follicle (A) and cultured for 7 days in medium containing 1 (B) and 10 ng/ml epidermal growth factor (C). Note the large nucleus of the granulosa cells in control follicles. Note the microvillos between the oocyte and granulosa cells in follicles cultured in 10 ng/ml of epidermal growth factor. O: oocyte; Nu: oocyte nucleus; nc: nucleolus; GC: granulosa cells; m: mitochondria; v: vesicles; mv: microvilli; arrow- oocyte membrane. (A: x4000, bar = 5µm; B: x6000, bar = 5 µm; C: x8000, bar = 2 µm).

DISCUSSION

This study showed the benefic effect of different concentrations of EGF in the caprine follicular survival and in the transition from primordial to primary follicles. The low concentrations (1 and 10 ng/mL) used in this experiment were chosen based on physiological concentrations of EGF in follicles from swine and mouse,[28,29] while 50 and 100 ng/mL showed satisfactory results after in vitro culture of preantral follicles in other species: bubaline, ovine, and caprine.[23-25,30-32]. In addition, we studied the effect of a high concentration of EGF (200 ng/mL) that had not been tested yet.

After 7 days of culture, an elevated follicular survival was observed after the addition of 1, 10, or 50 ng/mL of EGF in the culture medium. Epidermal growth factor is known as an in vivo and in vitro survival factor.[33] Previous studies have demonstrated that some ovarian growth factors, such as EGF, can inhibit apoptosis and thus warrant follicular survival.[34] In accordance with our results, some authors showed that low concentrations of EGF promote an inhibition of granulosa cells apoptosis or the reduction of follicular atresia levels in swine, bovine, and caprine.[19,20,23] Furthermore, low concentrations of EGF (0.75, 1.5, or 3 ng/mL) associated with follicle stimulating hormone (FSH) improved the quality of swine oocytes, leading to higher embryonic development rates.[35] However, our study shows that higher concentrations of EGF (100 or 200 ng/mL) decrease follicular viability after 7 days of culture. Similar results were observed by Talmimani et al,[36] which demonstrated that higher concentrations of EGF, that is 75 or 100 ng/mL induced degeneration of all 6 days cultured preantral follicles. In addition, Silva et al[25] did not observe a significant effect of EGF (100 ng/mL) in the survival of caprine preantral follicles, after 5 days of culture.

Regarding follicular activation (ie the transition from primordial to intermediate follicles), in the current study after the first day of culture, we observed a greater follicular activation in all treatments compared to control. Follicular activation in MEM^+ is feasible probably because this medium is rich in nutrients, such as amino acids and carbohydrates.[37] Despite this result, the addition of EGF to the culture medium promoted a further increase in activation rates compared to MEM^+. Moreover, low concentrations of EGF (1, 10, or 50 ng/mL) augment the percentage of primary follicles after 7 days of culture. This result is likely due to the fact that EGF is considered a mitogenic factor for different kinds of cells,[4] including ovarian granulosa cells.[6,7] In addition, the expression of EGF and its receptor in all development stages of caprine ovarian follicles[5] suggests that this growth factor plays a role in follicular activation. Moreover, EGF regulates the expression of conexin 43, which is an important protein for gap junction production, thus being important for preantral follicle development from swine and rabbit.[38,39] Some in vitro studies have reported that EGF can promote ovine primordial follicle activation[24] as well as granulosa cell proliferation and improvement of DNA synthesis.[31,40] However, Silva et al[25] did not observe any effect of 100 ng/mL of EGF on the proliferation of caprine granulosa cells. Furthermore, a recent study showed the negative effects of EGF on the steroidogenesis regulated by FSH and in the differentiation of granulosa cells from preantral follicles.[41] These contradictions may be due to differences related to species, culture conditions, and different follicular stages analyzed.

Similar to other studies,[42,43] we observed a few secondary follicles probably due to the small period of culture and the absence of any other growth factor or hormone in the culture medium, such as activin,[44] bone morphogenetic[45]protein-15, and growth differentiation[46] factor-9.

Although there was an increase in the percentage of primary follicles after EGF addition, no effect of EGF on the follicular and oocyte diameters was observed. This can probably happen due to the fact that intermediate follicles were already large, thus follicular growth was not perceptive but only the changes in granulosa cells morphology from flattened to cuboidal. Similar to our results, Zhou and Zhang[23,32] also observed that the use of EGF, in concentration 50 ng/mL, showed no effect on the caprine oocyte growth. Nevertheless, Silva et al[25] showed that EGF (100 ng/mL) increased the diameter of intermediate and primary follicles.

Our results of follicular viability after classical histology were confirmed by ultrastructural analysis. This technique is considered an important tool because it provides more information about the follicular and oocyte quality and is able to detect undiscernible damage to cellular membranes and organelles.[47] From the TEM studies, it was observed that besides basal and nuclear membranes, important organelles such as mitochondria, endoplasmic reticulum, and granulosa cells were preserved even after 7 days of culture in the presence of 1 or 10 ng/mL of EGF. Normal follicles had their ultrastructure similar to those described earlier in caprine[47] and subsequently in bovine and ovine.[37,48] In this study, we observed microvilli and vesicles, which are important for the oocyte and granulosa cells communication, allowing the exchange of substances and, consequently, warranting an adequate follicular development.

In conclusion, this study showed that the low concentrations of EGF (ie 1 or 10 ng/mL) are able to promote the maintenance of follicular viability and the transition from primordial to primary follicles after 7 days of in vitro culture.

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13 CAPÍTULO 8

Níveis de RNAm para o fator de crescimento epidermal (EGF) e efeito do EGF no cultivo in vitro de folículos pré-antrais caprinos

Steady-state level of mRNA for epidermal growth factor (EGF) and effect of EGF on in vitro culture of caprine preantral follicles

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Resumo

Os objetivos desse estudo foram verificar os níveis de RNAm para o fator de crescimento epidermal (EGF) em folículos caprinos nos diferentes estádios de desenvolvimento, e investigar a influência do EGF na sobrevivência, formação de antro e crescimento de folículos secundários cultivados por seis dias. Para tanto, folículos primordiais, primários e secundários de cabras, bem como pequenos e grandes folículos antrais foram obtidos, e os níveis de RNAm para o EGF foram quantificados pela RT-PCR em tempo real. A influência do EGF na presença ou ausência de FSH sobre o desenvolvimento de folículos secundários, e a expressão do RNAm para o EGF e R-FSH foram avaliadas após seis dias de cultivo. Além disso, a sobrevivência, a formação de antro e o diâmetro folicular foram avaliados a cada dois dias. Os níveis de RNAm para EGF em folículos secundários foram significativamente superiores que em folículos primordiais. Em pequenos e grandes folículos antrais, os níveis de RNAm para EGF em CCOs foram significativamente superiores que os níveis nas células da granulosa/teca. Durante o cultivo, o EGF na presença ou na ausência de FSH aumentou a taxa de crescimento diário folicular de folículos secundários quando comparados ao α-MEM^{+}. Em adição, FSH, EGF ou ambos reduziram os níveis de RNAm para o EGF, enquanto o EGF reduziu os níveis de RNAm para o R-FSH após cultivo dos folículos por seis dias. Concluindo, os níveis de RNAm para o EGF foram superiores em folículos secundários, e ambos FSH e EGF promoveram o crescimento de folículos secundários caprinos. Além disso, EGF e/ou FSH reduziram os níveis de RNAm para o EGF, e o EGF diminuiu os níveis de RNAm para R-FSH em folículos secundários cultivados.

Palavras-chave: EGF. Folículos secundários. FSH-R. Cabra. Cultivo.

Steady-state level of epidermal growth factor (EGF) mRNA and the effect of EGF on the in vitro culture of caprine preantral follicles

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Abstract

The aims of this study were to verify the steady-state level of epidermal growth factor (EGF) mRNA in goat follicles at different developmental stages and to investigate the influence of EGF on the survival, antrum formation and growth of secondary follicles cultured for six days. For this purpose, primordial, primary and secondary goat follicles as well as small and large antral follicles were obtained, and EGF mRNA levels were quantified by real-time RT-PCR. The influence of EGF in the presence or absence of FSH on the development of secondary follicles and on the EGF and FSH-R mRNA expression was evaluated after six days of culture. Furthermore, the survival, the antrum formation and the follicular diameter were evaluated every other day of culture. The EGF mRNA levels in secondary follicles were significantly higher than in primordial follicles. In small and large antral follicles, the EGF mRNA levels in cumulus–oocyte complexes (COCs) were significantly higher than the levels in granulosa/theca cells. During the culture, EGF in the presence or absence of FSH increased the follicular daily growth rate of secondary follicles when compared to α-MEM^{+}. In addition, FSH, EGF or both reduced the EGF mRNA levels, whereas EGF reduced the FSH-R mRNA levels after culture for six days. In conclusion, the EGF mRNA levels were higher in secondary follicles and both FSH and EGF promoted growth of goat secondary follicles. Furthermore, EGF and/or FSH reduced the EGF mRNA levels and EGF decreased the FSH-R mRNA levels in cultured secondary follicles.

Keywords: EGF, secondary follicles, FSH-R, goat, culture.

1. Introduction

The goat preantral follicles are highly susceptible to atresia. Thus, there is an interest in developing a culture system that supports the in vitro growth of preantral follicles to the stage at which the oocytes were capable of being matured and fertilized in vitro. The implementation of this system could maximize the in vitro production of embryos by providing a large number of oocytes that are homogeneous and meiotically competent for several biotechnologies such as in vitro fertilization, cloning and transgenesis. In some animal species, the in vitro growth and development of preantral follicles has been successful until the embryonic stage (pig: Wu and Tian, 2007, buffalo: Gupta et al., 2008, sheep: Arunakumari et al., 2010 and caprine: Saraiva et al., *in press*). Nevertheless, the birth of live offspring from the preantral follicles grown in vitro in these species is still a great challenge. In addition, the maturation rates of oocytes obtained from preantral follicles are very low.

The follicular development is the result of the complex interactions between pituitary gonadotropins and numerous intra-ovarian factors, which act as promoters of survival, stimulating the growth and differentiation of follicular cells (Fortune, 2003; Miyoshi et al., 2010). Among these factors, the epidermal growth factor (EGF) has been highlighted; it has emerged as an important substance capable of inducing follicular development in vitro (Celestino et al., 2009). The EGF is a protein belonging to the EGF family, which consists of at least eight members (Riese and Stern, 1998). EGF is considered a mitogenic factor, and it is involved in the regulation of several ovarian processes (Silva et al., 2006), including proliferation and cellular differentiation, in addition to steroidogenesis (Saha et al., 2000; Wang et al., 2007). The expression of protein and mRNA for EGF has been demonstrated in the oocyte and granulosa cells of early and late-staged follicles (hamster: Roy and Greenwald, 1990, human: Maruo et al., 1993; Bennett et al., 1996, pig: Singh et al., 1995), while EGF mRNA has been described only in oocyte and granulosa cells from pig antral follicles (Singh et al., 1995). In caprine, the protein and mRNA for EGF were expressed in all developmental stages of ovarian follicles and in the ovarian epithelium surface (Silva et al., 2006). However, quantification of the steady-state level of EGF mRNA during different stages of follicular development has not been performed as yet. The action of EGF in the ovary is mediated by a membrane receptor, EGF-R (ErbB1), which belongs to the ErbB superfamily (Riese and Stern, 1998). EGF-R mRNA and protein have been identified in the oocyte and granulosa cells of early-and late-stage follicles (rat: Chabot et al., 1986; Feng et al., 1987, human:

Maruo et al., 1993; Bennett et al., 1996; Qu et al., 2000, pig: Singh et al., 1995, cattle: Lonergan et al., 1996, mouse: Hill et al., 1999, hamster: Garnett et al., 2002, caprine: Gall et al., 2004; Silva et al., 2006). In hamsters, it was demonstrated that the expression of the protein and mRNA for the EGF receptor was positively regulated by gonadotropins and steroids (Garnett et al., 2002).

In vitro studies showed that EGF promotes the proliferation of granulosa cells in the rodent, swine, caprine and human (Gospodarowicz and Bialecki, 1979; Morbeck et al., 1993; Rajarajan et al., 2006), increases the follicular diameter in the rodent, swine, bovine, caprine and human (Romano et al., 1994; Roy and Kole, 1998; Gutierrez et al., 2000; Silva et al., 2004a; Mao et al., 2004), reduces the atresia levels in the cow, swine, and caprine (Gutierrez et al., 2000; Mao et al., 2004; Zhou and Zhang 2005a,b; Celestino et al., 2009) and promotes ovine and caprine primordial follicle activation and maintenance of survival for up to six and seven days of culture, respectively (Andrade et al., 2005; Celestino et al., 2009). There is evidence that the regulation of EGF activity in granulosa cells in vitro may occur by stimulation of FSH receptor expression (Luciano et al., 1994). When the combination of EGF with FSH was tested in the in vitro culture of pig preantral follicles, the vast majority of follicles grew to the antral stage, with high secretion of estradiol, and the oocytes from these follicles could be matured, fertilized and developed until the blastocyst stage (Wu and Tian, 2007). In caprine, the interaction between EGF and FSH promoted follicular survival, although it had no effect on growth (Zhou and Zhang, 2005a,b). Although there have been some studies to date, little is known about the in vitro effects of EGF in the presence or absence of FSH on the development of isolated caprine secondary follicles. Moreover, the in vitro effects of EGF in the presence or absence of FSH on the expression of EGF and FSH receptor (FSH-R) after culture is still unknown.

Therefore, the present study aimed (1) to verify the steady-state level of EGF mRNA during different follicular stages in goat ovaries, (2) to investigate a possible influence of EGF on the survival, antral cavity formation and growth of secondary follicles after culture for six days, and (3) to evaluate the effects of EGF and/or FSH on the EGF and FSH-R mRNA levels after six days of culture.

2. Material and Methods

2.1. Chemicals

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Steady-state level of EGF mRNA in goat ovarian follicles

To evaluate the steady-state level of mRNA, 30 ovaries from 15 goats (*Capra hircus*) were collected at a local slaughterhouse and rinsed in Minimum Essential Medium (MEM) containing antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). After this preparation, 10 ovaries from 5 goats were utilized for the isolation of primordial, primary, and secondary follicles. The remaining ovaries were used for the collection of cumulus–oocyte complexes (COCs), mural granulosa cells, and thecal cells from small and large antral follicles. Primordial, primary, and secondary follicles were isolated using a previously described mechanical procedure (Lucci et al., 1999). After isolation, these follicles were washed several times to completely remove the stromal cells and were then placed by category into separate Eppendorf tubes in groups of 10. This procedure was completed within 2 h, and all samples were stored at -80°C until the RNA was extracted. From a second group of ovaries (n=15), COCs aspirated from small (1–3 mm) and large (3–6 mm) antral follicles were recovered. Compact COCs were selected from the follicle content as described by van Tol and Bevers (1998). Thereafter, groups of 10 COCs were stored at – 80°C until RNA extraction. To collect mural granulosa and theca cell complex, small (n=10) and large antral follicles (n=10) were isolated from ovaries (n=5) and dissected free from stromal tissue with forceps as previously described (van Tol and Bevers, 1998). The follicles were then bisected, and the granulosa and theca cell complexes were collected and stored at -80°C.

The isolation of total RNA was performed using a Trizol plus purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 mL of Trizol solution was added to each frozen sample, and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz

units/mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 µL RNAse-free water.

Prior to reverse transcription, the eluted RNA samples were incubated for 5 min at 70°C and chilled on ice. Reverse transcription was then performed in a total volume of 20 µl, which was comprised of 10 µl of sample RNA, 4 µl 5X reverse transcriptase buffer (Invitrogen), 8 U RNAseout, 150 U Superscript III reverse transcriptase, 0.036 U random primers (Invitrogen), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C and then for 5 min at 80°C, then it was stored at -20°C. Negative controls were prepared under the same conditions, but without the inclusion of the reverse transcriptase.

Quantification of the EGF mRNA was performed using SYBR Green. PCR reactions were composed of 1 µL cDNA as a template in 7.5 µL of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µL of ultra-pure water, and 0.5 µM of each primer. The primers were designed to perform amplification of EGF mRNA. Glyceraldehyde-2-phosphate dehydrogenase (GAPDH) and β-actin were used as endogenous controls for normalization of the steady-state level of mRNA of the genes (Table 1). The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 45 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany). The delta-delta-CT method was used to transform CT values into normalized relative steady-state level of mRNA.

Table 1. Oligonucleotide primers used for PCR analysis of goat cells and tissues.

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
GAPDH	TGTTTGTGATGGGCGTGAACCA	s	287-309	GI:27525390
	ATGGCGTGGACAGTGGTCATAA	as	440-462	
β-actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI:28628620
	TCCTTGATGTCACGGACGATTTCC	as	386-410	
UBQ	GAAGATGGCCGCACTCTTCTGAT	s	607-631	GI:57163956
	ATCCTGGATCTTGGCCTTCACGTT	as	756-780	
EGF	CCAGGTTCTCTTAAGTGC	s	48-65	GI: 1706938
	ACCAAGAGCTGCTCTCTG	as	151-168	
FSH-R	AGGCAAATGTGTTCTCCAACCTGC	s	250-274	GI:95768228
	TGGAAGGCATCAGGGTCGATGTAT	as	316-340	

s,sense; as, antisense

2.3. Effect of EGF on the survival and growth of goat secondary follicles and expression of FSH-R and EGF

2.3.1. Isolation and selection of caprine preantral follicles

Ovaries (n=40) were collected at a local slaughterhouse from 20 adult (1 - 3 years old) mixed-breed goats, and we made a total of 4 replicas (5 goats/replica). Immediately postmortem, the ovaries were washed in 70% alcohol, followed by two rinses in Minimum Essential Medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The ovaries were transported within 1 hour to the laboratory in MEM at 4°C (Chaves et al., 2008).

In the laboratory, the surrounding fat tissue and ligaments were stripped off from the ovaries. Ovarian cortical slices (1 - 2 mm in diameter) were cut from the ovarian surface using a surgical blade under sterile conditions. Then, the ovarian cortex was placed in a

fragmentation medium, consisting of MEM plus HEPES. Secondary follicles of approximately 200 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the strips of ovarian cortex using 26 gauge (26 G) needles. After isolation, follicles were transferred to 100 µL drops containing fresh medium under mineral oil to further evaluate the follicular quality. Follicles with a visible oocyte, surrounded by granulosa cells, an intact basement membrane and no antral cavity were selected for culture.

2.3.2. Caprine preantral follicles culture

For in vitro studies, after selection, follicles were individually cultured in 100 µL drops of culture medium in Petri dishes (60 x 15 mm, Corning, USA) under mineral oil. Control culture medium, called α-MEM^{+}, consisted of α-MEM (pH 7.2 - 7.4) supplemented with 3.0 mg/mL bovine serum albumin (BSA), ITS (insulin 10 µg/mL, transferrin 5.5 µg/mL and selenium 5 ng/mL), 2 mM glutamine, 2 mM hypoxanthine and 50 µg/mL of ascorbic acid under mineral oil. Incubation was conducted at 39°C, 5% CO_2 in air for six days. Fresh media were prepared and incubated for one hour prior to use. Preantral follicles obtained from each animal were randomly distributed in the following treatments: α-MEM^{+} alone or supplemented with 100 ng/mL recombinant bovine FSH (rbFSH: Nanocore, São Paulo, SP, Brazil), 10 ng/mL recombinant human epidermal growth factor (rhEGF: Cell Sciences, Canton, MA, USA) or both. These concentrations of rbFSH and rhEGF were those that promoted the best results in the in vitro development of goat preantral follicles in previous studies of our laboratory (Celestino et al., 2009; Saraiva et al., unpublished data). Every other day, 60 µL of the culture media were replaced for fresh medium. The culture was replicated four times, and a mean number of 37 follicles were used per treatment.

2.3.3. Morphological evaluation of follicle development

Follicles were classified according to their morphological aspect, and those showing morphological signs of degeneration, such as darkness of oocytes and surrounding cumulus cells or those with misshapen oocytes, were classified as degenerated. Follicular diameter was measured only in healthy follicles in the *x* and *y* dimensions (90°) by using an ocular micrometer (100x magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tóquio,

Japão) every other day of culture. Regarding the follicular growth, the mean increase in follicular diameter was calculated as follows: the diameter of viable follicles at day 6 minus the diameter of viable follicles at day 0 divided by the total number of viable follicles at day 6. In addition, the percentages of secondary follicles that reached the antrum formation in vitro were determined. Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers.

2.3.4. Steady-state level of FSH-R and EGF mRNA in goat ovarian follicles cultured in vitro

To evaluate the effect of EGF, FSH or both combined on the FSH-R and EGF mRNA expression after a six-day culture period, for each treatment, groups of ten follicles were collected at the end of the culture period and stored at -80°C until the extraction of total RNA. Quantification of mRNA was performed as described previously, and the primers for EGF and FSH-R are shown in Table 1. β-actin and ubiquitin (UBQ) were used as endogenous controls for normalization of gene expression (Table 1).

2.4. Statistical analysis

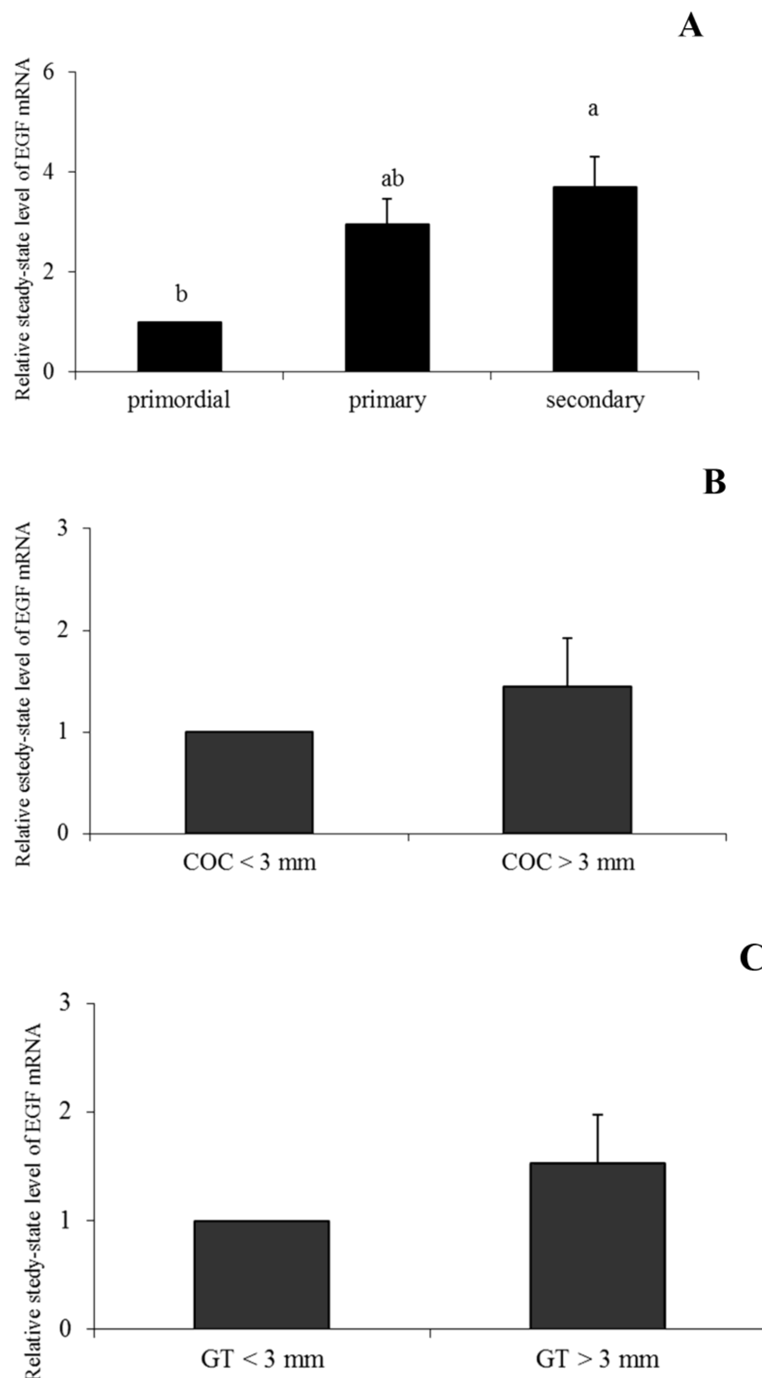
Data of mRNA expression in primordial, primary and secondary follicles were analyzed with the Kruskal-Wallis non-parametric test, while *t* test was used for paired comparisons of mRNA expression in small and large antral follicles ($P<0.05$). Data from the follicular survival and antrum formation after in vitro culture for each treatment were compared using the Chi-square test, and the results were expressed as percentages. Follicular diameter and growth rate, as well as EGF and FSH-R mRNA levels after culture did not show homoscedasticity, and these parameters were analyzed using the Kruskal-Wallis non-parametric test (SAS, 1999). The results were expressed as the mean ± standard error of the mean (SEM), and differences were considered to be significant when $P<0.05$.

3. Results

3.1. Steady-state level of EGF in goat ovarian follicles

Quantification of mRNA expression demonstrated that secondary follicles had

significantly higher levels of EGF mRNA than primordial follicles, but the levels did not differ significantly from that of primary follicles (P<0.05 – Fig. 1A). When the EGF mRNA levels in the primordial and primary follicles were compared, no significant difference was observed (P>0.05 – Fig. 1B). In addition, no significant difference was observed between COCs collected from small and large antral follicles (P>0.05 – Fig. 1B). Similar results were observed for granulosa/theca cells from small and large antral follicles (P>0.05 – Fig. 1C). On the other hand, real-time RT-PCR showed that COCs either from small or large antral follicles had significantly higher EGF mRNA levels than their respective granulosa/theca cells (P<0.05 – Fig. 1 D, E).



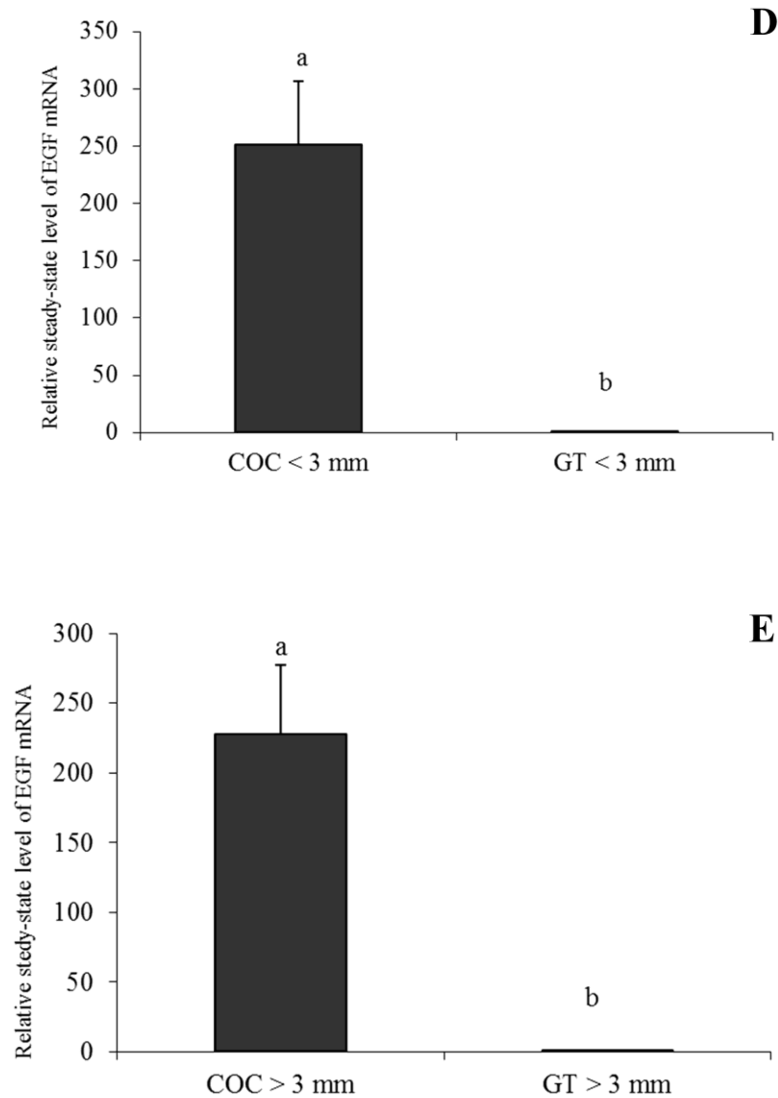


Figure 1. Expression of EGF mRNA in goat ovarian follicles (means ± SEM). A) primordial, primary and secondary follicles, B) COCs from small and large antral follicles, C) granulosa / theca cells from small and large antral follicles, D) COCs and granulosa / theca cells from small antral follicles, and E) COCs and granulosa / theca cells from large antral follicles
[a,b] ($P<0.05$)

3.2. Effect of EGF on survival, antrum formation and growth of goat secondary follicles

Preantral follicles selected for culture had a centrally located oocyte and normal granulosa cells, which were enclosed by an intact basal membrane (Fig. 2A, C). After six days of culture, follicle growth and antrum formation were observed (Fig. 2B, D). Effects of EGF

and/or FSH on follicular survival, antral cavity formation, follicular diameter and daily growth rate were evaluated at 0, 2, 4 and 6 days of culture and are shown in Table 2 and Figures 3, 4 and 5, respectively.

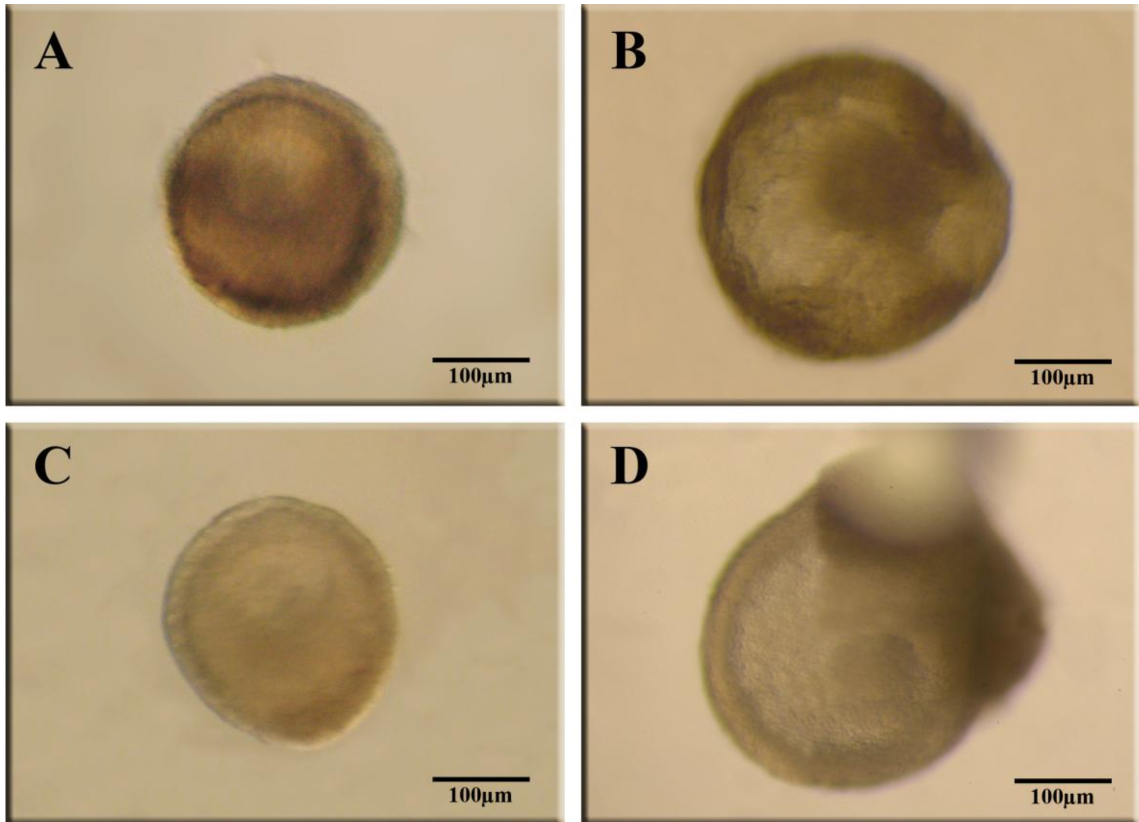


Figure 2. Caprine preantral follicles at day 0 (A, C) and antral follicles after six days of in vitro culture with EGF alone or EGF + FSH (B, D), respectively.

After six days of culture, all treatments promoted a high rate of follicular survival, which exceeded 90%. However, there was no significant difference among treatments and culture periods (Table 2).

Table 2: Percentage of survival of goat secondary follicles cultured for six days in α-MEM supplemented with FSH, EGF or both [a]

Days	**Treatments**			
	α-MEM^{+} (N = 37)	FSH (N = 37)	EGF (N = 37)	EGF + FSH (N = 38)
0	100	100	100	100
2	100	100	100	100
4	100	100	97.30	100
6	100	91.89	91.89	94.74

Abbreviations: EGF, epidermal growth factor; FSH, follicle stimulating hormone; α-MEM^{+}: enriched alpha minimal essential medium; N, number of follicles cultured for each treatment.
[a] No statistical difference was observed ($P>0.05$).

With regard to antrum cavity formation, on day 2 of culture, a positive effect of all of the treatments was observed (P<0.05), but the percentage of antral follicles was significantly higher when used EGF alone in comparison to FSH alone or EGF associated with FSH. After the progression of culture from day 2 to day 4, the percentage of antrum formation increased (P<0.05) and remained constant from day 4 to day 6 in all treatments (P>0.05), except when EGF + FSH treatment was used, in which a progressive increase in antrum formation was observed (P<0.05).

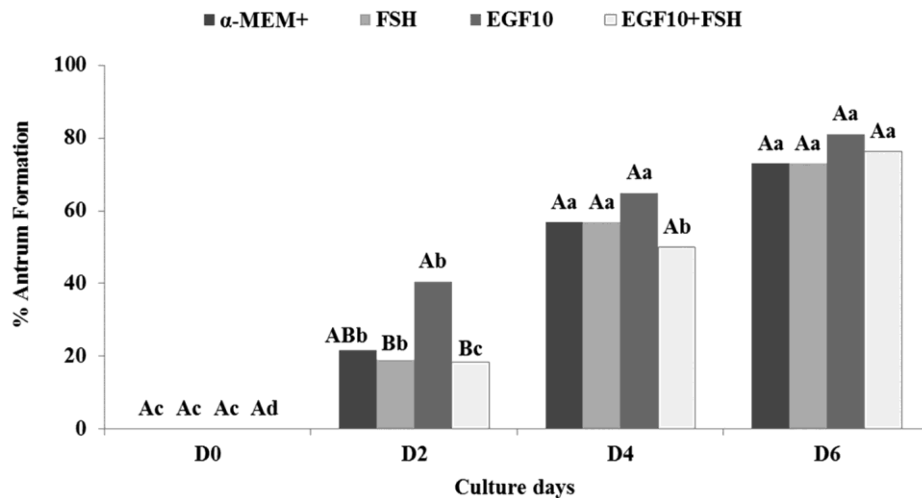


Figure 3. Percentage of antrum formation of goat secondary follicles cultured for six days in α-MEM$^+$ supplemented with FSH, EGF or both Percentage of antrum formation in goat secondary follicles cultured for six days in α-MEM$^+$ supplemented with FSH, EGF or both.

^{A,B} Differs among treatments (P<0.05)

^{a,b,c,d} Differs among days of culture (P<0.05)

The presence of EGF alone or associated with FSH caused a significant increase in follicular diameter as the culture progressed, and this increase was already observed from day 2 (P<0.05). Comparing the different treatments on the same days of culture, it was observed that from day 4 the use of EGF alone promoted a significant increase in follicular diameter when compared to α-MEM$^+$ (P<0.05). The addition of EGF to the culture medium, alone or associated with FSH, positively influenced the follicular daily growth rate compared to α-MEM$^+$ alone (higher than 20 μm/day) (P<0.05). However, it did not differ from the medium supplemented only with FSH (P>0.05).

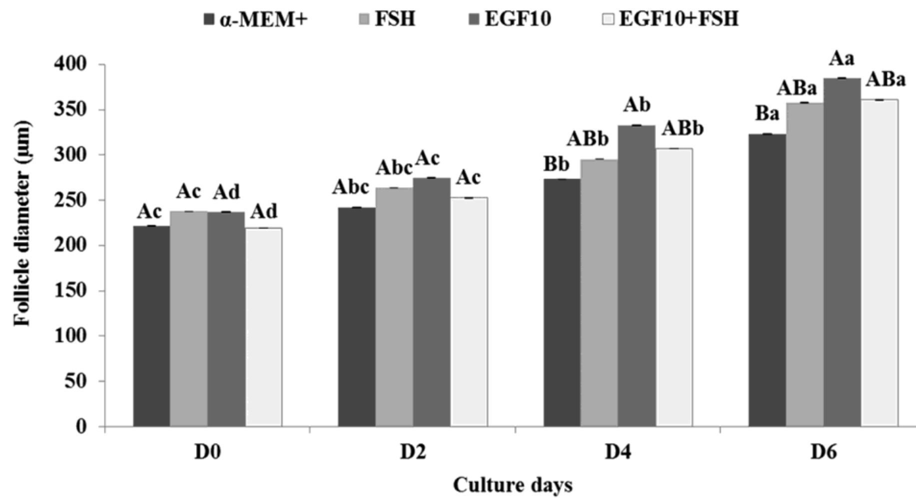


Figure 4. Diameter of morphologically normal follicles after in vitro culture for six days.

[A,B] Differs among treatments (P<0.05)

[a,b,c,d] Differs among days of culture (P<0.05)

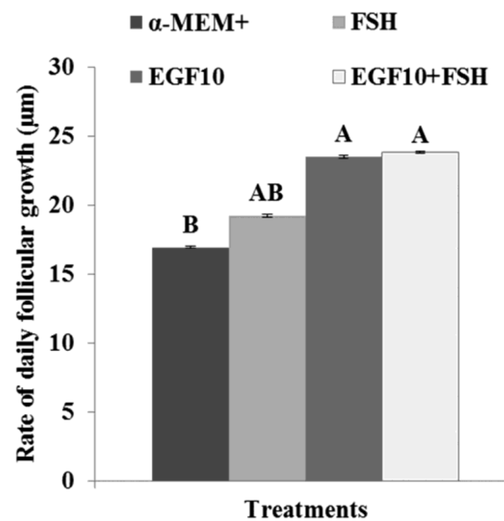


Figure 5. Daily growth rate of morphologically normal follicles during in vitro culture for six days

[A,B] Differs among treatments (P<0.05)

3.3. Expression of EGF and FSH-R in goat secondary follicles

After evaluation of EGF and FSH-R mRNA levels in cultured follicles, Figure 6 shows that the presence of FSH, EGF or both in culture medium significantly reduced the EGF mRNA levels. In addition, when EGF alone was present in the culture medium, a significant reduction in the FSH-R mRNA levels was observed after follicle culture for 6 days (Fig. 7).

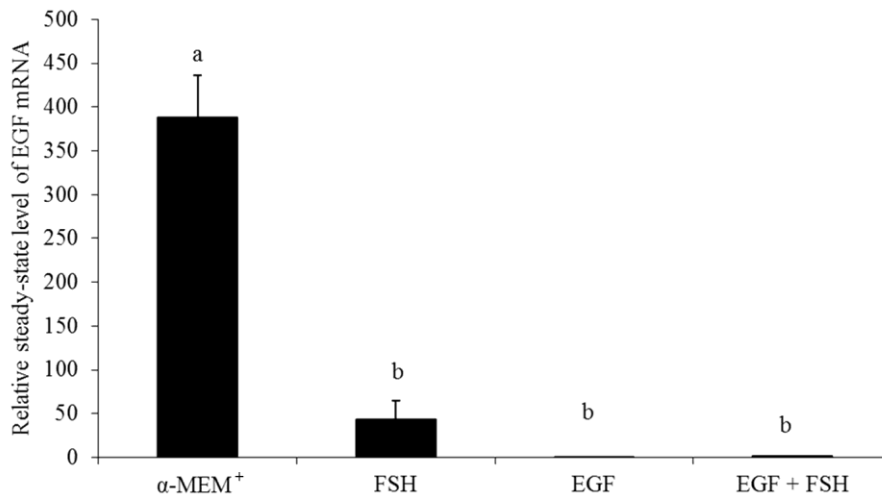


Figure 6. Steady-state level of EGF mRNA in goat secondary follicles cultured for six days in α-MEM+ supplemented with FSH, EGF or both

[a,b] ($P<0.05$)

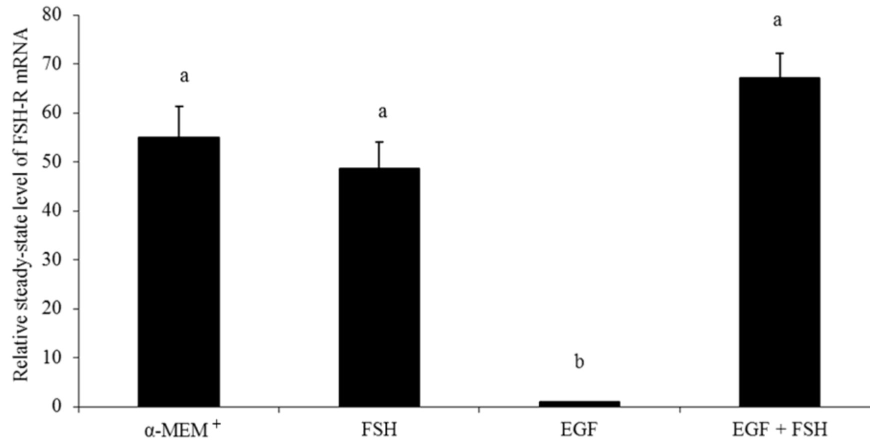


Figure 7 Steady-state level of FSH-R mRNA in goat secondary follicles cultured for six days in α-MEM$^+$ supplemented with FSH, EGF or both
[a,b] (P<0.05)

4. Discussion

This study showed for the first time that EGF mRNA levels increased from primordial to secondary follicles and that both FSH and EGF increased the secondary follicle diameter after six days of culture in goats. Furthermore, FSH, EGF or both reduced the EGF mRNA levels, while EGF reduced the FSH-R mRNA levels after follicle culture for six days.

Goat secondary follicles had higher EGF mRNA levels than primordial follicles. This finding confirms the results of previous studies showing that EGF acts on follicle development by promoting the oocyte growth of goat primary follicles in vitro and granulosa cell proliferation and differentiation (Saha et al., 2000; Silva et al., 2004a; Wang et al., 2007). Moreover, some studies have shown that although EGF is not essential for the activation of primordial follicles (Braw-Tal and Yossefi 1997; Fortune et al., 1998; Wright et al., 1999), it is important for the late follicular development (Gutierrez et al., 2000; Nayudu et al., 2002; Peng et al., 2010). According to Wu and Tian (2007), the production and the activity of EGF are more important in the granulosa cells of growing preantral follicles. In the antral follicles, COCs either from small or large antral follicles had higher EGF mRNA levels than their respective granulosa/theca cells. In porcine ovarian follicles, EGF mRNA and protein were detected in the oocyte (Singh et al., 1995). Besides its presence in pig oocytes, EGF was detected in bovine and human oocytes (Reeka et al., 1998; Glister et al., 2003). However, in other mammalian species, EGF has been shown to be expressed both in the granulosa and

theca cells (Singh et al., 1995; Park et al., 2004; Sekiguchi et al., 2004; Shimada et al., 2006). In goats, Gall et al. (2004) demonstrated that EGF can bind to its specific receptor located in the follicular cells or directly in the oocyte, therefore, being an important signal during the oocyte maturation process. In vitro studies with antral follicles have demonstrated that EGF stimulates the oocyte maturation (rat: Dekel and Sherizly, 1985, mouse: Smitz et al., 1998; De La Fuente et al., 1999, sheep: Guler et al., 2000, cattle: Lonergan et al., 1996; Sakaguchi et al., 2002, human: Goud et al., 1998, pig: Singh et al., 1997; Prochazka et al., 2000, 2003).

After six days of culture, a follicle survival rate exceeding 90% was observed in all treatments. Although different studies have demonstrated the importance of FSH and EGF substances for follicular survival, including goat follicles (Matos et al. 2007; Celestino et al., 2009), in this study, there were no effects of these substances when added to the culture medium. This probably occurred due to the use of an already rich culture medium, composed of amino acids, vitamins, antioxidants, inorganic salts and energetic substrates, which was able to sustain the survival after short-term culture regardless of the addition of hormones and/or growth factors. Besides the normal composition of this medium, important substances were also added such as hypoxanthine, pyruvate, glutamine and insulin-transferrin-selenium (ITS). Silva et al. (2004b) showed that the addition of pyruvate, glutamine, hypoxanthine and ITS to the culture medium (Minimum Essential Medium - MEM) increased the percentage of morphologically normal follicles in goats after five days of culture.

This study showed that EGF in the presence or absence of FSH increased the antrum formation and the diameter of caprine secondary follicles cultured in vitro, suggesting a considerable importance of both substances to the follicular development at this stage. The presence of mRNA for EGF and FSH receptors has been observed in caprine secondary follicles (EGF-R: Silva et al., 2006, FSH-R: Saraiva et al., unpublished data), and possibly the binding of FSH or EGF to their respective receptors stimulated the follicular growth. EGF is considered a mitogenic factor for granulosa cells (Saha et al., 2000; Wang et al., 2007). It has been implicated in the regulation (Roy, 1993; Campbell, 1999) and stimulation of in vitro preantral follicle growth in hamsters (Roy, 1993), mice (Boland and Gosden, 1994), humans (Roy and Kole, 1998), cows (Gutierrez et al., 2000), sheep (Hemamalini et al., 2003) and goats (Silva et al., 2004a; Rajarajan et al., 2006; Celestino et al., 2009). When EGF was tested in the in vitro culture of pig preantral follicles, besides promoting a suppression of apoptosis in granulosa cells, it increased the antrum formation (Mao et al., 2004). Regarding FSH, studies with preantral follicles in the pig (Hirao et al., 1994; Wu et al., 2001), cattle (Gutierrez

et al., 2000; Itoh et al., 2002), mouse (Gao et al., 2007) and goats (Saraiva et al., unpublished data) showed that this hormone was able to induce the growth and antrum formation. Furthermore, FSH can induce the follicular growth by interacting with different growth factors, particularly EGF (Demeestere et al., 2005). In hamsters, EGF has been shown to be a potent mitogen for cells of preantral follicles and is able to mediate the mitogenic action of FSH (Roy and Greenwald, 1991; Greenwald and Roy, 1994). In caprine, EGF alone or when associated with FSH stimulated the oocyte growth in vitro during the transition of primordial to primary follicles (Silva et al., 2004a). More recently the importance of EGF and FSH was demonstrated for the in vitro growth of ovine preantral follicles, especially for the more advanced stages (Peng et al., 2010).

After culture of caprine secondary follicles for six days, EGF and FSH reduced the EGF mRNA levels. The self-reduced expression of EGF probably occurred due to the overstimulation of follicular cells by the addition of exogenous EGF, triggering a primary regulatory mechanism that led to the reduction of its endogenous production, which was reflected by reduced mRNA levels. Expression analysis after culture revealed that EGF and FSH showed no synergistic effect on the pattern of mRNA expression for EGF and FSH receptor. Thus, this suggested that the action of EGF is not totally dependent on FSH and that the action of FSH appears to be strongly influenced by EGF. This reciprocal regulation between EGF and FSH was also observed in studies with hamster ovaries; these studies found that the follicle cells express the EGF gene, and that its expression is controlled by FSH, which in turn is partially influenced by EGF (Roy and Harris, 1994).

EGF reduced the FSH-R mRNA expression in goat follicles cultured in vitro. However, when EGF was associated with FSH, this reduction was inhibited. There is evidence for the action of EGF in regulating the activity of granulosa cells in vitro by inhibiting LH receptor expression and estradiol production induced by FSH, or by stimulating the FSH-R expression and progesterone production induced by FSH, or by binding affinity (Pulley and Marrone, 1986, Tapanainen et al., 1987, May et al. 1987; Hiramatsu et al., 1992, Luciano et al., 1994, Hattori et al., 1995). Studies suggest that both EGF and FSH can activate efficiently the cascade of mitogen-activated protein kinase (MAPK) in granulosa cells (Maizels et al., 1998). However, increases in cyclic adenine monophosphate synthesis (cAMP) induced by FSH interfered with the activation of MAPK signaling pathway in response to EGF (Wu et al., 1993) and its mitogenic effects in rat fibroblasts (Cook and McCormick, 1993).

In conclusion, the present study provides evidence that the EGF mRNA levels are higher in secondary follicles and that both FSH and EGF promote the growth of goat secondary follicles. Furthermore, EGF and FSH reduce the EGF mRNA levels, and EGF decreases the FSH-R mRNA levels in cultured secondary follicles. The results of the steady-state level of EGF e FSH-R mRNA and the culture system established in this work may contribute to future investigations on the mechanisms and substances involved in the regulation of follicular development. However, new studies are still necessary for a better understanding of the ovarian regulatory mechanisms.

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Disclosures

The authors declare that there is no potential conflict of interest that can be perceived as prejudicing the impartiality of the research reported.

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14 CONCLUSÕES

- Os RNAm para o KL, a BMP-15 e o EGF foram expressos nos compartimentos foliculares em todos os estádios de desenvolvimento, sugerindo assim o envolvimento destes fatores de crescimento no processo de desenvolvimento folicular na espécie caprina.

- A adição de 50 ng/mL de KL ao meio de cultivo promoveu a manutenção da integridade morfológica e ultraestrutural, o crescimento e a transição folicular em caprinos para o estádio primário após 7 dias de cultivo *in vitro.*

- A utilização de BMP-15 promoveu a sobrevivência e o crescimento folicular, e quando adicionado na concentração de 100 ng/mL estimulou o desenvolvimento dos folículos caprinos até o estádio secundário, assegurando ainda a manutenção da viabilidade folicular durante 7 dias de cultivo *in vitro*.

- A utilização de EGF promoveu a sobrevivência e a ativação folicular, e quando adicionado nas concentrações de 1 ou 10 ng/mL, estimulou o desenvolvimento dos folículos caprinos até o estádio primário, assegurando ainda a manutenção da integridade ultraestrutural folicular durante 7 dias de cultivo *in vitro*.

- A utilização de EGF promoveu o crescimento dos folículos secundários caprinos e a diminuição dos níveis de RNAm para o EGF e para o FSH-R após 6 dias de cultivo.

15 PERSPECTIVAS

Por meio do cultivo *in vitro* de folículos pré-antrais é possível se estudar o processo de foliculogênese, evento esse complexo e ainda não totalmente elucidado. Um conhecimento aprofundado desse processo pode ser empregado no desenvolvimento de biotecnologias reprodutivas, aumentando o sucesso das mesmas, inclusive em mamíferos selvagens ameaçados de extinção, e otimizando as ferramentas já utilizadas para o melhoramento genético de animais de interesse zootécnico, como os caprinos, ou até mesmo para o tratamento de infertilidade humana. Existem fortes evidências, conforme demonstradas neste trabalho, da regulação da foliculogênese inicial por diferentes substâncias, como hormônios e fatores de crescimento, sendo a completa elucidação dessa regulação um dos maiores desafios científicos.

Os fatores de crescimento KL, BMP-15 e EGF utilizados nesta pesquisa foram promissores no cultivo *in situ* de folículos pré-antrais iniciais caprinos, podendo ainda ser verificado efeitos positivos do EGF no cultivo de folículos secundários isolados. Entretanto, estudos complementares poderão ser realizados visando a utilização destas substâncias sobre o desenvolvimento *in vitro* de folículos pré-antrais isolados em estádios tardios, inclusive em cultivos *in vitro* de longa duração, ou ainda, em cultivos de dois passos (cultivo *in situ* seguido por um cultivo dos folículos isolados crescidos *in vitro*). Com base nos resultados alcançados nesse estudo sugere-se então a utilização sequencial das substâncias testadas, em que se propõe iniciar um cultivo de folículos *in situ* utilizando inicialmente o KL para promover a ativação dos folículos primordiais e garantir a sobrevivência folicular. Em um segundo momento, o emprego da BMP-15 poderá ser eficiente para a promoção da transição dos folículos primários para secundários. Uma vez obtido um grande número de folículos secundários, estes poderão ser isolados e cultivados *in vitro* na presença do EGF, o qual se mostrou como um fator importante para o crescimento dos folículos secundários e posterior desenvolvimento até os estádios antrais mais avançados. Além disso, são numerosas as possibilidades de se estudar a influência de tais substâncias sobre a expressão de outras que estejam envolvidas no controle do desenvolvimento folicular e diferentes vias de sinalização.

Diante das conclusões do trabalho, as informações obtidas poderão ser utilizadas para aperfeiçoar a elaboração e o fornecimento de meios de cultivo capazes de propiciar ótimas condições para um completo crescimento folicular e posterior produção de oócitos maturos a partir de folículos pré-antrais, revolucionando assim a produção *in vitro* de embriões.

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