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DEBORAH DE MELO MAGALHÃES PADILHA

EFEITO DO HORMÔNIO DE CRESCIMENTO (GH) E DO FATOR DE CRESCIMENTO SEMELHANTE À INSULINA I (IGF-I) NO DESENVOLVIMENTO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS E PERFIL DE EXPRESSÃO GÊNICA EM FOLÍCULOS SECUNDÁRIOS E ANTRAIS INICIAIS EM CAPRINOS

FORTALEZA
2012

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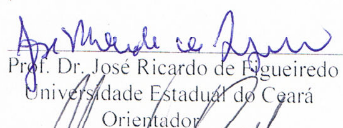
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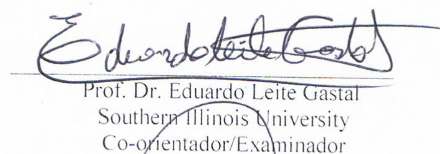
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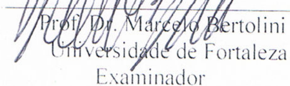
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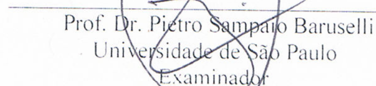
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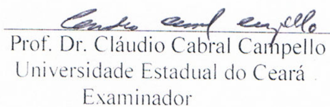
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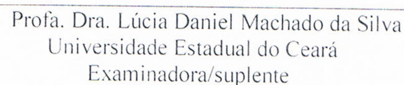
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Ao meu marido e filho, Rodrigo Tenório Padilha e Gustavo Magalhães Padilha por me fazerem descobrir o amor incondicional;
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Com amor, dedico

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“O cavalo prepara-se para o dia da batalha; mas
é do Senhor que vem a vitória” (Pv 21:31).

RESUMO

Os experimentos desta tese objetivaram avaliar: 1) o papel do IGF-I (Fase I) e do GH (Fase II), associados ou não ao FSH, em meio sequencial, sobre a sobrevivência, ativação e crescimento de folículos pré-antrais caprinos cultivados *in situ*, bem como expressão de RNAm para IGF-IR e FSHR em tecido cortical ovariano antes e após o cultivo *in vitro* (Fase I); 2) o efeito de diferentes intervalos de troca de meio de cultivo sobre a viabilidade e o desenvolvimento de folículos pré-antrais isolados de caprinos e ovinos (Fase III); 3) os efeitos do IGF-I (Fase IV) e do GH (Fase V), associados ao FSH, sobre a sobrevivência, formação de antro, crescimento *in vitro* de folículos secundários caprinos isolados, retomada da meiose de seus oócitos e a produção de embriões; 4) o perfil de expressão gênica de folículos secundários e terciários, através da técnica de microarranjo de DNA (Fase VI). Para o cultivo *in situ* (Fases I e II), fragmentos ovarianos foram cultivados por 16 dias e analisados por histologia, microscopia eletrônica de transmissão, microscopia de fluorescência e Azul de Trypan, sendo os folículos mensurados e classificados em primordiais, primários e secundários, bem como em normais ou degenerados. Quanto ao cultivo de folículos isolados, na Fase III, folículos secundários de cabras e ovelhas foram individualmente cultivados por 24 dias utilizando dois intervalos de troca de meio (dois ou seis dias). Nas Fases IV e V, folículos caprinos foram cultivados por 18 dias em diferentes concentrações de IGF-I (50 ou 100 ng/ml) ou GH (10 ou 50 ng/ml), na presença de FSH. Após 16 dias de cultivo *in situ*, observou-se que a associação de IGF-I e FSH durante todo o período de cultivo, bem como o meio sequencial contendo FSH até o dia 8 e GH do dia 8 ao dia 16 manteve a integridade ultraestrutural e a expressão de RNAm para FSHR, induziu a ativação, o crescimento folicular e a formação de folículos secundários. Na Fase III, para a espécie caprina e ovina, um maior percentual de folículos viáveis, taxa de formação de antro e oócitos destinados à MIV foram observados na troca de meio de cultivo a cada 2 e 6 dias, respectivamente. Nas Fases IV e V, a adição de IGF-I e GH ao cultivo promoveu a sobrevivência e melhorou a formação de antro, crescimento oocitário e retomada da meiose. Além disso, o GH resultou na produção de um embrião. A análise de microarranjo mostrou que existem importantes genes que são específicos para cada categoria folicular estudada (folículos secundários e terciários). Em conclusão, para caprinos e ovinos recomenda-se que o intervalo de troca de meio seja a cada 2

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Palavras-chave: IGF-I. GH. Folículos pré-antrais. Cultivo *in vitro*. Perfil de expressão gênica.

ABSTRACT

The aims of this thesis were to evaluate: 1) the role of IGF-I (Phase I) and GH (Phase II), alone or associated with FSH, in a sequential medium on the survival, activation and growth of caprine preantral follicles cultured *in situ*, as well as to quantify the levels of mRNA for IGFR-1 and FSHR in goat ovarian cortical tissue during *in vitro* culture (Phase I); 2) the effects of different medium replacement intervals on the viability and development of caprine and ovine preantral follicles (Phase III); 3) the effects of IGF-I (Phase IV) and GH (Phase V) associated with FSH on *in vitro* survival, antrum formation and growth of isolated caprine secondary follicles, as well as meiotic resumption of oocytes and embryo production; 4) the gene expression profile of secondary and tertiary follicles by microarray analysis (Phase VI). For the *in situ* culture (Phases I and II), ovarian fragments were cultured for 16 days and analyzed using histology, transmission electron microscopy, fluorescence microscopy and Trypan Blue dye and the follicles were measured and classified as primordial, primary or secondary, as well as normal or atretic. For the isolated follicles in Phase III, caprine and ovine secondary follicles were individually cultured for 24 days using two different medium replacement intervals (two or six days). In phases IV and V, caprine secondary follicles were cultured for 18 days with different concentrations of IGF-I (50 or 100 ng/mL) or GH (10 or 50 ng/mL) in the presence of FSH. After 16 days of *in situ* culture, the association of IGF-I and FSH during the entire culture period, as well as the sequential medium containing FSH until day 8 and GH from day 8 to day 16, maintained ultrastructural integrity and induced activation, follicular growth and secondary follicle formation. In Phase III, for the caprine and ovine species, higher percentages of viable follicles, antrum formation and oocytes acceptable for IVM were observed when the medium was replaced every 2 and 6 days, respectively. In phases IV and V, the addition of IGF-I and GH, respectively, to the culture medium promoted follicle survival and improved antrum formation, oocyte growth and meiotic resumption. Moreover, 50 ng/mL of GH resulted in embryo production. The microarray analysis showed important genes specific to each category analyzed (secondary and tertiary follicles). In conclusion, for isolated caprine and ovine preantral follicles, it is recommended medium replacement at intervals of two and six days, respectively. The use of FSH and GH in a sequential medium for in situ culture, as well as the association of IGF-I and FSH for a long-term in situ culture, and the use of IGF-I and GH for the culture of isolated secondary follicles, promoted the

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Keywords: IGF-I. GH. Preantral follicles. *In vitro* culture. Gene expression profiling.

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LISTA DE ABREVIATURAS E SIGLAS

AKR1C4	: Aldo-keto reductase family 1, member C4
ANA I	: Anaphase I (Anáfase I)
ANOVA	: Análise de variância
AT	: Azul de Tripan
ATP	: Adenosina Trifosfato
as	: Anti senso
Bak	: Bcl-2 antagonist killer (Antagonista assassino de Bcl-2)
Bax	: Bcl-2 associated X protein (Proteína x associada ao Bcl-2)
Bcl-2	: B-cell lymphoma 2 (Linfoma de células B2)
Bcl-xL	: Bcl-2 related protein, long isoform (Proteína relacionada ao Bcl-2 de longa isoforma)
Bid	: BH3 interacting domain death agonist (Agonista de morte com domínio de interação BH3)
Bik	: Bcl-2 interacting killer (Proteína exterminadora que interage com Bcl-2)
BMP	: Bone morphogenetic protein (Proteína morfogenética óssea)
BSA	: Bovine serum albumin (Albumina sérica bovina)
°C	: Graus Celsius
CaCl2	: Cloreto de cálcio
Calceína - AM	: Calceína acetoximetil
CAPES	: Coordenação de Aperfeiçoamento do Pessoal de Nível Superior

cDNA	:Complementary deoxyribonucleic acid (Ácido desoxirribonucleico complementar)
CGP	: Células Germinativas Primordiais
CNP	: C-type natriuretic peptide
CNPq	: Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	: Dióxido de Carbono
COCs	: Cumulus–oocyte complexes (Complexos cumulus-oócito)
CT	: Cycle threshold
CV	: Coefficient of variation (Coeficiente de variação)
D / d	: Day (dia)
Da	: Dalton
Dr.	: Doutor
Dra.	: Doutora
EGF	: Epidermal growth factor (Fator de crescimento epidermal)
EthD-1	: Ethidium Homodimer-1
FGF	: Basic fibroblast growth factor (Fator de crescimento fibroblástico)
FGF-2	: Basic fibroblast growth factor (Fator de crescimento fibroblástico básico)
FSH	: Hormônio Folículo Estimulante
Fig.	: Figura
Foxo 3a	: Forkhead box-O transcription factors 3a
G	: Gauge

g : Gravidade

gc : Granulosa cells (Células da granulosa)

GAPDH :Glyceraldehydes-2-phophate dehydrogenase (Gliceraldeído-2-fosfato desidrogenase)

GDNF :Glial cell-derived neurotrophic factor (Fator neurotrófico derivado da célula glial)

GDF-9 : Growth diferentiation factor-9 (Fator de crescimento e diferenciação-9)

GH : Growth hormone (Hormônio do crescimento)

GHR/R- GH : Receptor para o Hormônio do Crescimento

GnRH :Gonadotropin-Releasing Hormone (Hormônio liberador de gonadotrofinas)

GLM : General linear models (Modelos lineares generalizados)

GTPBP1 : GTP-binding protein 1

GV : Germinal vesicle (Vesícula germinal)

h : Horas

HC : Histologia clássica

IAA : Ácido 3-indol acético

IA : Inseminação artificial

i.e. : that is (isto é)

IBGE : Instituto Brasileiro de Geografia e Estatística

IGF : Insulin-like growth factor (Fator de crescimento semelhante à insulin

IGFBPs :Insulin-like growth factor-binding proteins (Proteínas de ligação dos fatores de crescimento semelhantes à insulina)

IGFR : Insulin-like growth factor receptor (Receptor para o fator de crescimento semelhante à insulina)

IL-1b : Interleucina 1b

IPA : Ingenuity Pathway Analysis

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

IVM : In vitro maturation (Maturação in vitro)

JAK-2 : Janus kinase-2

kDa : Quilodaltons

kg : Quilograma

KGF : Keratinocyte growth factor (Fator de crescimento keratinócito)

KL : Kit ligand

Kv : Quilovolts

LH : Hormônio Luteinizante

LAMOFOPA : Laboratório de Manipulação de Oócitos e Folículos Pré-Antrais

m : Mitochondria (mitocôndria)

M : Molar

MAPK : Mitogen-activated protein kinase (Proteína quinase ativada por mitógenos)

MEM^+ : Meio Essencial Mínimo suplementado

MET/TEM : Microscopia eletrônica de transmissão (TEM: sigla em inglês)

mg : Miligrama

MI : Metaphase I (Metáfase I)

MII	: Metaphase II (Metáfase II)
min	: Minutos
mL	: Mililitro
mm	: Milímetro
mm^3	: Milímetro cúbico
mM	: Milimolar
M	: Molar
mOsm/L	: Miliosmol/Litro
MOIFOPA	: Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-antrais
mRNA	: Messenger ribonucleic acid (Ácido ribonucléico mensageiro)
Na+	: Íon sódio
ng	: Nanograma
n	: Nucleus
nm	: Nanômetro
NOBOX	: Newborn ovary homeobox gene
NPPC	: Natriuretic peptide precursor type C
o	: Oocyte (Oócito)
O.D.	: Optical density
PAPPA-A	: Proteína Plasmática Associada à Gestação
PAS	: Ácido periódico de Schiff

P<0.05	: Probabilidade de erro menor do que 5%
P>0.05	: Probabilidade de erro maior do que 5%
p.	: Página
PCR	: Reação em Cadeia da Polimerase
pH	: Potencial Hidrogeniônico
PI3K	: Phosphoinositide 3-kinase (Fosfatidilinositol 3-quinase)
PIK3R6	: Phosphoinositide-3-kinase, regulatory subunit 6
PM	: Peso molecular
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)
pv	: Provérbios
qPCR	: Quantitative reverse transcription polymerase chain reaction (Reação em cadeia de polimerase via transcrição reversa quantitativa)
RT-PCR	: Reação em Cadeia de Polimerase-Transcriptase Reversa em tempo real
RENORBIO	: Rede Nordeste de Biotecnologia
rFSH	:Recombinant follicle stimulating hormone (Hormônio folículo estimulante)
s	: Sense (Senso)
SAS	: Statistical analysis system (Sistema de análise estatística)
sec	: Segundo
SD	: Standard deviation (Desvio padrão)

SEM	: Standard error of means (Erro padrão da média)
SLIT3	: Slit homolog 3
SIU	: Southern Illinois University
TGF-β	:Transforming growth factor beta (Fator de crescimento transformante beta)
S	: Supplemental table
SNK	: Student–Newman–Keuls
STAT	: Signal Transducer and Activator of Transcription
T	: Treatment (tratamento)
TE	: Transferência de embriões
U	: Unidade
UECE	: Universidade Estadual do Ceará
UNB	: Universidade de Brasília
x^2	: Qui-quadrado
v.	: Volume
VGBD	: Germinal vesicle breakdown
vs.	: Versus
wk	: Week
y	: Year
ZP	: Zona pelúcida
µg	: Microgramas

μL	: Microlitro
μm	: Micrômetro
%	: Porcentagem
~ :	:Aproximadamente

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1 INTRODUÇÃO

O rebanho nacional caprino e ovino é da ordem de 9.450.312 e 14.672.366 de cabeças, respectivamente, as quais estão distribuídas em todas as regiões do Brasil, porém com maior concentração na região Nordeste, representando aproximadamente 90% (caprino) e 54% (ovino) do rebanho brasileiro (IBGE, 2009). Apesar do grande efetivo do rebanho caprino e ovino nordestino, estas espécies são marcadas por índices insatisfatórios de desempenho produtivo, relacionado à deficiência no manejo sanitário e nutricional, criações de subsistência do pequeno criador, bem como pela ausência de biotecnologias de reprodução que caracterize seus sistemas. Neste sentido, a utilização de biotécticas reprodutivas como a inseminação artificial (IA), fecundação *in vitro*, transferência de embrião (TE) e clonagem podem contribuir para melhoria genética destas espécies, bem como preservação do material genético de raças em vias de extinção (MACHADO et al., 1995).

Atualmente, uma biotécnica reprodutiva que vem se destacando merecidamente é a manipulação de oócitos inclusos em folículos ovarianos pré-antrais (MOIFOPA), recentemente conhecida como "Ovário Artificial". A fêmea possui sua fertilidade mantida na forma de um pool de oócitos imaturos inclusos em folículos pré-antrais, os quais irão, em sua grande maioria, se tornar atrésicos durante a vida reprodutiva. A MOIFOPA objetiva resgatar esses folículos, previnindo-os da atresia e, por meio do cultivo *in vitro*, permitir que estes folículos alcancem sua completa maturação (FIGUEIREDO et al., 2007). Levando em consideração que a MOIFOPA ainda é uma técnica bastante onerosa, seu principal foco, hoje, é contribuir para elucidação dos mecanismos implicados na foliculogênese, conservação e multiplicação de animais em vias de extinção, bem como servir como modelo para estudos na reprodução humana, já que a foliculogênese em cabras e ovelhas possui uma alta similaridade com relação a mulheres. Futuramente, a MOIFOPA poderá tornar-se capaz de contribuir de forma efetiva para produção *in vitro* de embriões em larga escala, visando a multiplicação de animais de alto valor genético (FIGUEIREDO et al., 2008).

As informações disponíveis sobre as substâncias que controlam o desenvolvimento folicular e maturação oocitária *in vitro*, bem como a atresia em ovários caprinos ainda não propiciam resultados promissores, especialmente nas fases iniciais do crescimento folicular.

Sabe-se que em sistemas *in vivo* hormônios e fatores de crescimento agem harmonicamente controlando os mecanismos envolvidos na foliculogênese pré-antral. Dentre estes, deve-se citar o Fator de Crescimento Semelhante à Insulina I (IGF-I) e o Hormônio do Crescimento (GH). Experimentos *in vivo* têm revelado que o GH atua promovendo o desenvolvimento de folículos ovarianos de bovinos (GONG; BRAMLEY; WEBB, 1991), aumentando as concentrações periféricas de insulina e/ou IGF-I em novilhas (GONG et al., 1997). O IGF-I, adicionado durante o cultivo *in vitro* de folículos pré-antrais, estimulou o crescimento folicular em humanos (LOUHIO et al., 2000), bovinos (GUTIERREZ et al., 2000), ratos (ZHAO et al., 2001) e camundongos (LIU et al., 1998) em sinergia com o hormônio folículo estimulante (FSH), além de promover a ativação de folículos primordiais em caprinos (MARTINS et al., 2010).

Para uma melhor compreensão da relevância deste trabalho, a revisão de literatura a seguir abordará aspectos relacionados ao ovário mamífero, foliculogênese, população e atresia folicular, regulação da foliculogênese, cultivo *in vitro* de folículos pré-antrais e métodos de avaliação da qualidade folicular antes e após o cultivo *in vitro*. Ênfase será dada às substâncias avaliadas na presente tese. O Capítulo 1 da presente tese constitui um artigo de revisão (publicado) intitulado “Hormônio do crescimento (GH) e fator de crescimento semelhante à insulina-I (IGF-I): importantes reguladores das foliculogêneses *in vivo* e *in vitro*”. Os demais capítulos que compõem a tese são artigos técnicos envolvendo: efeito do IGF-I e GH para folículos cultivados inclusos no córtex ovariano (Capítulos 2 e 3 – artigos publicados) ou secundários na forma isolada (Capítulos 5 e 6 – artigos publicados); teste de intervalos de troca de meio para o cultivo *in vitro* de folículos pré-antrais caprinos e ovinos (Capítulo 4 – artigo publicado); e, por fim, estudo do perfil de expressão gênica de folículos isolados secundários e terciários de caprino, através da técnica de microarranjo de DNA (Capítulo 7 – artigo aceito para publicação).

2 REVISÃO DE LITERATURA

2.1 Ovário mamífero

O ovário, principal órgão do sistema reprodutor feminino, é dividido em duas regiões distintas, a saber: a medular e a cortical. Além disso, externamente este órgão é circundado pela superfície epitelial, denominada epitélio germinativo. Na maioria das espécies domésticas, exceto a equina, a região medular consiste na porção interna do ovário e é composta por tecido conjuntivo (fibroblastos, colágeno e fibras reticulares), nervos, artérias e veias, responsáveis pela sustentação e nutrição deste órgão (SILVA, 2005). Já a região cortical, também chamada de parenquimatosa, é considerada a porção funcional, sendo composta por células germinativas (oócitos) e somáticas (células da granulosa, da teca e do estroma), as quais formam os folículos (MCGEE; HSUEH, 2000). Após a ovulação do folículo, é formado o corpo lúteo, estrutura também presente no córtex que, assim como o folículo, possui vários estádios de desenvolvimento (Figura 1). O ovário exerce duas importantes funções fisiológicas: 1) liberação de oócitos maturos (ovulação) aptos a serem fecundados (BARNETT et al., 2006) e 2) produção de hormônios, fatores de crescimento e peptídeos (HIRSHFIELD, 1991). Estas duas funções são exercidas pela interação de dois fenômenos que ocorrem no ovário, a oogênese e a foliculogênese.

A oogênese consiste na formação e diferenciação das células germinativas primordiais (CGP) culminando com a formação do oócito haplóide fecundado (VAN DEN HURK; ZHAO, 2005). No embrião, as células germinativas presentes na parede do saco vitelínico, deslocam-se para as gônadas em desenvolvimento, sofrem extensiva proliferação mitogênica e redistribuição das organelas citoplasmáticas, transformando-se em oogônias (SADEU et al., 2006). Estas entram em mitose, seguida de meiose e diferenciam-se em oócitos primários, os quais iniciam a primeira divisão meiótica, passando pelos estádios da prófase I (leptóteno, zigóteno, paquíteno e diplóteno) da primeira divisão meiótica (HIRSHFIELD, 1991). No estádio de diplóteno ou vesícula germinativa da prófase I, ocorre a primeira interrupção da divisão meiótica e os oócitos permanecem neste estádio até a puberdade. A partir deste período, com o pico do FSH e do hormônio luteinizante (LH) durante a puberdade, os oócitos crescidos retomam a meiose e o

núcleo passa do estádio de vesícula germinativa para diacinese. Em seguida, ocorre a ruptura da vesícula germinativa, progressão para metáfase I, anáfase I, telófase I, expulsão do primeiro corpúsculo polar e formação do oócito secundário, iniciando a segunda divisão meiótica, em que o núcleo do oócito evolui até o estádio de metáfase II, quando ocorre a segunda interrupção da meiose (GORDON, 1994). O oócito permanece neste estádio até ser fecundado pelo espermatozóide, quando então completa a meiose e expulsa o segundo corpúsculo polar, formando o oócito haplóide fecundado.

A foliculogênese, por sua vez, é definida como o processo de formação, crescimento e maturação folicular, iniciando-se com a formação do folículo primordial e culminando com o estádio de folículo pré-ovulatório (VAN DEN HURK E ZHAO, 2005). O folículo é considerado a unidade morfológica e funcional do ovário mamífero, cuja função é proporcionar um ambiente ideal para o crescimento e maturação do oócito (CORTVRINDT E SMITZ, 2001), bem como produzir hormônios e peptídeos (ADASHI, 1992). De acordo com o grau de evolução folicular, a foliculogênese pode ser classificada em pré-antral ou antral, conforme será abordada no tópico seguinte.

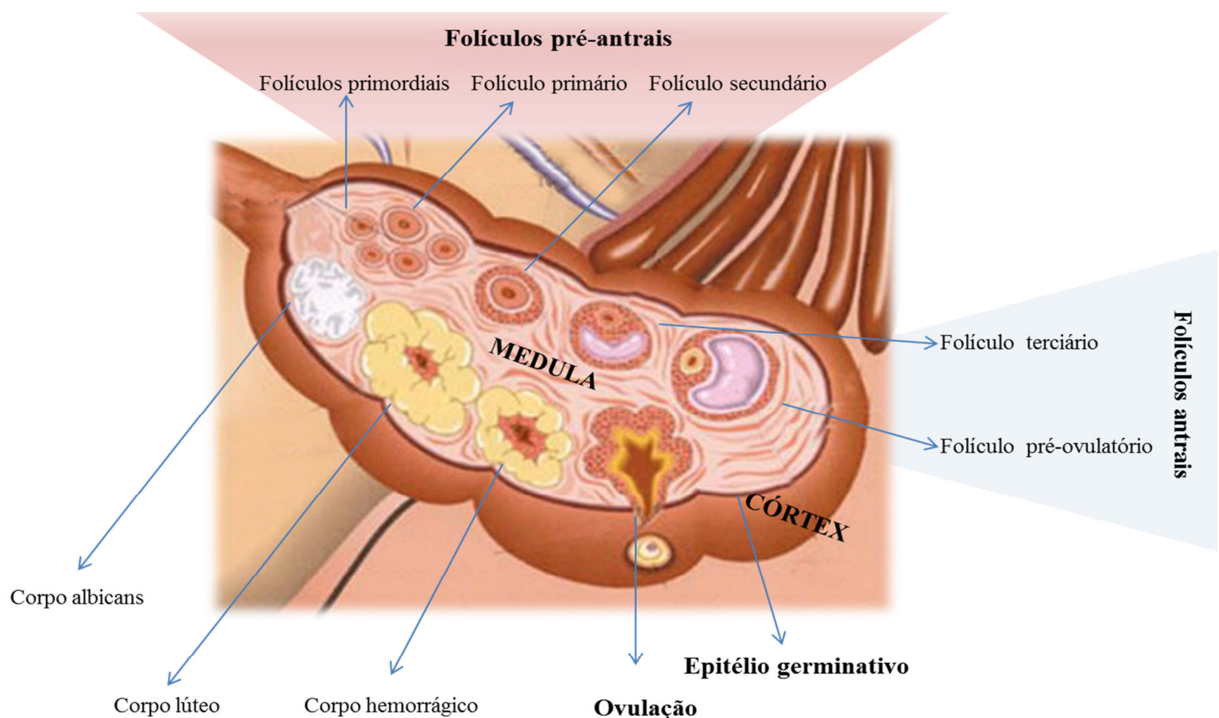

Figura 1. Esquematização do ovário mamífero e as estruturas que o compõem. Adaptado de http://reproducaohuman.blogspot.com.br/

2.2 Foliculogênese

2.2.1 Pré-antral

A fase pré-antral da foliculogênese compreende os folículos primordiais, primários e secundários, também chamados de folículos não-cavitários. Os folículos primordiais representam 95% da totalidade de folículos presentes no ovário e são os menores folículos, constituídos por um oócito quiescente, imaturo e circundado por uma camada de células da pré-granulosa de morfologia pavimentosa (SILVA, 2005), medindo aproximadamente 33 µm em caprinos (BEZERRA et al., 1998). O núcleo do oócito é relativamente grande e ocupa uma posição central à excêntrica. As proteínas que irão formar a zona pelúcida já começam a ser sintetizadas (LEE, 2000).

O pool de folículos primordiais é mantido em dormência e serve como reserva para a ativação folicular (KIM, 2012). A ativação, primeiro sinal do início do desenvolvimento de folículos primordiais, é definida como a retomada da proliferação das células da granulosa, com a mudança na morfologia dessas células, de pavimentosa para cúbica, bem como o crescimento oocitário, o que pode acontecer dias, meses ou anos após a sua formação (HIRSHFIELD, 1991). O controle da ativação de folículos primordiais envolve uma comunicação bidirecional entre o oócito e suas células somáticas circundantes (EPPIG et al., 2001).

Quando uma única camada de células da granulosa cúbicas circunda o oócito, forma-se o folículo primário (SILVA, 2005). Nesse folículo, a membrana plasmática do oócito já apresenta projeções que penetram entre as células da granulosa adjacentes, e algumas microvilosidades aparecem na superfície oocitária. Em caprinos, o folículo primário possue o diâmetro médio de aproximadamente 49,8 µm (BEZERRA et al., 1998).

Com a proliferação das células da granulosa, ocorre a formação do folículo secundário, caracterizado por um oócito circundado por duas ou mais camadas de células da granulosa de morfologia cúbica (SILVA, 2005), com as quais o oócito mantém íntimo contato. O núcleo do oócito assume uma posição excêntrica e as organelas movem-se para a periferia. A zona pelúcida já pode ser bem identificada (PARROTT; SKINNER, 1999). Nessa fase, inicia-se a formação das células da teca interna a partir do estroma intersticial (VAN DEN HURK; ZHAO, 2005). Em caprinos, os pequenos folículos secundários têm diâmetro médio de aproximadamente 83 µm

(BEZERRA et al., 1998).

2.2.2 Antral

À medida que o folículo cresce e se desenvolve, uma cavidade repleta de fluido folicular denominada antro começa a se formar e, a partir deste estádio, os folículos passam a ser classificados como antrais. Os folículos antrais, por sua vez, podem ainda ser classificados em terciário e pré-ovulatório (Figura 1). O fluido antral é um composto rico em substâncias reguladoras derivadas do sangue ou secreções das células foliculares, como por exemplo, gonadotrofinas, esteróides e fatores de crescimento. A produção desse fluido é intensificada pelo aumento da vascularização folicular e permeabilidade dos vasos sangüíneos (VAN DEN HURK e ZHAO, 2005) que ocorre com o desenvolvimento do folículo. Neste estádio, as células da granulosa são diferenciadas em células do cumulus (mais próximas ao oócito) e células murais.

O desenvolvimento dos folículos antrais é caracterizado por uma fase dependente de gonadotrofinas, as quais irão desencadear os mecanismos de recrutamento, seleção e dominância folicular (VAN DEN HURK e ZHAO, 2005), sendo a formação de folículos pré-ovulatórios um pré-requisito para a ovulação e formação do corpo lúteo, bem como para a manutenção da fertilidade. Além das gonadotrofinas, peptídeos sintetizados localmente desempenham papel chave na regulação da fase antral, tanto por meio de mecanismos parácrinos como endócrinos (FORTUNE, 2003).

2.3 População e atresia folicular

Desde 1950, tratamentos para falhas e insuficiência ovariana foram bastante restritos por se acreditar que o pool de células germinativas presentes ao nascimento (primatas e ruminantes), ou logo após o nascimento (roedores), era incapaz de ser renovável (EPIFANO; DEAN, 2002). Entretanto, estudos posteriores foram realizados em mulheres (BUKOVSKY et al., 2004) e camundongas (JOHNSON et al., 2005) durante a vida adulta sugeriram pela primeira vez a existência da neofoliculogênese (formação de novos folículos), desafiando o “dogma” instituido na literatura sobre a existência de uma reserva finita de oócitos ao nascimento. Recentemente, estudos associando técnicas *in vivo* e *in vitro* comprovaram a existência de células-tronco

presentes na superfície do epitélio ovariano capazes de serem transformadas em oócitos tanto em ovários de camundongas (ZOU et al., 2009) como de mulheres (WHITE et al., 2012). Além disso, esses estudos demonstraram ainda o nascimento de filhotes de camundongas após a fecundação in vitro desses oócitos oriundos das células-tronco (ZOU et al., 2009).

O número de folículos por ovário, independente da neofoliculogênese, varia entre espécies e indivíduos (KATSKA-KSIAZKIEWICZ, 2006), sendo de aproximadamente 1.500 na camundonga (SHAW et al., 2000); 33.000 na ovelha (AMORIM et al., 2000); 235.000 na vaca (BETTERIDGE et al., 1989), 35.000 na cabra (LUCCI et al., 1999) e, aproximadamente, 2.000.000 na mulher (ERICKSON, 1986). Apesar da grande população folicular presente no ovário mamífero, a quase totalidade dos folículos, ou seja, 99,9%, não chega à ovulação, mas morre por um processo natural denominado atresia, o qual ocorre por via degenerativa e/ou apoptótica (FIGUEIREDO et al., 2008). Mesmo sendo um fenômeno natural, a atresia reduz significativamente o número de oócitos que seriam ovulados, diminuindo assim o potencial reprodutivo das fêmeas. A atresia, independente da via em que ocorra, é um processo que pode acometer qualquer estádio do desenvolvimento folicular, sendo predominante na fase antral. Além de ser regulada principalmente por fatores endócrinos, como o FSH e o LH, fatores parácrinos, incluindo o Kit Ligand (KL), IGF-I, fator de crescimento epidermal (EGF), fator de crescimento fibroblástico-2 (FGF-2), ativina e interleucina-1b (IL-1b), também influenciam no processo de morte celular nos diferentes estádios foliculares. Desta forma, é provável que o balanço entre os fatores que promovem sobrevivência e aqueles que induzem a morte decidirá se um determinado folículo continuará o seu desenvolvimento ou sofrerá atresia (HSU; HSUEH, 2000).

No que se refere à atresia por degeneração, esta tem como uma das principais causa a isquemia folicular, em que a falha no fornecimento de oxigênio e nutrientes para o ovário provoca a morte celular. Este processo é iniciado por uma perda da homeostase celular e é caracterizado pela lise e desintegração da célula (BRAS et al., 2005). Já a atresia por apoptose, é um processo de morte celular individual e ativo, caracterizado pela fragmentação do DNA a cada 180-200 pares de base e formação de corpos apoptóticos, sendo um processo altamente dependente da expressão gênica, em que o desbalanço entre os genes pró (Bax, Bid, Bik; HUSSEIN, 2005; GREENFELD et al., 2007) e anti-apoptóticos (Bcl-2, Bcl-xL) determinam a morte celular (RACHID; VASCONCELOS; NUNES, 2000). A apoptose pode ocorrer por duas

vias, de acordo com o estímulo inicial: intrínseca (por meio da mitocôndria) ou extrínsica (por intermédio de receptores de superfície celular, também chamados de receptores de morte; ELMORE, 2007) .

Diante disso, visando evitar a enorme perda folicular que ocorre naturalmente *in vivo* pela atresia, nas últimas décadas têm sido desenvolvidos vários sistemas de cultivo *in vitro* de folículos pré-antrais e antrais que possibilitam o estudo dos fatores que controlam a atresia e o crescimento folicular.

2.4 Regulação da foliculogênese

A fase pré-antral da foliculogênese é, predominantemente, regulada por fatores intraovarianos (GONG et al., 1997). A comunicação parácrina e a autócrina entre oócitos e células da granulosa são mediadas por fatores de crescimento produzidos por ambas as células, destacando-se, dentre eles, o KL, o FGF, o fator de crescimento e diferenciação 9 (GDF-9) e a proteína morfogenética óssea 15 (BMP-15). O IGF-I nesta fase pré-antral tem uma ação endócrina, tendo em vista que os folículos pré-antrais expressam apenas os receptores (IGFR-1) e as proteínas ligantes (IGFBPs), mas não os ligantes. As IGFBPs regulam a biodisponibilidade local de IGF-I produzida no fígado (WEBB et al., 2003). Na fase pré-antral, os folículos são responsivos às gonadotrofinas (FSH e/ou LH), porém pouco dependentes destes hormônios. Alguns trabalhos demonstraram que o FSH e o LH podem atuar nesta etapa promovendo a sobrevivência, a ativação e o crescimento de folículos iniciais (SARAIVA et al., 2008; MAGALHÃES et al., 2009a,b). Até alguns anos atrás, acreditava-se que os folículos primordiais não possuíam receptores para FSH, mas que este hormônio poderia atuar de forma indireta, por meio da regulação da expressão de vários fatores de crescimento essenciais à ativação e ao posterior crescimento folicular (JOYCE et al., 1999; THOMAS et al., 2005). Entretanto, um estudo recente em caprinos demonstrou a presença de receptor para FSH no oócito de folículos primordiais, possibilitanto a existência da atuação direta deste hormônio nesta fase folicular (SANTOS et al., 2011).

Recentemente, foi reportada a importância da via fosfatidilinositol 3 quinase (PI3K) no controle da ativação folicular. Em ovário de camundongo deficientes para a proteína forkhead box O3 (Foxo3a), todo o pool de folículos primordiais foi ativado de forma prematura

(CASTRILLON et al., 2003). Além disso, também foi demonstrado que as vias de sinalização PI3K/phosphatase e fosfatase e tensina homolog (PTEN-regulador negativo do padrão PI3K) estão envolvidas na regulação da quiescência e ativação de folículos primordiais (JOHN et al., 2008; REDDY et al., 2008).

Os folículos ovarianos antrais se desenvolvem em ondas foliculares na maioria das espécies, incluindo humano, ruminantes e equinos. A foliculogênese em animais de laboratório tem a duração bem inferior (21 dias) a de animais domésticos, como por exemplo nos ruminantes (cerca de 6 meses) (LUSSIER; MATTON; DUFOUR, 1987). O destino de um folículo depende de um mecanismo complexo que envolve hormônios e fatores de crescimento atuando sinergicamente de maneira autócrina, parácrina e endócrina. Embora diversos fatores envolvidos no desenvolvimento e maturação folicular já tenham sido caracterizados, ainda não se conhece o porquê da seleção de um (espécies mono-ovulares) ou mais (espécies multi-ovulares) folículo(s) emergem como dominante, enquanto os demais tornam-se atrésicos. Os hormônios e fatores de crescimento mais importantes envolvidos na foliculogênese são aqueles produzidos pela hipófise (principalmente FSH, LH and GH), membros da superfamília do fator de crescimento transformante β (TGF β) (incluindo activina, inibina e folistatina), membros da família FGF, EGF, sistema dos fatores de crescimento semelhante a insulina (IGF), incluindo os IGFs e suas proteínas de ligação (IGFBPs).

A fase antral é conhecida como sendo dependente de gonadotrofinas. O FSH e o LH aumentam a atividade esteroidogênica nas células da granulosa e da teca, resultando em um aumento na síntese e no acúmulo de esteroides, especialmente o estradiol. Nesta fase, além das gonadotrofinas, peptídeos produzidos localmente desempenham um papel crucial na regulação das exigências foliculares, atuando por meio de mecanismos parácrinos e endócrinos. Um aumento nas concentrações plasmáticas de FSH estimula o recrutamento folicular e a emergência da onda folicular (FORTUNE, 1994). Em espécies monovulatórias, um folículo é selecionado (dominante) e adquire capacidade ovulatória, enquanto os demais subordinados entram em atresia. O folículo dominante atua de forma ativa na supressão do crescimento dos subordinados pela secreção de estradiol e inibina (FORTUNE, 1994; GINTHER et al., 1996). Até certa fase do desenvolvimento folicular antral, os folículos podem crescer independentemente do suporte do LH, mas o crescimento subsequente requer a presença de LH (GONG et al., 1997). Além das gonadotrofinas, peptídeos sintetizados localmente desempenham papel-chave na regulação da

fase antral, tanto por meio de mecanismos parácrinos como endócrinos (WEBB et al., 2003; FORTUNE; RIVERA; YANG, 2004). Dentre esses peptídeos, merecem destaque o sistema IGF, incluindo os fatores IGF-1, IGF-2 e as IGFBPs, além de alguns membros da família FGF, como o FGF-2, FGF-7 (ou KGF), FGF-8 e FGF-10 (FORTUNE; RIVERA; YANG, 2004; BURATINI et al., 2004, 2005; BURATINI, 2007). Há fortes evidências de que o sistema IGF desempenha um papel crítico na seleção do folículo dominante. Os IGFs agem de forma sinérgica com o FSH na promoção do crescimento folicular e na produção de estradiol (FORTUNE; RIVERA; YANG, 2004). Para a ovulação do folículo dominante, ocorre o aumento da pulsatilidade, seguido do pico de LH. Este pico também é responsável pela retomada da meiose para que ocorra a maturação oocitária.

Nos tópicos seguintes desta revisão serão abordadas as principais características dos diferentes sistemas de cultivo folicular *in vitro*, destacando-se também as diversas pesquisas com cultivo *in vitro* de folículos pré-antrais que vêm sendo utilizadas no intuito de elucidar a foliculogênese inicial.

2.5 Cultivo *in vitro* de folículos pré-antrais (Ovário Artificial)

Diferentes sistemas de cultivo têm sido desenvolvidos para manter a viabilidade e promover o crescimento de folículos pré-antrais *in vitro* (VAN DEN HURK et al., 2000). Nesses sistemas de cultivo, os folículos ovarianos podem ser cultivados dentro do próprio tecido ovariano (cultivo *in situ*) ou na forma isolada. Em roedores, devido à pequena dimensão da gônada feminina, os ovários são cultivados por inteiro no meio de cultivo (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, ricos em folículos primordiais, com o objetivo de estudar a ativação folicular e o posterior crescimento de folículos primários (bovinos: BRAW-TAL; YOSSEFI, 1997; humanos: ZHANG et al., 2004; caprinos: MARTINS et al., 2008; equinos: KEITH et al., *in press*). Além da praticidade, o cultivo de fragmentos de córtex ovariano tem a vantagem de manter o contato entre as células foliculares e as do estroma ovariano (ABIR et al., 2006).

Visando a realização de cultivo *in vitro* de folículos isolados, métodos mecânicos e/ou enzimáticos têm sido utilizados para a obtenção de um grande número de folículos primários e/ou

secundários intactos provenientes de ovários de diferentes espécies (cabras: LUCCI et al., 1999; ovelhas: CECCONI et al., 1999; vacas: FIGUEIREDO et al., 1993, ratas: ZHAO, 2001; camundongas: LENIE et al., 2004, PESTY et al., 2007; equinos: KEITH et al., *in press*). Esta forma de cultivo apresenta como vantagens o acompanhamento individual dos folículos, além de favorecer a maior perfusão do meio para o folículo (ABIR et al., 2006). Os folículos isolados podem ser cultivados diretamente sobre o suporte de plástico (placa de cultivo), sobre uma matriz de colágeno (DEMEESTERE et al., 2005) ou inclusos em gotas de alginato (WEST et al., 2007; XU et al., 2009). O modelo de cultivo tridimensional evita a aderência das células foliculares ao suporte plástico, mantendo integridade morfológica do folículo (SMITZ et al., 2010) e mimetizando o microambiente do estroma ovariano (XU et al., 2009).

Atualmente, o sistema que apresenta resultados mais satisfatório no cultivo *in vitro* de folículos pré-antrais de camundongos (produção de crias viáveis) é aquele conhecido como cultivo “em dois passos” (O’BRIEN; PENDOLA; EPPIG, 2003). Esse método associa os dois sistemas acima citados: *in situ* e isolado. Nesse modelo, inicialmente é efetuado o cultivo dos folículos no córtex ovariano (*in situ)* para maximizar a ativação folicular e o crescimento dos folículos primordiais até o estádio de secundário, na sequência o isolamento e cultivo dos folículos secundários crescidos *in vitro* (O’BRIEN; PENDOLA; EPPIG, 2003; TELFER et al., 2008).

O objetivo do cultivo *in vitro* é tentar ao máximo mimetizar as condições *in vivo* do ambiente folicular. Neste contexto, diversos estudos têm investigado os efeitos de hormônios e fatores de crescimento, adicionados isoladamente ou em associação, e em diferentes concentrações. No caso das substâncias adicionadas em associação, são utilizados tanto meios de cultivo não-sequenciais (quando as substâncias são adicionadas simultaneamente) quanto sequenciais (quando cada substância ou combinação de substâncias é adicionada em momentos diferentes do cultivo para suprir as necessidades específicas dos diferentes estádios foliculares). Dentre os fatores de crescimento e hormônios que atuam regulando a ativação e o crescimento dos folículos ovarianos, destacam-se o IGF-I e o GH. Ambos os fatores, em associação ou não com o FSH, correspondem ao foco da presente tese e terão suas características e funções detalhamente descritas no artigo de revisão intitulado: Hormônio do crescimento (GH) e fator de crescimento semelhante à insulina-I (IGF-I): importantes reguladores das foliculogêneses *in vivo* e *in vitro* (ver Capítulo 1).

2.5.1 Principais avanços no cultivo *in vitro* de folículos ovarianos

Um considerável progresso tem sido observado com o cultivo *in vitro* de folículos pré-antrais de diferentes espécies nas últimas décadas. O resultado mais satisfatório foi obtido por O'BRIEN; PENDOLA; EPPIG (2003), o qual obteve crias saudáveis a partir do cultivo de oócitos oriundos de folículos pré-antrais de camundongas. A produção de embriões proveniente de oócitos derivados de folículos pré-antrais crescidos *in vitro* foi relatada em ratas (DANIEL; ARMSTRONG; GORELANGTON, 1989), porcas (WU; EMERY; CARRELL, 2001) e, recentemente, em búfalas (GUPTA et al., 2008), ovelhas (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010) e cabras (MAGALHÃES et al., 2011a; estudo apresentado na presente tese – Capítulo 6). Em outras espécies, observou-se apenas o desenvolvimento até o estádio antral a partir do cultivo de folículos secundários avançados (bovina: GUTIERREZ et al., 2000; humana: ROY; TREACY, 1993; canina: SERAFIM et al., 2010). Entretanto, em gatas (JEWGENOW; STOLTE, 1996) e gambás (BUTCHER; ULLMAN, 1996), foi observado apenas o crescimento de folículos pré-antrais isolados após o cultivo *in vitro* sem a formação de antro. Esses diferentes resultados refletem a dificuldade da técnica de MOIFOPA e a diferença entre as espécies.

A dificuldade de alcançar nascimento após o cultivo de folículos pré-antrais oriundos de animais domésticos pode ser devida a alguns fatores como: 1) duração da foliculogênese ser menor em animais de laboratório (21 dias) quando comparada a de animais domésticos (6 meses em bovinos) (LUSSIER; MATTON; DUFOUR, 1987); 2) menor disponibilidade de ovários nas espécies domésticas; 3) maior homogeneidade genética em animais de laboratório. Além disso, o ambiente em que os animais ficam alojados possibilita um melhor controle com relação à temperatura, umidade e alimentação quando comparado a animais domésticos. Desta forma, o sucesso na obtenção de oócitos de boa qualidade após cultivo *in vitro* de folículos pré-antrais ainda é limitado, especialmente em ruminantes, sendo necessários mais esforços para melhorar as condições de cultivo.

2.6 Métodos de avaliação da qualidade folicular antes e após o cultivo *in vitro*

Com a finalidade de se avaliar mudanças morfo-fisiológicas ocorridas durante o cultivo in

vitro de folículos pré-antrais, diferentes técnicas de avaliação folicular antes e após o cultivo podem ser utilizadas. Dentre as técnicas disponíveis, pode-se destacar aquelas que permitem a avaliação da qualidade folicular, como a histologia clássica (HC), a microscopia eletrônica de transmissão (MET) e a microscopia de fluorescência (MATOS et al., 2007a). Além disso, existem ainda as técnicas que permitem o estudo da expressão e quantificação de genes, transcritos e proteínas envolvidos na foliculogênese. Dentre essas técnicas, merece destaque a análise de microarranjo de DNA, que embora onerosa, permite a análise de todo o genoma, contribuindo ricamente para uma melhor elucidação desse processo de biologia molecular. A seguir, será abordada brevemente cada uma dessas técnicas.

2.6.1 Histologia clássica

Por permitir uma análise quantitativa, a histologia clássica (HC) é uma valiosa técnica de análise folicular antes e após o cultivo *in vitro,* principalmente quando é utilizada em associação com outras técnicas. Além disso, essa análise permite verificar a mudança na morfologia das células da granulosa de pavimentosa para cúbica, fato que caracteriza a ativação folicular, bem como analisar a integridade morfológica do oócito, das células da granulosa e tecais. A HC permite, dessa forma, a classificação dos folículos quanto ao seu estádio de desenvolvimento (primordial, primário, secundário ou antral), bem como quanto às suas características morfológicas (normais ou atrésicos). Apesar das inúmeras vantagens, a HC não permite a avaliação da integridade das membranas plasmática e nuclear, bem como das organelas citoplasmáticas. A HC técnica pode ser executada não só em folículos isolados, como também naqueles inclusos em fragmentos de córtex ovariano (MATOS et al., 2007a). Nesse último caso, para que os folículos isolados não sejam perdidos durante o processamento histológico, pode-se encapsulá-los previamente em uma matriz de colágeno ou alginato e corá-los com azul de alcian para marcação de glicoproteínas (PARSHAD; KAURR; NATT, 2008).

Estudos utilizando a HC (coloração com hematoxilina-eosina ou ácido períodico de Schiff-hematoxilina-PAS) como ferramenta para avaliação folicular demonstraram que esta técnica pode apresentar resultados similares aos de viabilidade folicular utilizando marcadores fluorescentes como a calceína e o corante Azul de Tripan (AMORIM et al., 2003; ROSSETTO et al., 2009). Além disso, alguns autores demonstraram que a HC pode ainda, sem a mesma

precisão, mostrar resultados de morfologia folicular equivalentes aos obtidos nas análises sob microscópio eletrônico (ROSSETTO et al., 2009; CELESTINO et al., 2011; CHAVES et al., 2011). A HC pode ainda ser utilizada para avaliar morte celular devido à necrose, por meio da visualização da presença de vacúolos (MARTINEZ-MADRID et al., 2004).

2.6.2 Microscopia eletrônica de transmissão

A MET, técnica qualitativa e acurada, baseia-se em uma análise minuciosa de amostras por meio de um equipamento versátil (microscópio eletrônico de transmissão). Esta técnica é capaz de fornecer informações estruturais e químicas, produzindo imagens de elevada resolução. Esse sistema é baseado em um feixe de elétrons que atravessa uma amostra ultrafina e gera partículas e radiação que podem ser usadas para formar uma imagem ampliada (superiores a 10^6 vezes) ou realizar análise química da amostra (JUNQUEIRA; CARNEIRO, 2004). Por meio destes recursos, a MET é capaz de permitir uma precisa avaliação da integridade de membranas celulares e ainda avaliar importantes características de organelas citoplasmáticas, sendo comumente utilizada como uma técnica complementar à HC. (SALEHNIA; MOGHADAM; VELOJERDI, 2002).

Estudos com o cultivo *in vitro* de folículos pré-antrais demonstraram que os resultados encontrados na histologia clássica foram confirmados por MET (MATOS et al., 2007b; MARTINS et al., 2008, ROSSETTO et al., 2009; MAGALHÃES et al., 2009a,b). Entretanto, MATOS et al. (2006), embora tenham encontrado uma elevada percentagem de folículos histologicamente normais após 5 dias de cultivo em meio MEM suplementado com IAA, os estudos ultraestruturais não confirmaram a manutenção da integridade morfológica desses folículos.

2.6.3 Microscopia de fluorescência e corantes vitais

Uma técnica também bastante utilizada para avaliar a viabilidade folicular antes ou após o cultivo *in vitro* é a microscopia de fluorescência. O princípio desta técnica é a utilização de marcadores fluorescentes que, ao serem excitados com radiação de baixo comprimento de onda, absorvem energia e emitem luz de comprimento de onda maior (JUNQUEIRA; CARNEIRO,

2004). Por ser uma técnica rápida, confiável e prática para avaliação da viabilidade folicular antes ou após estudos de cultivo *in vitro* (CORTVRINDT; SMITZ, 2001; LOPES et al., 2009), a microscopia de fluorescência vem sendo comumente adotada para análise de folículos pré-antrais e oócitos após a maturação *in vitro* (MIV) (ROSSETTO et al., 2009; BRUNO et al., 2009; CELESTINO et al., 2011; MAGALHÃES-PADILHA et al., 2012a). Os folículos e/ou oócitos analisados por fluorescência nesses estudos tiveram como base a detecção simultânea de células vivas e mortas marcadas por calceína acetoximetil (calceína-AM) e pelo etídio homodímero-1 (EthD-1), respectivamente. A calceína-AM é um diéster fluorogênico que atravessa passivamente a membrana celular, quando então é convertido por esterases intracelulares (ativas somente em células íntegras), a seu análogo negativo, impermeável e de fluorescência verde. No tocante ao EthD-1, com a perda da integridade ou aumento da permeabilidade da membrana, a atividade esterásica cessa e este corante reage com os ácidos nucléicos, produzindo fluorescência vermelha (VAN DEN HURK et al., 1998). Desta forma, a calceína permite a verificação da viabilidade celular através da atividade esterásica, enquanto EthD-1 permite constatar as alterações físicas e químicas na membrana celular danificada, com elevação de 40 vezes em sua fluorescência à medida que se associa a ácidos nucléicos (LOPES et al. 2009).

Outro marcador que também pode ser utilizado na microscopia de fluorescência, juntamente coma calceína e o EthD-1, para avaliar os folículos cultivados *in vitro* é o Hoescht 33342. Esse marcador penetra nas células e se intercala entre as bases nitrogenadas do DNA e marca a cromatina, permitindo assim a avaliação do estádio meiótico oocitário (JEWGENOW, 1996; MATOS et al., 2007a).

O corante vital, que não utiliza a microscopia de fluorescência, mas também é uma ferramenta bastante útil é o Azul de Tripan (AT). Este corante atravessa membranas celulares apenas em células mortas, tornando-as azuis. Células vivas não são coradas devido aos seus controles de transporte transmembrana que não permitem a ligação do Azul de Tripan. O AT é utilizado em rotina de microscopia para corar e avaliar a viabilidade de folículos pré-antrais antes e após cultivo *in vitro* (LUZ et al., 2009; MAGALHÃES-PADILHA et al., 2012b).

2.5.4 Biologia molecular

Na tentativa de compreender algumas mudanças ocorridas durante o cultivo *in vitro*, bem

como o processo de foliculogênese ovariana, técnicas de biologia molecular vêm sendo comumente empregadas. Através dessas técnicas, é possível detectar alterações nos padrões de expressão de RNAm, tanto de ligantes quanto de receptores de diferentes substâncias, dentre eles fatores de crescimento e hormônios, que ocorrem em resposta a fenômenos relacionados à sobrevivência, ao crescimento e à diferenciação celular (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). Esta última, embora seja onerosa e de necessitar de profissionais qualificados para a interpretação dos resultados, é uma ferramenta valiosa no estudo da foliculogênese, pois permite não só a análise de todo o genoma, ou seja, milhares de genes simultaneamente presentes no folículo, como também estudar as principais vias de sinalização as quais esses genes estão associados.

Atualmente, a técnica mais utilizada para quantificar a expressão de RNAm é a RT-PCR em tempo real (qPCR) (KREUZER; MASSEY, 2002). Esta técnica permite uma análise da quantificação relativa 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 qPCR e da RT-PCR convencional, tem sido possível identificar diferentes substâncias, como hormônios e fatores de crescimento, presentes em folículos ovarianos caprinos (CELESTINO et al., 2011; FROTA et al., 2011; ALMEIDA et al., 2012; MAGALHÃES et al., 2012a).

3 JUSTIFICATIVA

As espécies caprina e ovina são de grande interesse sócio-econômico no Brasil, especialmente para a região Nordeste, onde se concentra aproximadamente 90% do rebanho nacional. Além disso, o uso destas espécies como modelo biológico para estudos na reprodução humana abre novas perspectivas de pesquisa. Entretanto, deficiências no manejo sanitário e nutricional, aliado a sistemas de reprodução ineficazes, faz com que as espécies caprina e ovina apresentem baixa produtividade. Desta forma, é de grande importância o desenvolvimento de biotécnicas que visem otimizar a eficiência reprodutiva nestas espécies, como por exemplo a MOIFOPA. No tocante à pesquisa fundamental, esta biotécnica poderá contribuir para a elucidação dos mecanismos implicados na foliculogênese na fase pré-antral.

A maior parte da população ovariana é constituída de folículos pré-antrais (aproximadamente 90%), os quais armazenam a quase totalidade dos oócitos presentes no ovário. Apesar dos milhares de folículos presentes no ovário mamífero, a maioria deles (99,9%) não chegam à fase ovulatória, sendo eliminados por atresia. Esse processo resulta em uma diminuição drástica no número de oócitos que possívelmente ovulariam e produziriam embriões viáveis. Neste contexto, a biotécnica de MOIFOPA visa resgatar, cultivar *in vitro*, bem como criopreservar os oócitos inclusos nos folículos pré-antrais que naturalmente iriam sofrer atresia. Em associação com outras tecnologias reprodutivas, como a FIV e a TE, a MOIFOPA poderá não somente otimizar, como também conservar o material genético de animais de alto valor zootécnico e/ou em vias de extinção, bem como para a implantação de bancos de germoplasma animal. Adicionalmente, esta biotécnica vem sendo utilizada com sucesso na indústria farmacêutica para testes de eficiência/toxicidade de substâncias, bem como oferecendo subsídios para compreensão da foliculogênese inicial. A MOIFOPA aliada à análise da expressão dos fatores que controlam a foliculogênese é de grande importância para a pesquisa fundamental ou básica e para a reprodução animal.

O conhecimento acerca da expressão de fatores e hormônios envolvidos no processo de desenvolvimento folicular, bem como das interações entre estas substâncias em cada uma das fases da foliculogênese, é extremamente necessário para que possam ser desenvolvidas estratégias de cultivo que busquem otimizar a produção *in vitro* de embriões a partir de folículos

pré-antrais. Além disso, o conhecimento do melhor intervalo de troca de meio em um sistema de cultivo *in vitro* é essencial para o sucesso da viabilidade e desenvolvimento folicular. Dentre as substâncias que vêm sendo utilizadas com bastante êxito no cultivo folicular em diversas espécies, destacam-se o IGF-I e o GH. Entretanto, a importância desses fatores adicionados na forma sequencial em um cultivo *in situ* de longa duração sobre características estruturais e ultraestruturais de folículos pré-antrais caprinos ainda não tinham sido estudadas. Além disso, não eram conhecidos os efeitos de diferentes concentrações de IGF-I e GH sobre o cultivo *in vitro* de folículos secundários caprinos isolados, bem como sobre a maturação e a fecundação dos oócitos oriundos desses folículos. Por fim, na espécie caprina, nunca tinha sido realizado nenhum estudo que permitisse avaliar simultaneamente todos os genes envolvidos na transição de folículos pré-antrais para antrais iniciais.

A fim de se alcançar os objetivos propostos nesta tese, foram empregadas técnicas eficientes não só de morfologia, como também de viabilidade e análise ultra-estrutural, tais como: histologia clássica, fluorescência, coloração com Azul de Trypan e microscopia eletrônica de transmissão. Além disso, técnicas de biologia molecular também foram utilizadas como ferramentas, dentre elas a RT-PCR em tempo real (qPCR) e a atual e valiosa análise de microarranjo de DNA, a qual permite a avaliação simultânea de milhares de genes, contribuindo para desvendar os mecanismos e vias de sinalização envolvidos na foliculogênese.

4 HIPÓTESES CIENTÍFICAS

Diante do exposto, as seguintes hipóteses científicas foram formuladas:

1) Folículos pré-antrais caprinos e ovinos requerem diferentes intervalos de troca de meio, durante o cultivo *in vitro*.

2) Os níveis de expressão de RNAm para os receptores de IGF-I e FSH em folículos caprinos variam em função do desenvolvimento folicular.

3) A utilização de meios sequenciais contendo IGF-I ou GH, associados ou não ao FSH, mantém a viabilidade folicular e promove a ativação e o crescimento de folículos pré-antrais caprinos após cultivo *in situ* de longa duração.

4) A adição de IGF-I ou GH associados ao FSH ao meio de cultivo *in vitro* influencia positivamente a sobrevivência, a formação de antro, o crescimento de folículos secundários e o desenvolvimento de oócitos meioticamente competentes, permitindo a produção de embriões caprinos provenientes de folículos pré-antrais isolados e cultivados *in vitro*.

5) O perfil de expressão gênica é modificado de acordo com a transição de folículos secundários para terciários iniciais.

5 OBJETIVOS

A presente tese foi dividida em 6 fases, sendo os respectivos objetivos gerais e específicos apresentados abaixo.

5.1 OBJETIVOS GERAIS

1) Verificar os efeitos do IGF-I e GH em um cultivo folicular *in situ* (Fases I e II) ou isolado (Fase IV e V), associados ou não ao FSH, sobre o desenvolvimento *in vitro* de folículos pré-antrais caprinos.

2) Avaliar o efeito de diferentes intervalos de troca de meio sobre o desenvolvimento *in vitro* de oócitos oriundos de folículos pré-antrais caprinos e ovinos cultivados *in vitro* (Fase III).

3) Investigar o perfil de expressão gênica de folículos ovarianos caprinos secundários e terciários isolados (Fase VI).

5.2 OBJETIVOS ESPECÍFICOS

1) Estudar o efeito do IGF-I e GH, associados ou não ao FSH em meio sequencial, sobre a morfologia, viabilidade, ultraestrutura, ativação e crescimento *in vitro* de folículos pré-antrais caprinos submetidos a um cultivo *in situ* de longa duração (16 dias).

2) Quantificar, através de RT-PCR em tempo real, a expressão de RNAm para receptores de IGF-I e FSH em folículos pré-antrais caprinos isolados e em tecido cortical ovariano antes e após o cultivo *in vitro*.

3) Avaliar o efeito de diferentes intervalos de troca de meio (a cada 2 ou 6 dias) sobre a morfologia, crescimento e maturação *in vitro* de oócitos oriundos de folículos pré-antrais caprinos e ovinos cultivados *in vitro*.

4) Verificar a influência de duas concentrações de IGF-I (50 ou 100 ng/mL) e GH (10 ou 50 ng/mL), na presença FSH, sobre a sobrevivência, formação de antro, crescimento e maturação *in vitro* de folículos secundários caprinos isolados e cultivados por 18 dias, bem como a influência do GH associado ao FSH na produção *in vitro* de embriões oriundos do cultivo de folículos pré-antrais.

5) Verificar detalhadamente, através de microarranjo de DNA, o perfil de expressão gênica de folículos ovarianos caprinos secundários e terciários, bem como as principais vias de sinalização que esses genes estão envolvidos.

Nas páginas seguintes, serão apresentados sete capítulos referentes aos artigos ténico-científicos (seis) e de revisão (um) que compõem esta tese. Vale salientar que seis dos artigos já se encontram publicados e um aceito para publicação. A revisão está disponível em periódico incluso no Qualis CAPES “B3” e seis artigos científicos publicados em periódicos inseridos no Qualis CAPES “A1” (dois artigo) e “A2” (quatro artigos). Além disso, dois resumos dos artigos técnicos que compreendem a presente tese foram premiados em competição de estudante: 1) SBTE 2010: “Produção de embrião após o crescimento, maturação e fecundação *in vitro* de oócitos oriundos de folículos pré-antrais caprinos cultivados na presença do hormônio de crescimento (GH)” – **terceiro lugar (ver apêndice)**. 2) Simpósio SIU 2011 - Departament of Plant Biology - Southern Illinois University, USA: “Gene expression during early folliculogenesis in goats using microarray analysis” – **primeiro lugar (ver apêndice)**.

6 CAPÍTULO 1

Hormônio do crescimento (GH) e fator de crescimento semelhante à insulina-I (IGF-I): importantes reguladores das foliculogêneses *in vivo* e *in vitro*

(Growth Hormone (GH) and Insulin-like growth factor I (IGF-I): major regulators of folliculogenesis in vivo and in vitro)

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Resumo

A foliculogênese é caracterizada pelo desenvolvimento folicular, envolvendo as etapas de ativação, crescimento e maturação. Durante este evento, hormônios e fatores de crescimento agem em conjunto controlando os complexos mecanismos envolvidos na fisiologia reprodutiva. Dentre estes, destacam-se o hormônio do crescimento (GH) e o fator de crescimento semelhante à insulina I (IGF-I), que estão presentes nas diversas etapas da foliculogênese, atuando de forma direta e/ou indireta a fim de proporcionar o desenvolvimento folicular. Durante as últimas décadas, pesquisas acerca do efeito destas substâncias, isoladas ou em associação, têm sido amplamente realizadas. Neste sentido, esta revisão irá abordar o papel do GH e do IGF-I na regulação da foliculogênese, bem como a interação destes fatores nos desenvolvimentos foliculares *in vivo* e *in vitro*.

Palavras-chave: Foliculogênese. GH. IGF-I. Reprodução *in vivo* e *in vitro*.

Hormônio do crescimento (GH) e fator de crescimento semelhante à insulina-I (IGF-I): importantes reguladores das foliculogêneses in vivo e in vitro

Growth Hormone (GH) and Insulin-like growth factor I (IGF-I): major regulators of folliculogenesis in vivo and in vitro

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Abstract

The folliculogenesis is characterized by follicular development, involving the steps of activation, growth and maturation. During this event, hormones and growth factors act jointly managing the complex mechanisms involved in reproductive physiology. Among these, we highlight the Growth Hormone (GH) and Insulin-like Growth Factor I (IGF-I) that are present at different stages of folliculogenesis, acting in ways direct or indirect to provide follicular development. During recent decades, searches on the effect of these substances alone or in combination have been widely performed. Therefore, this review will address the role of GH and IGF-I in regulation of folliculogenesis, as well as the interaction of these factors in follicular development in vivo and in vitro.

Keywords: foliculogenesis, GH, IGF-I, reproduction in vivo and in vitro.

Introdução

A foliculogênese pode ser definida como o desenvolvimento folicular desde o estádio primordial até o pré-ovulatório, envolvendo as etapas de ativação, crescimento e maturação. O desenvolvimento folicular é controlado por uma complexa interação entre fatores endócrinos, parácrinos e autócrinos (Gougeon, 1996). O destino de um folículo, portanto, depende do equilíbrio entre os fatores estimulantes e inibitórios no ovário. Os fatores estimulantes são responsáveis pela sobrevivência e pelo desenvolvimento folicular, enquanto os inibitórios são aqueles responsáveis pela atresia. Dentre os fatores envolvidos nessa regulação da foliculogênese, pode-se destacar o hormônio do crescimento (GH) e o fator de crescimento semelhante à insulina I (IGF-I), os quais são importantes reguladores das diversas etapas do desenvolvimento folicular.

O GH é um hormônio somatotrófico secretado pelo lobo anterior da hipófise na circulação, o qual se liga a receptores nos tecidos-alvo com o objetivo de estimular o crescimento (Herrington e Carter-Su, 2001). Estudos in vitro e in vivo têm revelado a importância deste hormônio durante o desenvolvimento folicular (Hutchinson et al., 1988; Gong et al., 1997; Sirotkin e Makarevich, 2002).

Os IGFs (IGF-I e IGF-II) podem ser produzidos na maioria dos órgãos e tecidos do organismo. Por não existir um órgão de armazenamento, a secreção destes fatores ocorre à medida que eles são produzidos (Yakar et al., 2002). No ovário, o IGF-I possui sua origem nas células da granulosa e tem como principal função estimular o desenvolvimento folicular nas fases pré-antral e antral (Armstrong e Benoit, 1996). Tendo em vista a grande importância do GH e do IGF-I na fisiologia da reprodução, esta revisão irá abordar o papel destas duas substâncias na regulação da foliculogênese, bem como a interação destes fatores nos desenvolvimentos foliculares in vivo e in vitro.

Fatores reguladores da foliculogênese

A foliculogênese pode ser dividida em duas fases: pré-antral e antral. A fase pré-antral inclui a ativação dos folículos de primordiais para primários e o crescimento destes para secundários. A fase antral está relacionada com o desenvolvimento dos folículos secundários até o estádio pré-ovulatório (Saumande, 1991). Os fatores autócrinos, parácrinos e endócrinos

envolvidos no desenvolvimento e na diferenciação folicular incluem fatores de crescimento, peptídeos e hormônios (van den Hurk e Zhao, 2005). Entretanto, as exigências foliculares são diferentes para cada fase da foliculogênese, diferindo com relação à substância requerida e às concentrações necessárias.

Na fase pré-antral, os folículos são responsivos às gonadotrofinas (hormônio folículo estimulante - FSH e/ou luteinizante - LH), porém pouco dependentes destes hormônios. Alguns trabalhos demonstraram que o FSH e o LH podem atuar na fase pré-antral promovendo a sobrevivência, a ativação e o crescimento de folículos iniciais (Saraiva et al., 2008; Magalhães et al., 2009). Embora os folículos primordiais não possuam receptores para FSH, alguns trabalhos demonstraram que este hormônio pode atuar por meio da regulação da expressão de vários fatores de crescimento essenciais à ativação e ao posterior crescimento folicular (Joyce et al., 1999; Thomas et al., 2005). A fase pré-antral é, predominantemente, regulada por fatores intraovarianos (Gong et al., 1996). A comunicação parácrina e a autócrina entre oócitos e células da granulosa são mediadas por fatores de crescimento produzidos por ambas as células, destacando-se, dentre eles, o kit ligant (KL), o fator de crescimento fibroblástico (FGF), o fator de crescimento e a diferenciação 9 (GDF-9) e a proteína morfogenética óssea 15 (BMP-15). O IGF-I nesta fase pré-antral tem uma ação endócrina, tendo em vista que os folículos expressam apenas os receptores (IGFR-1) e as proteínas ligantes (IGFBPs), mas não os ligantes. As IGFBPs regulam a biodisponibilidade local de IGF-I produzida no fígado (Webb et al., 2003).

A fase antral é conhecida como sendo dependente de gonadotrofinas. O FSH e o LH aumentam a atividade esteroidogênica nas células da granulosa e da teca, resultando em um aumento na síntese e no acúmulo de esteroides, especialmente o estradiol. Nesta fase, além das gonadotrofinas, peptídeos produzidos localmente desempenham um papel crucial na regulação das exigências foliculares, atuando por meio de mecanismos parácrinos e endócrinos. Um aumento nas concentrações plasmáticas de FSH estimula o recrutamento folicular e a emergência da onda folicular (Fortune, 1994). Em espécies monovulatórias, um folículo é selecionado (dominante) e adquire capacidade ovulatória, enquanto os demais subordinados entram em atresia. O folículo dominante atua de forma ativa na supressão do crescimento dos subordinados pela secreção de estradiol e inibina (Fortune, 1994; Ginther et al., 2003). Até certa fase do desenvolvimento folicular antral, os folículos podem crescer independentemente do suporte do LH, mas o crescimento subsequente requer a presença de LH (Gong et al., 1996). Há fortes

evidências de que o sistema IGF desempenha um papel crítico na seleção do folículo dominante. Os IGFs agem de forma sinérgica com o FSH na promoção do crescimento folicular e na produção de estradiol (Fortune et al., 2004). Para a ovulação do folículo dominante, ocorre o aumento da pulsatilidade, seguido do pico de LH. Este pico também é responsável pela retomada da meiose para que ocorra a maturação oocitária (Monniaux et al., 1997).

Hormônio do crescimento (GH)

Características estruturais e sítios de expressão do hormônio GH

O GH é um hormônio produzido pelos somatotrofos no lobo anterior da hipófise, é liberado na circulação e liga-se a receptores nos tecidos-alvo com o objetivo de estimular o crescimento (Herrington e Carter-Su, 2001). Este hormônio é constituído por uma cadeia única de 198 aminoácidos com duas pontes dissulfídricas internas, o que confere a esta estrutura um peso molecular de 22 kDa (Rosenfeld e Cohen, 2002). A glicina, particularmente, é o aminoácido mais importante para a atividade biológica do GH (Chen et al., 1991).

A secreção de GH ocorre em pulsos e é controlada pelo hipotálamo por meio do hormônio liberador do GH (GHRH), da somatostatina e da ghrelina. A somatostatina exerce um efeito inibitório, enquanto o GHRH e a ghrelina estimulam a secreção de GH por intermédio de receptores específicos distintos acoplados à proteína G (Rosicka et al., 2002). O GH possui dois locais de interação com seu receptor (GHR), podendo, em algumas espécies, ligar-se ao receptor da prolactina (Bramley et al., 1987).

Receptores e vias de sinalização celular do GH

Os GHRs pertencem à família dos receptores de citocinas, apresentando um domínio extracelular, uma porção transmembranária e um domínio citoplasmático (Sjiogren et al., 1999). No organismo, o GHR se apresenta na forma de dímero e exibe alterações conformacionais quando ligado ao GH, permitindo a transfosforilação dos hemirreceptores e, consequentemente, das proteínas responsáveis pela sinalização intracelular (Brown et al., 2005). A molécula de GH possui dois sítios de ligação na sua estrutura, cada um deles vai se ligar de modo sequencial a

duas moléculas de GHR (Carter-Su et al., 1996). Trabalhos recentes sugerem que alguns GHR possam também sinalizar por meio da dissociação da membrana plasmática e translocação para o núcleo, dirigindo-se à maquinaria transcripcional (Swanson et al., 2007). Conway-Cambel et al. (2007) demonstraram que o GHR nuclear está correlacionado com o alto status proliferativo tanto in vivo quanto in vitro.

A transmissão do sinal ocorre mediante a ativação e a fosforilação da enzima JAK2 (Janus kinase 2) e de resíduos do domínio intracelular do GHR, o que resulta no engajamento de diversas proteínas de sinalização intracelular, incluindo os STAT (signal transducters and activators of transcription) -1, -3 e -5, e componentes da via das MAP (mitogen-activated protein) quinases. A fosforilação do STAT-5 é importante nas ações somatotróficas do GH, pois participa da regulação da secreção do IGF-I e da IGFBP-3 (Kofoed et al., 2003).

Funções do GH nas foliculogênese in vitro e in vivo

Os efeitos do GH no ovário podem ser diretos ou indiretos. Os efeitos indiretos estão relacionados à atuação do IGF-I, já que o GH estimula a liberação deste fator, enquanto os efeitos diretos estão relacionados com a expressão para os receptores de GH (R-GH) que foram detectados em ovários humanos (Sharara e Nieman, 1994).

O RNA mensageiro (RNAm) para R-GH foi detectado em ovários bovinos e ovinos por hibridização in situ (Eckery et al., 1997). Esses autores demonstraram que este RNAm é abundante no oócito e em células da granulosa de folículos pré-antrais e pequenos folículos antrais de ovelhas. Em ovários bovinos, o RNAm já foi localizado no oócito de folículos primordiais e primários, e começa a ser expresso em células da granulosa de folículos primários, permanecendo durante o estádio secundário (Kölle et al., 1998).

O GH age sobre as células da granulosa de ratas acelerando o processo de diferenciação das células foliculares em células luteínicas (Hutchinson et al., 1988). Alguns trabalhos demonstraram que o GH, na concentração de 1 mg/mL, aumentou o diâmetro folicular durante quatro dias de cultivo em folículos pré-antrais de fêmeas de camundongos medindo 100-105 µm (Liu et al., 1998; Kikuchi et al., 2001). Kobayashi et al. (2000), utilizando essa mesma concentração em seus experimentos com camundongos, mostraram que o GH promoveu a produção de estradiol, a secreção de inibina e a proliferação das células da granulosa e da teca.

Em bovinos, a utilização de 100 ng/mL de GH associado à insulina aumentou a síntese de progesterona e a proliferação das células da granulosa cultivadas por quatro dias (Langhout et al., 1991). Em ovinos, o FSH e o GH atuaram como reguladores da secreção de IGF-I de forma dose-dependente, além de estimularem a produção de estrógeno (Khalid et al., 2000).

Recentemente, Shimizu et al. (2008) sugeriram que o aumento da expressão de GHR em células da granulosa pode ser um ponto de desvio para que o folículo antral entre no estádio pré-ovulatório durante o final do desenvolvimento folicular, podendo, dessa forma, auxiliar no processo de maturação de folículos pré-ovulatórios. Além disso, o GH também pode estar agindo indiretamente, via sistêmica ou pela produção local de IGF-I. Um grande número de estudos in vitro mostrou que o GH afeta a maturação do oócito, aumenta os receptores de gonadotrofinas e, desta forma, auxilia na foliculogênese (Sirotkin e Makarevich, 2002).

Experimentos in vivo têm revelado que o GH atua promovendo o desenvolvimento de folículos ovarianos de bovinos (Gong et al., 1991), aumentando as concentrações periféricas de insulina e/ou IGF-I em novilhas (Gong et al., 1997). Também nesta mesma espécie, Kaiser et al. (2006) observaram que, in vivo, o GH pode modular a formação das junções Gap no estádio inicial da foliculogênese. Estudos realizados por Swanchara et al. (1999) concluíram que, em bovinos, a imunoneutralização contra o fator liberador do GH diminuiu os níveis séricos de IGF-I e influenciou no desenvolvimento reprodutivo em novilhas púberes. Em ovinos, o cotratamento de ovelhas superovuladas com GH aumentou tanto a taxa de ovulação quanto o número de embriões transferidos (Folch et al., 2001).

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

Características estruturais e sítios de expressão da proteína IGF-I

O IGF-I, também conhecido como somatomedina C, pertence ao sistema IGF, o qual é composto por diferentes elementos, a saber: IGF-I e IGF-II, dois tipos de receptores (IGFR-1 e IGFR-2) e seis proteínas de ligação (IGFBP-1, -2, -3, -4, -5, e -6). Os IGFs (I e II) são fatores de crescimento peptídicos com elevado grau de homologia estrutural com a pró-insulina e se apresentam como moléculas de cadeia única com pesos moleculares de 7.649 e 7.471 Da, respectivamente, compartilhando resíduos idênticos em 45 posições e 62% de homologia entre si.

Esses fatores exercem atividade sobre o metabolismo intermediário, a proliferação, o crescimento e a diferenciação celular (Jones e Clemmons, 1995).

O IGF-I é produzido predominantemente no fígado exercendo a função de um hormônio endócrino, entretanto ele também pode ser sintetizado por tecidos-alvo atuando de forma parácrina e autócrina, sendo sua produção estimulada pelo GH. A secreção dos IGFs ocorre à medida que eles são produzidos, não existindo um órgão de armazenamento.

Receptores e vias de sinalização celular do IGF-I

Os IGFs (I e II) possuem dois tipos de receptores, IGFR-1 e IGFR-2. Esses receptores são do tipo tirosina quinase transmembrana e possuem uma elevada homologia com o receptor de insulina. O IGFR-1 é composto por duas subunidades extracelulares α e duas subunidades β de 135 kDa e 90 kDa, respectivamente. Cada subunidade α é ligada a uma subunidade β por uma ponte dissulfídrica, formando um α-β hemirreceptor que se liga a outro hemirreceptor por ponte dissulfídrica entre as subunidades α, dando origem ao o receptor completo (Martinelli e Aguiar-Oliveira, 2005). A subunidade β é constituída por uma parte extracelular, um segmento transmembranário e um domínio citoplasmático e, uma vez que esta subunidade é ativada, ela promove a fosforilação de resíduos de tirosina do próprio receptor e de proteínas-substrato associadas (Izadyar et al., 1998). As vias de sinalização ativadas são várias, dentre elas a fosfoinositol-3-quinase (PI3K) e as MAP quinases.

O IGFR-1 medeia a maioria das ações, tanto do IGF-I quanto do IGF-II. Entretanto, a afinidade deste receptor pelo IGF-I é maior do que pelo IGF-II ou pela insulina. O IGFR-2 se liga ao IGF-II, com menor afinidade ao IGF-I, mas não à insulina. Os efeitos do IGF-II são mediados via IGFR-1 (Izadyar et al., 1998). O papel fisiológico do IGFR-2 ainda não está bem elucidado. Há indícios de que o IGFR-2 possa participar da remoção do IGF-II do ambiente extracelular (Jones e Clemmons, 1995).

Interação IGF-I/IGFBPs

Os IGFs associam-se às proteínas transportadoras denominadas insulin-like growth factor binding proteins (IGFBPs). Nos fluidos biológicos, as IGFBPs estão presentes e atuam inibindo

ou potencializando a ação dos dois tipos de IGFs nas células-alvo. A biodisponibilidade de IGF pode ser aumentada por meio da atividade de enzimas específicas, as IGFBPs proteases. Os níveis de IGFBPs no líquido folicular alteram-se dramaticamente durante a foliculogênese (Monget et al., 1996). Essas proteínas intrafoliculares desempenham uma função-chave na regulação do desenvolvimento folicular por modularem os IGFs e, portanto, as ações das gonadotrofinas (Monget et al., 1989).

Durante o desenvolvimento folicular, o conteúdo de IGFBPs no líquido folicular tem sido estudado e quatro tipos de IGFBP foram identificados: IGFBP-3 (PM entre 39 e 49KDa); IGFBP-2 (PM de 35 KDa); IGFBP-5 (PM entre 30 e 32Kda) e a IGFBP-4 (PM entre 22 e 28 KDa), em bovinos (De La Sota et al., 1996) e ovinos (Monget et al., 1996). No soro, as concentrações de IGFBP-1 e -2 são reguladas negativamente ou não afetadas pelo GH, enquanto a concentração de IGFBP-3 está positivamente regulada pelo GH e pelo IGF-I (Monget et al., 1996).

De forma geral, as IGFBPs possuem quatro funções essenciais na regulação das atividades dos IGFs: 1) atuar como proteínas de transporte no plasma; 2) prolongar a meia-vida dos IGFs por regular sua depuração metabólica; 3) proporcionar um meio de tecido de células-alvo de tipo específico; 4) modular diretamente a interação dos IGFs com seus receptores e, assim, indiretamente, controlar a sua biorreatividade.

Funções do IGF-I nas foliculogêneses in vitro e in vivo

O envolvimento do IGF-I nos estádios iniciais da foliculogênese foi evidenciado por estudos em que o nocaute do gene comprometeu severamente o desenvolvimento pré-antral e o antral inicial em camundongos (Elvin e Matzuk, 1998). Em ovários de suínos e roedores, o IGF-I tem sido localizado nas células da granulosa de folículos antrais saudáveis, enquanto o IGF-II foi encontrado nas células da granulosa de folículos saudáveis e atrésicos (Zhou et al., 1996). Ambos os receptores de IGF estão presentes em células da granulosa de folículos primários, secundários e antrais (Monget et al., 1989).

O IGF-I, adicionado durante o cultivo in vitro de folículos pré-antrais nas concentrações de 20 e 50 ng/mL, estimulou o crescimento folicular em humanos (Louhio et al., 2000), bovinos (Gutierrez et al., 2000), ratos (Zhao et al., 2001) e camundongos (Liu et al., 1998), em sinergia com o FSH. Atuando em associação com o FSH, o IGF-I (100 ng/mL) também aumentou a

proliferação e a atividade esteroidogênica de células da granulosa de camundongos (Liu et al., 1998). Em suínos, a utilização de 50 ng/mL de IGF-I resultou no crescimento folicular, estimulou a proliferação das células da granulosa e preveniu a apoptose de folículos pré-antrais cultivados por quatro dias na presença de soro (Guthrie et al., 1998). Experimentos mostraram que o IGF-I, na concentração de 100 mg/L, proporcionou o crescimento e a viabilidade de oócitos inclusos em folículos pré-antrais caprinos (Zhou e Zhang, 2005b). Em camundongos, o IGF-I (10, 50 e 100 ng/mL) aumentou a esteroidogênese de folículos pré-antrais cultivados in vitro por seis, 10 e 12 dias (Demeestere et al., 2004). Em ratas, o IGF-I aumentou significativamente o diâmetro folicular e o conteúdo do DNA (Zhou et al., 1996). Fortune (2003) demonstrou que o IGF-I regula o desenvolvimento folicular nos estádios iniciais da foliculogênese, controlando o processo de maturação oocitária. A adição de IGF-I (100 ng/mL) ao meio de cultivo manteve a sobrevivência dos oócitos e estimulou o crescimento in vitro de folículos pré-antrais caprinos (Zhou e Zhang, 2005b). Nesta mesma espécie, o IGF-I estimulou o crescimento de oócitos de folículos pré-antrais, tendo sua ação reforçada pelo EGF (Zhou e Zhang, 2005b). Em bovinos, o IGF-I (10 ng/mL) promoveu o crescimento de pequenos folículos antrais in vitro e aumentou a viabilidade de oócitos (Walters et al., 2006), além de ter estimulado a proliferação e a sobrevivência das células da granulosa, prevenindo a apoptose (Quirk et al., 2004). Além disso, pesquisas demonstraram que a ação de IGF-I em grandes folículos secundários promoveu o aumento da incidência de dominância folicular e ovulação (Ginther et al., 2008).

Em um experimento realizado in vivo, Velazquez et al. (2009) observaram que o IGF-I exerce importante função no desenvolvimento folicular, na qualidade oocitária, e no posterior desenvolvimento embrionário em novilhas não superovuladas. O IGF-I desempenha um papel essencial na reprodução de mamíferos, como observado por meio de falhas na atividade ovariana e no desenvolvimento embrionário em modelos de camundongos com gene knockout para este fator (Elvin e Matzuk, 1998).

Considerações finais

O GH e o IGF-I desempenham funções essenciais na regulação do desenvolvimento folicular, tanto na fase antral como na pré-antral. Desta forma, um conhecimento aprofundado dessas duas substâncias e de suas atuações nas reproduções in vivo e in vitro permitirá maior

compreensão da fisiologia reprodutiva, principalmente na fase da foliculogênese pré-antral, a qual é menos elucidada. Uma compreensão maior das substâncias envolvidas no desenvolvimento folicular também permitirá a elaboração de um meio sequencial para o cultivo folicular, respeitando as exigências requeridas para cada fase folicular. Em grande parte das espécies, o GH e o IGF-I parecem não ser necessários para a ativação folicular, entretanto eles atuam promovendo o crescimento de folículos secundários e a formação de antro. O GH atua melhorando o desenvolvimento folicular, principalmente na fase antral inicial, e estimula a maturação oocitária, enquanto o IGF-I auxilia na esteroidogênese e na proliferação das células da granulosa, bem como no crescimento oocitário.

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7 CAPÍTULO 2 (Fase I)

Cultivo *in vitro* de longa duração de tecido cortical ovariano caprino: efeitos do FSH e IGF-I no desenvolvimento folicular pre-antral e na expressão de RNAm para receptores de FSH e IGF-I

(Long-term in vitro culture of ovarian cortical tissue in goats: effects of FSH and IGF-I on preantral follicular development and FSH and IGF-I receptor mRNA expression)

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Resumo

O cultivo *in vitro* de longa duração (16 dias) de tecido cortical ovariano caprino foi realizado para testar o efeito do FSH e IGF-I na viabilidade e desenvolvimento de folículos pré-antrais e expressão de RNAm para receptores de FSH e IGF-I. Os fragmentos ovarianos foram cultivados em α-MEM$^+$ sozinho ou suplementado com diferentes combinações de FSH e IGF-I (meio sequencial). O período de cultivo foi dividido em duas etapas (Dia 0 a 8 e Dia 8 a 16). Os folículos foram isolados e classificados em normais ou degenerados e primordiais, primários e secundários. A viabilidade dos folículos isolados foi determinada através do corante Azul de Trypan. A expressão de RNAm para FSHR e IGFR-1 foi avaliada através de qPCR. No Dia 8 de cultivo, mais ($P<0,05$) folículos normais e viáveis (média geral, 81% e 79%, respectivamente) foram observados nos tratamentos contendo IGF-I sozinho ou associado com FSH quando comparado aos tratamentos cultivados com FSH ou α-MEM$^+$ sozinho (68% e 63%). No Dia 16 de cultivo, tratamentos com FSH e/ou IGF-I tiveram mais ($P<0,05$) folículos viáveis (69%) do que o α-MEM$^+$ (38%). Os percentuais de desenvolvimento folicular observados nos tratamentos IGF-I/FSH, FSH+IGF-I/FSH+IGF-I e FSH/IGF-I foram similares, porém superiores ($P<0,05$) aos demais tratamentos. O tratamento que associou FSH e IGF-I durante todo o periodo de cultivo promoveu os maiores ($P<0,05$) diâmetros folicular e oocitário e percentual de folículo secundário (28%). A expressão de RNAm para FSHR no controle não-cultivado foi similar ao tratamento suplementado com FSH e IGF-I, porém superior ($P<0,05$) ao α-MEM$^+$. A expressão de IGFR-1 não diferiu entre os tratamentos. A associação de FSH e IGF-I em um cultivo *in vitro* de longa duração promoveu o desenvolvimento folicular, mantendo a expressão de RNAm para receptores de FSH.

Palavras-chave: Cabra. Folículos pré-antrais. FSH. IGF-I. Expressão de RNAm.

Long-term in vitro culture of ovarian cortical tissue in goats: effects of FSH and IGF-I on preantral follicular development and FSH and IGF-I receptor mRNA expression

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Abstract

Long-term in vitro culture (16 days) of caprine ovarian cortical tissue was performed to test the effect of FSH and IGF-I on the viability and development of preantral follicles and mRNA expression for FSH and IGF-I receptors. Fragments were cultured in α-MEM$^+$ alone or supplemented with different combinations of FSH and IGF-I (sequential medium). The culture period was divided into two parts. Follicles were isolated and classified as normal or abnormal and primordial, primary or secondary. Viability of isolated follicles was determined by staining with Trypan Blue dye. Expression of FSHR and IGFR-1 mRNA was evaluated by qPCR. At Day 8 of culture, more (P<0.05) follicles in treatments containing IGF-I alone or associated with FSH were normal and viable (overall mean, 81% and 79%, respectively) than the treatments cultured with FSH or α-MEM$^+$ alone (68% and 63%). At Day 16 of culture, treatments with FSH and/or IGF-I had more (P<0.05) viable follicles (69%) than α-MEM$^+$ (38%). The percentages of follicular development observed in the IGF-I/FSH, FSH+IGF-I/FSH+IGF-I, and FSH/IGF-I treatments were similar but higher (P<0.05) than the other treatments. FSH and IGF-I during the entire culture period maximized (P<0.05) follicular and oocyte diameters and percentage of secondary follicles (28%). FSHR mRNA expression in the non-cultured control was similar to the treatment supplemented with FSH and IGF-I, but higher (P<0.05) than α-MEM$^+$. IGFR-1 expression did not differ among treatments. Association of FSH and IGF-I in long-term in vitro culture promoted follicular development, maintaining FSHR mRNA expression.

Keywords: Goat. Preantral follicles. FSH. IGF-I. mRNA expression.

Introduction

The majority of follicles within the ovary remain as dormant primordial follicles and only a limited number are recruited from the resting follicle reservoir into the growing follicle pool. The activation of primordial follicles (transition from primordial to primary) is a progressive and highly regulated process, and during the stages of activation and growth most of the follicles undergo atresia (Skinner 2005). In vitro follicle culture is a tool used for understanding the underlying mechanisms of oocyte growth and development. Although several studies have tested hormones and growth factors on in vitro culture of preantral follicles (Lima et al. 2011; Magalhães-Padilha et al. 2011), the exact mechanisms responsible for the activation and subsequent growth and development of primordial follicles remain unclear. Some of the substances involved in the regulation of in vitro folliculogenesis are FSH and IGF-I (Magalhães et al. 2009; Martins et al. 2010, respectively). However, there is no information regarding the exact moment during early folliculogenesis when each of these substances are required. Recent studies (Lima et al. 2011; Magalhães-Padilha et al., unpublished data) have shown that different substances have to be added to the culture media sequentially for proper early follicle development.

FSH acts by binding to its receptor expressed in granulosa cells (O'Shaughnessy et al. 1996; Ulloa-Aguirre et al. 2003) from the primary follicle stage onward (Oktay et al. 1997). Although FSH receptors are not apparently present at the primordial stage, the possibility that FSH has an indirect effect on very early follicular development cannot be excluded. This indirect effect may occur via paracrine factors, such as IGF-I and activin released by larger follicles or ovarian stroma cells (van den Hurk and Zhao 2005). Some in vitro studies have verified that supplementation of the culture medium with FSH promotes the maintenance of preantral follicular viability and growth in goats (Matos et al. 2007; Magalhães et al. 2009) as well as steroidogenesis and antrum formation in different species (mouse: Cortvrindt et al. 1998; sheep: Cecconi et al. 1999; cattle: Gutierrez et al. 2000; human: Wu et al. 2002; pig: Wu and Tian 2007).

The involvement of IGF-I during the early stages of folliculogenesis was demonstrated when IGF-I induced the development of mouse preantral follicles in IGF-I gene knockout animals (Baker et al. 1996). Both mRNA and proteins for IGF-I are expressed in different follicle

compartments (cumulus-oocyte complex, mural granulosa and theca cells) at various stages (primordial, primary, secondary and antral follicles) of follicular development in goats and the presence of this growth factor in the medium promoted follicular survival and development (Martins et al. 2010). A recent study (Magalhães-Padilha et al. 2011) reported that caprine secondary follicles had a tendency for higher IGFR-I mRNA expression when the medium was supplemented with FSH during long-term in vitro culture and IGF-I had a positive effect on survival and antrum formation. Furthermore, some studies demonstrated that IGF-I in synergy with FSH stimulated preantral follicular growth in mice, cows, humans and rats (Liu et al. 1998; Gutierrez et al. 2000; Louhio et al. 2000; Zhao et al. 2001, respectively).

Therefore, in several species, FSH and IGF-I are known to be essential substances for adequate folliculogenesis. However, the association of FSH with IGF-I in a sequential medium used for long-term in vitro culture of early caprine preantral follicles has not been studied. In addition, there is a lack of information about mRNA expression for FSH and IGF-I receptors before and after in vitro culture of ovarian cortical tissue in goats. The aims of this study were to investigate the effect of a sequential medium containing FSH and IGF-I, in different combinations, on the survival, viability, development and in vitro growth of caprine preantral follicles during a long-term culture period (16 days; experiment 1) and to test the hypothesis that the combination of FSH and IGF-I would increase FSHR and IGFR-1 mRNA expression during in vitro culture of preantral follicles (experiment 2).

Materials and Methods

Chemicals

All chemicals and culture media used for this study were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Experiment 1. In vitro culture of preantral follicles

Source of ovaries

Goat ovaries (*n*=16) were obtained at a local slaughterhouse from eight non-pregnant adult (1–3 years old), crossbred goats (*Capra hircus*). Immediately postmortem, the ovaries were washed in 70% alcohol for 10 s and then washed two times in α-Minimum Essential Medium (α-MEM) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin. The pairs of ovaries were transported to the laboratory in α-MEM within 2 h at 4°C (Chaves et al. 2008).

Experimental protocol

The organ culture system utilized herein has been previously described in detail (Magalhães et al. 2009; Celestino et al. 2010). Briefly, the ovaries from each animal were stripped of surrounding fat tissues and ligaments. Ovarian cortex tissue samples from each ovarian pair were cut into 13 slices (3 mm x 3 mm x 1 mm) using a scalpel under sterile conditions. The samples were immediately submitted to mechanical isolation (non-cultured control) or placed in culture for 8 or 16 days. The cortex tissue samples were transferred to 24-well culture dishes containing 1 ml of culture medium. Culture was performed at 39°C in 5% CO_2 in a humidified incubator and all media were incubated for 1 h prior to use. The basic culture medium consisted of α-MEM (pH 7.2 to 7.4) supplemented with ITS (10 µg/ml insulin, 5.5 µg/ml transferrin and 5 ng/ml selenium), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml bovine serum albumin (BSA) and 50 µg/ml ascorbic acid, which was called α-MEM^+ (cultured control). For experimental conditions, the medium was supplemented with human recombinant IGF-I (Human IGF-I 50 ng/ml; PeproTech, Inc., Rocky Hill, NJ, USA) and/or human recombinant FSH (rFSH 50 ng/ml; BioVision, Mountain View, CA, USA) at different combinations (Fig. 1).

Treatment identification	α–MEM$^+$ supplementation	
	Day 0 → Day 8	Day 8 → Day 16
α–MEM$^+$/α–MEM$^+$	none	none
FSH/FSH	FSH	FSH
FSH/IGF-I	FSH	IGF-I
FSH + IGF-I/FSH + IGF-I	FSH + IGF-I	FSH + IGF-I
IGF-I/ IGF-I	IGF-I	IGF-I
IGF-I/FSH	IGF-I	FSH

Figure 1. Treatments according to medium supplementation for the in vitro culture of caprine ovarian tissue. Days 0 to 8 and Days 8 to 16 intervals are the first and second halves of the culture period, respectively. Identification of each sequential medium used in each half of the culture period is shown as well as the full composition of each treatment.

Each treatment was replicated eight times and the culture media were replenished every other day. The concentrations of FSH and IGF-I used in this work were chosen based on previous studies in our laboratory (Magalhães et al. 2009; Martins et al. 2010, respectively).

Assessment of follicular morphology, development and in vitro growth before and after in vitro culture ovarian tissue

Before culture (non-cultured control) and after 8 or 16 days in culture, preantral follicles were isolated from ovarian fragments using a mechanical method described by Lucci et al. (1999). Briefly, a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) was adjusted at a sectioning interval of 75 µm and samples were cut into small pieces. The fragments were placed in α-MEM$^+$, repeatedly aspirated 40 times using a large Pasteur pipette

(approximately 1600 µm in diameter) and aspirated again 40 more times with a smaller Pasteur pipette (approximately 600 µm in diameter) in order to dissociate preantral follicles from the ovarian stroma. The material was passed through 500 and 100-µm nylon mesh filters, resulting in a suspension containing preantral follicles less than 100 µm in diameter. This procedure was carried out at room temperature within a 10-min time frame.

Follicular stage and morphology were assessed using a microscope (Nikon, Tokyo, Japan) under 400× magnification. The developmental stages of follicles have been defined previously (Hulshof et al. 1994) as primordial (one layer of flattened granulosa cells around the oocyte) or developing, which includes primary (one layer of cuboidal granulosa cells) and secondary (two or more layers of cuboidal granulosa cells around the oocyte) follicles. To evaluate the quality of the isolated preantral follicles, variables such as integrity of the basement membrane, cellular density, presence or absence of picnotic bodies and integrity of the oocyte were observed. Overall, 3,120 follicles were evaluated for morphology (30 follicles per treatment x 8 replicates x 2 days of evaluation + 240 follicles for the non-cultured control).

To evaluate follicular development, the percentages of normal primordial and developing follicles (primary and secondary) were calculated before (non-cultured control) and after culture in each treatment. In addition, follicle and oocyte diameters were measured in normal follicles only. Follicular diameter was recorded from edge to edge of the outer layer of granulosa cells or from the outer layer of the theca cell layer when present. Oocyte diameter was recorded from edge to edge of the ooplasm. Two perpendicular diameters were recorded in each measurement and the average of these two values was calculated.

Preantral follicle live/dead status by Trypan Blue

To confirm the morphological findings for follicular health status, the follicle live/dead ratio for each treatment was analyzed using a more accurate method of assessment based on Trypan Blue staining. The operator did not have knowledge of the treatments when evaluating viability. Isolated early-stage follicles were classified as viable (not stained) or non-viable (stained) using Trypan Blue dye (Santos et al. 2008). Aliquots of 100 µl of suspension containing preantral follicles received 5 µl of Trypan Blue 0.4%. Overall, 3,120 follicles were evaluated for viability status.

Experiment 2. Expression of FSH and IGF-I receptor mRNA in ovarian cortical tissue

For this experiment, ten additional goat ovaries were used in three replicates. Ovarian fragments were cultured in α-MEM^{+} alone or supplemented with FSH and IGF-I (best treatment in experiment 1). Before (non-cultured control) and after in vitro culture for 16 days, fragments from each treatment were processed using a tissue chopper and mRNA isolation was performed using an RNeasy Mini Kit (Qiagen, Maryland, USA) for animal tissue. The elutes of total follicle RNA were treated with 1 µl DNase (Fisher Scientific, 1u/µl) for 10 min at 37°C and 2 min at 65°C to remove genomic DNA. Quality of the extracted RNA was evaluated by gel electrophoresis, while quantity was evaluated by ND 1000 (NanoDrop 1000 spectrophotometer). The entire total mRNA was intact with high quality, i.e. optical density (O.D.) 260/280 and 260/230 ratios were between 1.8 and 2.0 and 1.8 or greater, respectively.

The mRNA was reverse transcribed into first-strand cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) with random primers. The relative expression of mRNA for IGFR-1 and FSHR was determined by qPCR (quantitative Polymerase Chain Reaction) technique. Aliquots of 2 µl of cDNA were used as a template in 12.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 7.5 µl of ultra-pure water and 2 µM of each primer. The primers were designed to perform amplification of mRNA for IGFR-1 and FSHR. β-actin, Ubiquitin and GAPDH (Table 1) were used as endogenous controls for normalization of steady-state levels of mRNA of genes. The amplifications were carried out by one initial denaturation and activation of the polymerase for 10 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 (Bio-Rad, Hercules, California, USA). The delta-delta-CT method was used to transform CT values into normalized relative steady-state levels of mRNA (Saraiva et al. 2010; Magalhães-Padilha et al. 2011).

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

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
β-ACTIN	ACCACTGGCATTGTCATGGACTCT	S	188-211	GI:28628620
	TCCTTGATGTCACGGACGATTTCC	As	363-386	
GAPDH	AGGCAAATGTGTTCTCCAACCTGC	S	287-309	GI:27525390
	TGGAAGGCATCAGGGTCGATGTAT	As	440-462	
UBQ	GAAGATGGCCGCACTCTTCTGAT	S	607-631	GI:57163956
	ATCCTGGATCTTGGCCTTCACGTT	As	756-780	
IGFR-1	TCTGTTGATACTGGGAGGCTTGGT	S	6-30	GI:110347755
	AATACTCCGGGTTCACAGACGCAT	As	103-127	
FSHR	AGGCAAATGTGTTCTCCAACCTGC	S	250-274	GI:95768228
	TGGAAGGCATCAGGGTCGATGTAT	As	316-340	

S, sense; AS, antisense

Statistical analyses

Data that were not normally distributed, according to Shapiro-Wilk tests, were transformed to logarithms, arc sin or ranks. Sequential data were analyzed by SAS MIXED procedure (SAS version 9.2; SAS Institute, Cary, NC, USA). If an effect of treatment (control vs. treatments) or an interaction of treatment and day were significant or approached significance, data were further examined by Duncan's test to locate differences among treatments within each day and differences between days within a treatment. A probability of $P<0.05$ indicated that a difference was significant. Data are given as the mean ± standard error of the mean (SEM) unless otherwise stated. ANOVA was used to test the effect of FSH and IGF-I on the relative expression of FSH and IGF-I receptors on non-cultured control and ovarian fragments submitted to culture for 16 days in α-MEM$^+$ alone and with FSH+IGF-I/FSH+IGF-I supplementation.

Results

Experiment 1

Preantral follicular normality and viability before and after in vitro culture

A total of 6,240 (3,120 for normality and 3,120 for viability) preantral follicles were analyzed. The percentage of morphologically normal and viable preantral follicles before and after 8 or 16 days of in vitro culture is shown (Fig. 2).

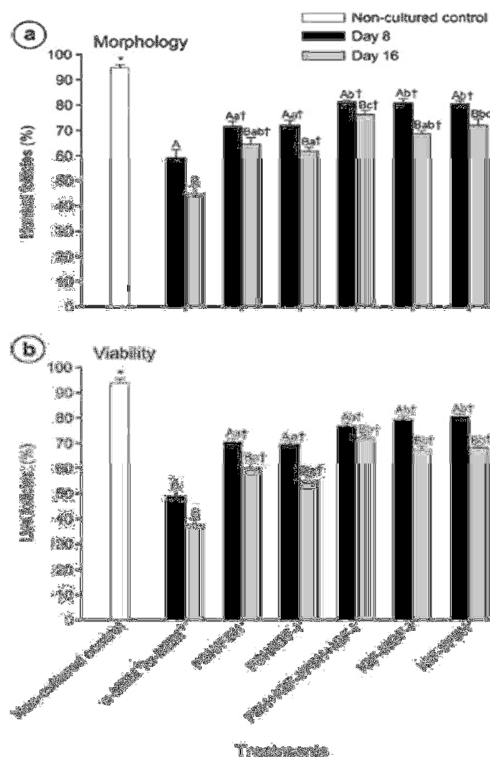

Figure 2. Percentage of **a** normal and **b** viable follicles in non-cultured ovarian cortical tissue (non-cultured control) and in tissue after 8 or 16 days of culture in α-MEM$^+$ alone or supplemented with different combinations of FSH and/or IGF-I. *The non-cultured control differs (P<0.05) from all treatments. † Differs significantly from α-MEM$^+$/α-MEM$^+$ treatment on the same day of culture (P<0.05). $^{a–c}$Within each day, values without a common superscript differ (P<0.05). ^{A,B}Within each treatment, values without a common superscript differ (P<0.05) between days.

Normal follicles from the non-cultured control and after culture in α-MEM$^+$ supplemented with FSH and IGF-I during the entire culture period (FSH+IGF-I/FSH+IGF-I) are illustrated (Fig. 3a and b, respectively). A viable follicle from the FSH+IGF-I/FSH+IGF-I treatment and a degenerated/dead follicle after 16 days of culture in α-MEM$^+$ alone are also illustrated (Fig. 3c and d, respectively). In the degenerated/dead follicle, disorganized granulosa cells were noticeable.

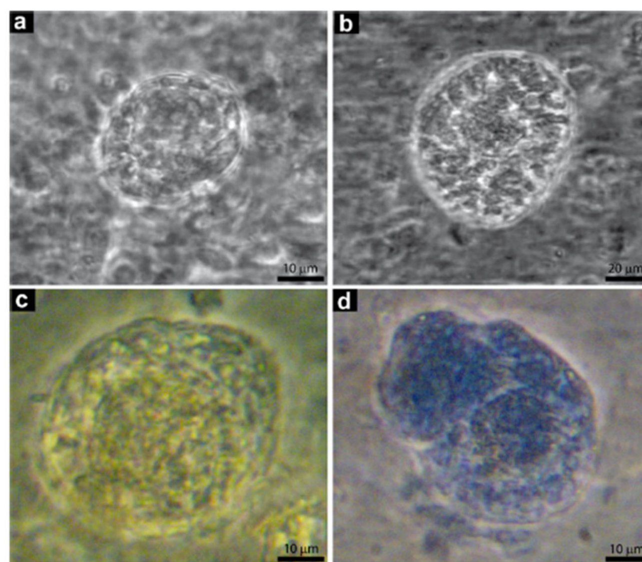

Figure 3. Mechanically isolated follicles visible by light microscopy. **a** Normal primordial follicle from the non-cultured control; **b** Normal secondary follicle cultured in the FSH+IGF-I/FSH+IGF-I treatment; **c** Live (viable) primary follicle cultured in the FSH+IGF-I/FSH+IGF-I treatment; and **d** Dead (non-viable) secondary follicle cultured in α-MEM$^+$ alone stained with Trypan Blue.

During the culture period (days 0 to 16), there was a progressive and significant (P<0.05) reduction in the percentage of normal and viable follicles in all treatments. At Day 8 of culture, follicles from the treatments cultured with IGF-I alone or associated with FSH had a higher (P<0.05) percentage of follicular normality and viability than the follicles from treatments cultured with FSH or α-MEM$^+$ alone. At Day 16 of culture, the FSH+IGF-I/FSH+IGF-I treatment had more (P<0.05) normal follicles than all other treatments, except for the IGF-I/FSH treatment. The percentages of viable follicles observed in the IGF-I/IGF-I, IGF-I/FSH and FSH+IGF-

I/FSH+IGF-I treatments were similar but higher ($P<0.05$) than the other treatments after 16 days of culture.

Development of primordial follicles after in vitro culture

The percentage of preantral follicular development in the non-cultured control and after 8 or 16 days of in vitro culture is shown (Fig. 4a). The percentage of follicular development was higher ($P<0.05$) at days 8 and 16 of culture in all treatments when compared to the non-cultured control. At day 8, the treatments cultured with FSH alone had a higher ($P<0.05$) percentage of follicular development than the treatments cultured with IGF-I or α-MEM$^+$ alone. In all treatments, the percentage of follicular development increased ($P<0.05$) from days 8 to 16, except the FSH/FSH treatment. At day 16, the percentages of follicular development observed in the IGF-I/FSH and FSH+IGF-I/FSH+IGF-I treatments were similar but higher ($P<0.05$) than the α-MEM$^+$/α-MEM$^+$ and the other treatments, except for the FSH/IGF-I treatment.

Percentage of secondary follicles after in vitro culture

The FSH+IGF-I/FSH+IGF-I and IGF-I/FSH treatments increased ($P<0.05$) the percentage of secondary follicles from days 8 to 16. At Day 16 of culture, the FSH/FSH, FSH+IGF-I/FSH+IGF-I and IGF-I/FSH treatments showed more ($P<0.05$) secondary follicles when compare to the α-MEM$^+$/α-MEM$^+$ treatment. Moreover, the highest ($P<0.05$) percentage of secondary follicles was observed in the FSH+IGF-I/FSH+IGF-I treatment (Fig. 4b).

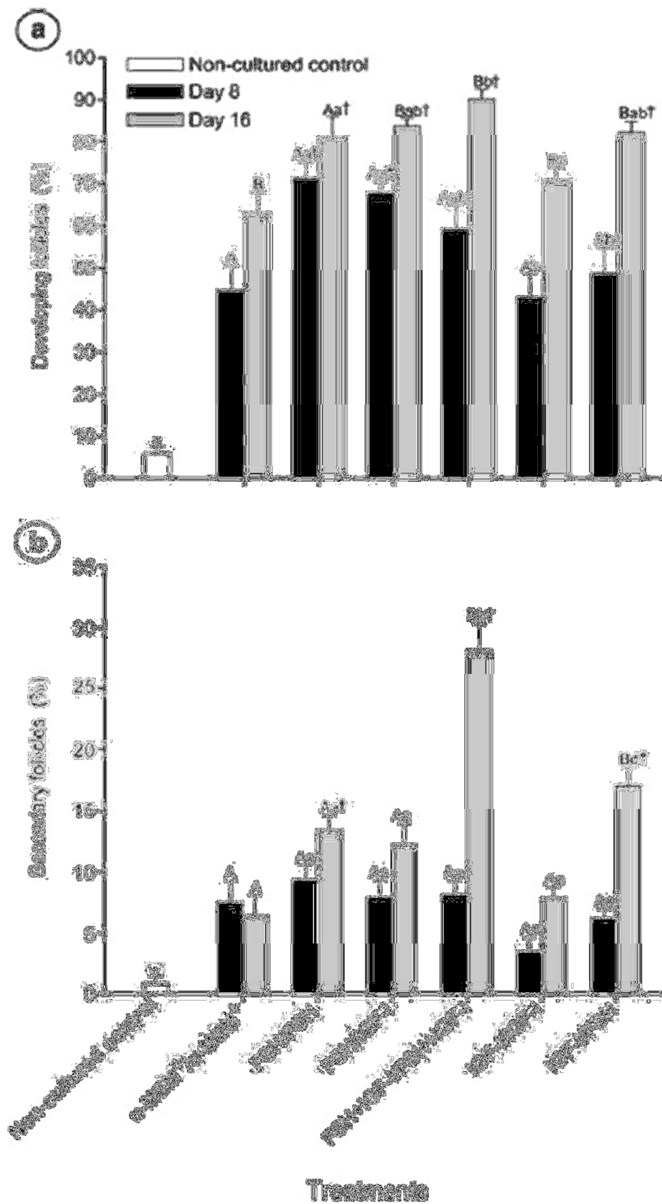

Figure 4. Percentage of **a** follicular development and **b** secondary follicles in non-cultured ovarian cortical tissue (non-cultured control) and in tissue after 8 or 16 days of culture in α-MEM$^+$ alone or supplemented with different combinations of FSH and/or IGF-I. * The non-cultured control differs (P<0.05) from all treatments. † Differs significantly from α-MEM$^+$/α-MEM$^+$ treatment on the same day of culture (P <0.05). $^{a–d}$Within each day, values without a common superscript differ (P<0.05). ^{A,B}Within each treatment values without a common superscript differ (P<0.05) between days.

Follicular and oocyte diameters

At days 8 and 16 of culture, all treatments had an increase in follicular and oocyte diameters when compared to the non-cultured control (Table 2). At day 8 of culture, all treatments resulted in an increase in follicular and oocyte diameters when compared to the α-MEM$^+$/α-MEM$^+$ treatment, except IGF-I/IGF-I and IGF-I/FSH treatments. At day 8 of culture, the treatments cultured with FSH alone or associated with IGF-I had greater (P<0.05) follicular and oocyte diameters than the other treatments. At day 16 of culture, the FSH+IGF-I/FSH+IGF-I treatment had the greatest (P<0.05) follicular and oocyte diameters. Moreover, an increase in follicular and oocyte diameters was observed from days 8 to 16 in the α-MEM$^+$/α-MEM$^+$, FSH+IGF-I/FSH+IGF-I and IGF-I/FSH treatments.

Table 2. Follicle and oocyte diameters (mean ± SEM) in non-cultured tissues (non-cultured control) and in tissues cultured for 8 or 16 days in α-MEM^{+} alone or α-MEM^{+} containing IGF-I and/or FSH.

Treatment	Day 0	Day 8	Day 16
Follicle diameter (µm)			
Non-cultured control	31.5 ± 1.1	-	-
α-MEM^{+}/α-MEM^{+}	-	42.7 ± 1.0 ^{Aa*}	47.2 ± 1.2 ^{Ba*}
FSH/FSH	-	49.0 ± 1.2 ^{Ac*}	50.8 ± 1.1 ^{Ab*}
FSH/IGF-I	-	48.3 ± 1.2 ^{Abc*}	49.7 ± 1.0 ^{Aab*}
FSH+IGF-I/FSH+IGF-I	-	49.6 ± 1.3 ^{Ac*}	59.6 ± 1.4 ^{Bc*}
IGF-I/IGF-I	-	43.9 ± 1.1 ^{Aa*}	48.4 ± 1.1 ^{Bab*}
IGF-I/FSH	-	45.5 ± 1.4 ^{Aab*}	51.5 ± 1.2 ^{Bb*}
Oocyte diameter (µm)			
Non-cultured control	26.8 ± 0.7	-	-
α-MEM^{+}/α-MEM^{+}	-	34.5 ± 0.5 ^{Aa*}	35.9 ± 0.5 ^{Ba*}
FSH/FSH	-	37.8 ± 0.5 ^{Ac*}	37.6 ± 0.5 ^{Abc*}
FSH/IGF-I	-	37.4 ± 0.5 ^{Abc*}	37.0 ± 0.4 ^{Aabc*}
FSH+IGF-I/FSH+IGF-I	-	38.2 ± 0.6 ^{Ac*}	41.0 ± 0.6 ^{Bd*}
IGF-I/IGF-I	-	35.5 ± 0.5 ^{Aa*}	36.4 ± 0.5 ^{Aab*}
IGF-I/FSH	-	36.1 ± 0.6 ^{Aab*}	38.1 ± 0.5 ^{Bc*}

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

$^{a–d}$ Within a column, values without a common superscript differ (P<0.05).

^{A,B} Within a row, values without a common superscript differ (P<0.05).

Experiment 2

FSH and IGF-I receptor mRNA expression

Ovarian cortex tissue cultured for 16 days in the FSH+IGF-I/FSH+IGF-I treatment had similar (*P*>0.05) FSHR mRNA expression when compared to the non-cultured control. However, tissue cultured for 16 days in α-MEM$^+$ alone had a lower (*P*<0.05) relative expression of FSHR than the non-cultured control (Fig. 5a). No difference (*P*>0.05) was detected among treatments for IGFR-1 mRNA expression (Fig. 5b).

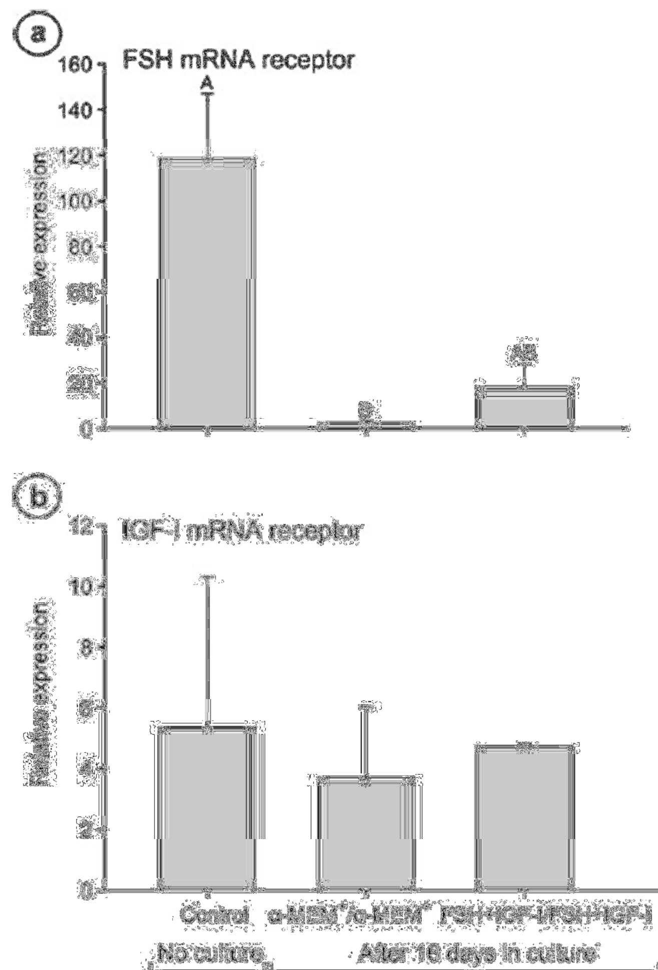

Figure 5. Relative expression of FSHR **a** and IGFR-1 **b** mRNA in ovarian cortical tissue at Day 0 (non-cultured control) and Day 16 of in vitro culture in the presence or absence of FSH and IGF-I. [A,B] Among treatments, values without a common superscript differ (P<0.05).

Discussion

Our results demonstrate, for the first time, the importance of FSH associated with IGF-I during an entire long-term culture period (16 days) on early folliculogenesis in goats. The association of these substances stimulated activation of primordial follicles as well as the development of primary to secondary follicles. The positive effect of the association between FSH and IGF-I in this study was further confirmed by the similarity of expression of FSH receptor mRNA in cultured ovarian tissue when compared with the non-cultured tissue and decreased expression of FSH receptor mRNA when the tissue was cultured without FSH and IGF-I.

The highest percentage of follicular viability observed in the IGF-I /IGF-I, IGF-I/FSH and FSH+IGF-I/FSH+IGF-I treatments at day 16 demonstrated the importance of IGF-I during the first half of the culture period. Recently, Martins et al. (2010) reported that IGF-I promoted the survival of primordial and primary follicles submitted to in situ ovarian culture for 7 days in goats. Moreover, the relevance of the IGF system in regulating the early stages of oocyte and preantral follicular development has also been demonstrated in goats by other researchers (Thomas et al. 2007; Magalhães-Padilha et al. 2011). In the current study, the presence of FSH associated with IGF-I was essential for maintaining follicular normality during the second half of the culture. This result is supported by the fact that FSH receptors only begin to be expressed in granulosa cells (Ulloa-Aguirre et al. 2003) at the primary follicular stage (Oktay et al. 1997), although FSH has an indirect effect on early follicular development via paracrine factors released by larger follicles or ovarian stroma cells, such as IGF-I, Kit Ligand and activin (van den Hurk and Zhao 2005).

The percentage of follicular development increased in all treatments at day 16 of culture. Activation of primordial follicles in vitro has been achieved thus far in rodents, cattle and primates, where it occurs spontaneously without the addition of growth factors or hormones (Cushman and Fortune 2003). In this study, although the culture medium without IGF-I and/or FSH promoted follicular development, IGF-I/FSH and FSH+IGF-I/FSH+IGF-I treatments showed more (P<0.05) developing follicles than the α-MEM^{+}/α-MEM^{+} and the other treatments, except for the FSH/IGF-I treatment. In addition, the FSH+IGF-I/FSH+IGF-I and IGF-I/FSH treatments were the only ones that increased the number of secondary follicles from days 8 to 16

of culture. In addition, the greatest follicular and oocyte diameters were observed in the FSH+IGF-I/FSH+IGF-I treatment. Therefore, this result demonstrated the importance of synergy between FSH and IGF-I for early folliculogenesis in goats. The activation and further development of primordial follicles is a highly regulated process and a large number of the hormones and growth factors involved in this process are still unknown. Some studies demonstrated that the use of IGF-I in synergy with FSH in the in vitro culture medium stimulated preantral follicular growth in mice, cows, humans and rats (Liu et al. 1998; Gutierrez et al. 2000; Louhio et al. 2000; Zhao et al. 2001, respectively). A recent study using goat secondary follicles cultured in vitro revealed a positive effect of IGF-I associated with FSH on follicular growth rate and antrum formation (Magalhães-Padilha et al. 2011).

The relative expression of mRNA for FSH receptors on tissue cultured for 16 days in the FSH+IGF-I/FSH+IGF-I treatment did not differ from the non-cultured control. However, tissue cultured for 16 days in α-MEM$^+$ alone had a lower relative expression of FSHR mRNA when compared to the non-cultured control. In this study, preantral follicles were cultured *in situ* in the ovarian cortex. The ovarian cortex might contain all follicular stages, even secondary and tertiary follicles, which have large amounts of mRNA for FSH receptors. During the in vitro culture process, some of the follicles become atretic, especially tertiary follicles (Meresman 2011). This may explain the decrease in FSH receptors mRNA expression in this study for the α-MEM$^+$ treatment. In this study, although follicular viability was reduced after 16 days of culture in the treatment with FSH and IGF-I compared to the non-cultured control, when the culture medium was supplemented with FSH and IGF-I, the relative expression of mRNA for FSHR was similar to the non-cultured control. This finding might be explained by the fact that the FSH+IGF-I/FSH+IGF-I treatment increased the formation of secondary follicles, as previously observed in Experiment 1.

No difference was detected among treatments for IGFR-1 mRNA expression. This result is in agreement with Armstrong et al. (2000), who detected IGFR-1 mRNA in both granulosa and theca cells of bovine preantral and antral follicles, with no difference in IGFR-1 mRNA expression among follicular stages. Therefore, the expression of IGFR-1 mRNA in preantral follicles but absence of endogenous IGF-I or –II mRNA expression, highlights the endocrine mechanism for the IGF regulation of preantral follicle growth (Armstrong et al. 2000). Whereas some studies have reported the presence of growth factors and hormone receptors in isolated

follicles during in vitro culture (Celestino et al. 2010; Lima et al. 2011), to our knowledge this seems to be the first study to demonstrate the expression of FSHR and IGFR-1 mRNA during in vitro culture of ovarian cortical tissue in goats. Although the IGFR-I mRNA expression observed in the present study represented the complete in situ culture system, further studies are required to investigate the expression of mRNA for IGFR-I in individual follicular cells.

In conclusion, a similarity of FSH receptor mRNA expression was observed in cultured ovarian tissue when compared with the non-cultured (control) tissue. This effect, detected in the tissue cultured with medium supplemented with FSH and IGF-I during long-term in vitro culture of preantral follicles, might be associated with the positive effect of these substances during early folliculogenesis, characterized herein by activation of primordial follicles and subsequent progression to secondary follicles in goats. Moreover, the association of FSH and IGF-I during 16 days of culture promoted a high percentage of normal and viable follicles.

Declaration of interest

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

Acknowledgments

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8 CAPÍTULO 3 (Fase II)

Efeito de um meio sequencial no cultivo *in vitro* de tecido cortical ovariano caprino

(Effect of sequential medium on in vitro culture of goat ovarian cortical tissue)

Periódico: *Animal Reproduction Science* 132: 159-168, 2012

Resumo

Um meio sequencial foi avaliado nas taxas de sobrevivência, ativação e crescimento de folículos pré-antrais caprinos submetidos a um cultivo de longa duração, com o objetivo de estabelecer um sistema de cultivo *in vitro* ideal. Fragmentos ovarianos foram cultivados por 16 dias em α-MEM^{+} sozinho ou suplementado com hormônios (GH e/ou FSH) adicionados sequencialmente em diferentes dias de cultivo. Os fragmentos foram cultivados na primeira (dias 0 a 8) e segunda (dias 8 a 16) parte do período de cultivo, gerando 10 tratamentos: α-MEM^{+}/α-MEM^{+}, FSH/FSH, FSH/GH, FSH/FSH+GH, GH/GH, GH/FSH, GH/FSH+GH, FSH+GH/FSH+GH, FSH+GH/FSH e FSH+GH/GH. Morfologia folicular, viabilidade e ultraestrutura foram analisadas. Após 1 dia de cultivo, os tratamentos com FSH mantiveram o percentual de folículos normais similar ao controle fresco. No dia 16 de cultivo, o tratamento FSH/GH mostrou o maior ($P < 0,05$) percentual de folículos normais. A ultraestrutura folicular foi preservada no controle fresco e no tratamento FSH/GH. Folículos cultivados com FSH/GH tiveram o percentual de viabilidade maior ($P < 0,05$) que o tratamento α-MEM^{+}; entretanto a viabilidade foi menor ($P < 0,05$) quando comparada ao controle fresco. O tratamento FSH/GH apresentou o maior ($P < 0,05$) percentual de ativação follicular e formação de folículos secundário, além de produzir a maior ($P < 0,05$) média de diâmetro folicular após 16 dias de cultivo. Em conclusão, o meio sequencial suplementado com FSH seguido de GH durante um cultivo de longa duração manteve a sobrevivência, a viabilidade e a ultraestrutura de folículos pré-antrais de cabras e promoveu a ativação e formação de folículos secundários.

Palavras-chave: Caprino. Foliculogênese. FSH. GH. Folículo pré-antral.

Effect of sequential medium on *in vitro* culture of goat ovarian cortical tissue

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ABSTRACT

A sequential medium was evaluated on the survival, activation and growth rates of caprine preantral follicles submitted to a long-term culture period, aiming to establish an ideal *in vitro* culture system. Ovarian fragments were cultured for 16 days in α-MEM$^+$ alone or supplemented with hormones (GH and/or FSH) added sequentially on different days of culture. Ovarian fragments were cultured in the first (days 0-8) and second (days 8-16) halves of the culture period, generating 10 treatments: α-MEM$^+$/α-MEM$^+$, FSH/FSH, FSH/GH, FSH/FSH+GH, GH/GH, GH/FSH, GH/FSH+GH, FSH+GH/FSH+GH, FSH+GH/FSH and FSH+GH/GH. Follicle morphology, viability and ultrastructure were analyzed. After day 1 of culture, FSH treatments maintained the percentage of normal follicles similar to the fresh control. At day 16 of culture, the treatment FSH/GH showed the highest ($P < 0.05$) percentage of normal follicles. The ultrastructure of follicles was preserved in the fresh control and FSH/GH treatment. Follicles cultured with FSH/GH had a higher ($P < 0.05$) viability than α-MEM$^+$; however the viability was lower ($P < 0.05$) when compared to the fresh control. The FSH/GH treatment showed the highest ($P < 0.05$) percentage of follicular activation and secondary follicle formation and produced the largest ($P < 0.05$) mean follicular diameter after 16 days of culture. In conclusion, a sequential medium supplemented with FSH followed by GH during a long-term culture maintains the survival, viability and ultrastructure of goat preantral follicles, and promotes activation and secondary follicles.

Keywords: Caprine; Folliculogenesis; FSH; GH; Preantral follicle

1. Introduction

The ovarian cortical tissue contains thousands of oocytes enclosed in preantral follicles at birth. The majority of those are primordial follicles, considered to be the resting follicle pool. However, most of these oocytes (99.9%) undergo atresia during the stages of activation, growth and maturation (Skinner, 2005). Due to this phenomenon, the ovary's ability to produce mature oocytes is quite poor. During recent decades, many researchers in the field of animal reproduction have contributed to an improvement in the biotechnological multiplication of animals of high economic value and endangered species. Among these biotechnologies is the *in vitro* culture of preantral follicles, which despite being a biotechnical complexity, has resulted in the birth of live mice (O'Brien et al*.,* 2003; Wang et al*.,* 2011) and the production of viable embryos in some species (rat: Daniel et al*.,* 1989; pig: Wu et al*.,* 2001; buffalos: Gupta et al*.,* 2008; sheep: Arunakumari et al*.,* 2010; goat: Magalhães et al*.,* 2011). Despite these promising results, the exact mechanisms responsible for the activation and subsequent growth and development of primordial follicles remain unclear.

Follicular development is controlled by a complex interaction among the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and local factors, such as steroid hormones and growth factors (Gougoen, 1996). The fate of a follicle, therefore, depends on the balance between stimulatory and inhibitory factors in the ovary. Several short-term *in vitro* culture studies in goats have achieved follicular activation in the presence of growth factors and/or gonadotropins (Martins et al*.,* 2008; Magalhães et al*.,* 2009; Celestino et al*.,* 2010). However, only one study in this species has achieved *in vitro* development from primordial to secondary follicles enclosed in ovarian tissue (Martins et al*.,* 2008). This suggests that an extended culture period and a sequential medium may be required for preantral follicle development according to different growth phases. Recently, the addition of different growth factors/hormones at different times of culture (i.e., sequential culture medium) stimulated the activation of primordial follicles to later developmental stages in ovine ovarian cortical tissue (Peng et al*.,* 2010).

Among the hormones involved in folliculogenesis are growth hormone (GH) and follicle stimulating hormone (FSH). GH has been shown to play a role in folliculogenesis through the detection of GH-R immunoreactivity and mRNA encoding GH-R in ovarian tissue in humans

(Carlsson et al*.,* 1992) and cows (Kölle et al*.,* 1998). Some *in vitro* studies suggest that GH plays a role in follicular growth during the early gonadotropin-independent stages of folliculogenesis and could have a direct inhibitory action on follicle apoptosis (Chun and Hsueh, 1998; Sirotkin and Makarevich, 1999). Other *in vitro* studies have demonstrated that the addition of FSH to the culture medium promotes the maintenance of viability and growth of preantral follicles in goats (Matos et al*.,* 2007; Magalhães et al*.,* 2009).

In spite of the importance of GH and FSH revealed by the aforementioned *in vitro* studies, there are no reports regarding the interaction of these hormones added on different days of long-term culture (sequential medium) in cultures of caprine preantral follicles. Therefore, the aim of this study was to investigate the effect of a sequential culture medium supplemented with GH and/or FSH on the survival, activation and *in vitro* growth of caprine preantral follicles during long-term (16 days) culture.

2. Materials and methods

2.1. Chemicals

All the chemicals and culture media used for this study were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2.2. Source of ovaries

Ovarian cortical tissue (*n* = 18 ovaries) was harvested at a local slaughterhouse from nine adult (1–3 years old), cross-breed goats (*Capra hircus*). Four goats were used for histological analysis and transmission electron microscopy (TEM) and five for fluorescence analysis. Immediately postmortem, the ovaries were washed in 70% alcohol followed by two washes in Minimum Essential Medium (MEM) supplemented with 100 µg/ml penicillin and 100µg/ml streptomycin. The pairs of ovaries were transported to the laboratory in MEM within 1 h at 4 °C (Chaves et al*.,* 2008).

2.3. Experimental protocol

The organ culture system utilized herein has been previously described in detail (Magalhães et al., 2009; Celestino et al., 2010). Briefly, in the laboratory, the ovaries were stripped off the surrounding fat tissues and ligaments. Subsequently, for histological and TEM analyses, ovarian cortex tissue from each ovarian pair from the same animal was cut into 19 slices (approximate size: 3mm x 3 mm, with 1 mm thickness) using a scalpel under sterile conditions. One slice was immediately fixed for histological and ultrastructural analysis (fresh control) and the other 18 were placed in culture for 1, 8 or 16 days in individual wells. The cortex tissue samples were transferred to 24-well culture dishes containing 1 ml of culture medium. Culture was performed at 39°C in 5% CO_2 in a humidified incubator and all the media were incubated for 1 h prior to use. The basic culture medium consisted of α-MEM (pH 7.2 - 7.4) supplemented with ITS (10 µg/ml insulin, 5.5 µg/ml transferrin and 5 ng/ml selenium), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml bovine serum albumin (BSA) and 50 µg/ml ascorbic acid, which was called α-MEM^+ (cultured control). For experimental conditions, the medium was supplemented with human recombinant GH (rGH 10 ng/ml; MP Biomedicals LLC, Eschwege, Germany), bovine recombinant FSH (rFSH 50 ng/ml; Nanocore, São Paulo, Brazil) or GH and FSH in different combinations (Fig. 1). Each treatment was repeated four times and applied to one slice from each of 4 goats and the culture media were replenished every other day. The concentrations of GH and FSH used in this work were chosen based on previous studies in our laboratory (Martins et al., 2008; Magalhães et al., 2009).

α–MEM⁺ supplementation Day 0	Day 8	Treatment identification
none	none	α–MEM⁺
FSH	FSH	FSH/FSH
FSH	GH	FSH/GH
FSH	FSH + GH	FSH/FSH + GH
GH	GH	GH/GH
GH	FSH	GH/FSH
GH	FSH + GH	GH/FSH + GH
FSH + GH	FSH + GH	FSH + GH/FSH + GH
FSH + GH	FSH	FSH + GH/FSH
FSH + GH	GH	FSH + GH/GH

Fig. 1. Tested treatments according to supplementation of the medium for the *in vitro* culture of caprine ovarian tissues.

2.4. Morphological analysis and assessment of in vitro follicular growth

Before culture (fresh control) and after 1, 8 or 16 days in culture, all tissue 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 ovarian cortex tissue samples were cut into 7-µm sections, which were mounted on glass slides and stained with periodic acid – Schiff (PAS) – hematoxylin. Follicle stage and morphology were assessed in serial sections using a microscope (Nikon, Tokyo, Japan) under 400× magnification. The developmental stages of follicles have been defined previously (Hulshof et al., 1994) as primordial (one layer of flattened and cuboidal granulosa cells) or growing follicles (primary – one layer of cuboidal granulosa cells, and secondary – two or more layers of cuboidal granulosa cells around the oocyte). Follicles were classified as histologically normal when an intact oocyte was present,

surrounded by granulosa cells that were well organized in one or more layers and lacked a pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. The percentage of morphologically normal follicles, before (Day 0) and after culture, indicated the percentage of follicle survival.

Overall, 120 follicles were evaluated for each treatment (30 follicles per treatment in one repetition x four repetitions = 120 follicles). To evaluate follicular activation, the percentages of normal primordial and growing follicles were calculated before (fresh control) and after culture in each treatment. In addition, follicle and oocyte diameters were measured in healthy follicles only. Follicle diameter was recorded from edge to edge of the outer layer of granulosa cells or from the outside edge of the theca cell layer when present. Oocyte diameter was recorded from edge to edge of the oocyte membrane. Two perpendicular diameters were recorded in each measurement and the average of these two values was calculated. Each follicle was examined in the section in which its oocyte appeared in the largest cross section and matched with the same follicle on adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once.

2.5. Ultrastructural analysis

To better evaluate follicular morphology, ultrastructural analysis was performed on preantral follicles from non-cultured tissue (control), follicles cultured in α-MEM^+ alone, as well as the treatments which demonstrated the best results for morphology, activation, and growth. Small pieces (1 mm^3) of tissue were cut off from caprine ovarian fragments before and after each period of culture for each treatment and fixed for 2 h in a solution containing 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After fixation, the fragments were washed three times with 0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in a 0.1 M sodium cacodylate buffer. Next, samples were contrasted with uranyl acetate, dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. Follicles classified as histologically viable in toluidine blue-stained semi-thin sections (3 µm) were submitted to ultrastructural analysis. For

that analysis, ultra-thin sections (60 nm) were cut on an ultramicrotome (Reichert Supernova, Germany) and examined using a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope, operating at 80 kV. For ultrastructural analysis, a total of, at least, 5 follicles were examined per group.

2.6. Assessment of preantral follicle viability by fluorescence microscopy

To confirm the results of the morphological and ultrastructural analyses, the viability of follicles from fresh control, follicles cultured in α-MEM$^+$ alone and follicles cultured in the treatment that provided the best outcome were further analyzed using a more accurate method of assessment based on fluorescent probes. Pairs of goat ovaries (n = 5) were cut into fragments; one of which was immediately processed for follicle isolation and the other fragments were cultured in α-MEM$^+$ alone and in the treatment that provided the best outcome. After the *in vitro* culture, the cortical slices from both treatments were subjected to follicle isolation.

Goat preantral follicles were isolated from ovarian fragments using a mechanical method described by Lucci et al. (1999). Briefly, a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) was adjusted for a sectioning interval of 75 µm and samples were cut into small pieces. The fragments were then placed in α-MEM$^+$, resuspended 40 times using a large Pasteur pipette (diameter ~ 1600 µm) and resuspended again 40 more times with a smaller Pasteur pipette (diameter ~ 600 µm) in order to dissociate preantral follicles from the ovarian stroma. The material obtained was passed through a 100-µm nylon mesh filter, resulting in a suspension containing preantral follicles less than 100 µm in diameter. This procedure was carried out at room temperature within a 10-min time frame.

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 detects intracellular esterase activity in viable cells, the latter labels nucleic acids in non-viable cells with disrupted plasma membranes. The test was performed by adding 4 µM calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) to a suspension of isolated follicles, which were then incubated at 37 °C for 15 min. After being labeled, the follicles were washed once by centrifugation at

100×g for 5 min and resuspended in α-MEM$^+$. The cells were then mounted on glass microscope slides in 5 μl of anti-fading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching, and examined using a fluorescence microscope (Nikon, Eclipse 80i, 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).

2.7. Statistical analyses

Initially, all data were submitted to Shapiro-Wilk and Bartlett tests to confirm normal distribution and homoscedasticity, respectively. The percentages of morphologically normal preantral follicles and different follicular classes (primordial and growing follicles) were then submitted to analysis of variance (ANOVA) using the GLM procedure of SAS (Version 1999; Cary, NC), followed by the Student-Newman-Keuls and Duncan tests for a comparison of the means. Follicle and oocyte diameters did not show homogeneity of variance, even after transformation, and were analyzed by Kruskal-Wallis non-parametric test. Data for follicular viability assessed through fluorescence microscopy were analyzed as dispersion of frequencies using the χ^2 test. Differences among groups were considered to be significant when $P < 0.05$ and results were expressed as the mean ± standard deviation (SD).

3. Results

3.1. Caprine preantral follicle survival before and after in vitro culture

A total of 2280 preantral follicles were analyzed by classical histology. Normal follicles from the fresh control (primordial follicles) and after culture in α-MEM$^+$ supplemented with FSH and GH (FSH/GH) (secondary follicle) are shown (Fig. 2A and B). Degenerated follicles after 16 days of culture in α-MEM$^+$ alone are also shown (Fig. 2C). In the degenerated follicles, retracted oocytes, pyknotic nuclei and disorganized granulosa cells are noticeable.

The percentage of morphologically normal preantral follicles in the fresh control and after 1, 8 or 16 days of *in vitro* culture is shown in Table 1. After 1 day of culture, there was a reduction ($P < 0.05$) in the percentage of normal follicles in all treatments, compared to the fresh control (91.7%), except when the culture was performed only with FSH (85.8%; $P > 0.05$). In contrast, after 8 and 16 days of culture, the percentage of normal follicles decreased ($P < 0.05$) in all treatments in comparison to the fresh control. When the treatments were compared, there was a higher percentage of normal follicles in all treatments performed only with FSH in the first half of culture period, i.e. day 8 (81.7%). Moreover, after 16 days of culture, the treatment FSH/GH showed the highest ($P < 0.05$) percentage of normal follicles (77.5%). With the progression of the culture period from 1 to 8 days, a decrease ($P < 0.05$) in the percentage of normal follicles was observed only in the treatment α-MEM$^+$. However, all treatments showed a further decrease ($P < 0.05$) in the percentage of normal follicles from 8 to 16 days of culture, except the treatment FSH/GH.

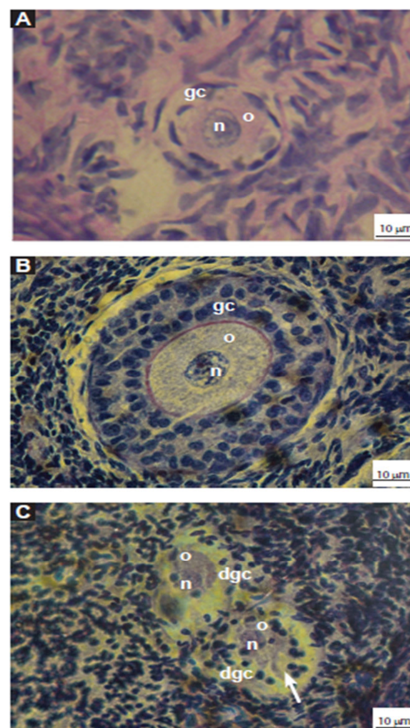

Fig. 2. Histological sections of (A) normal preantral follicle from the fresh control or (B) normal secondary follicle cultured for 16 days in FSH/GH treatment and (C) degenerated preantral follicles after 16 days of culture in α-MEM$^+$ alone. o = oocyte; n = oocyte nucleus; gc = granulosa cell. Note the retracted oocytes (arrow), and disorganized granulosa cells (dgc).

Table 1 Percentages (mean ± SD) of morphologically normal preantral follicles in non-cultured tissues (fresh control) and in tissues cultured for 1, 8 or 16 days in α-MEM^{+} alone or α-MEM^{+} containing GH and/or FSH.

	Normal preantral follicles (%)			
Treatments	Day 0	Day 1	Day 8	Day 16
Control	91.7 ± 1.9	–	–	–
α-MEM^{+}		70.0 ± 2.7^{*bA}	59.2 ± 3.2^{*dB}	42.5 ± 3.2^{*dC}
FSH/FSH§				59.2 ± 1.7^{*bC}
FSH/GH		85.8 ± 3.2^{aA}	81.7 ± 1.9^{*aAB}	77.5 ± 3.2^{*aB}
FSH/FSH+GH				62.5 ± 4.2^{*bC}
GH/GH§		68.3 ± 1.9^{*bA}		53.3 ± 2.7^{*cB}
GH/FSH			64.2 ± 3.2^{*cA}	50.0 ± 2.7^{*cB}
GH/FSH+GH				54.2 ± 1.7^{*cB}
FSH+GH/FSH+GH§		81.7 ± 7.9^{*aA}	75.0 ± 1.9^{*bA}	64.2 ± 3.2^{*bB}
FSH+GH/FSH				63.3 ± 2.7^{*bB}
FSH+GH/GH				60.0 ± 2.7^{*bB}

^{a,b,c,d}Different superscripts within the same day of culture indicate significant difference ($P < 0.05$).

^{A,B,C}Different superscripts within the same treatment indicate significant difference ($P < 0.05$).

§ Groups initially cultured with the same substances (FSH or GH or FSH+GH) have a common value on days 1 and 8.

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

3.2. Activation of caprine primordial follicle after in vitro culture

The percentage of activation of preantral follicles in the fresh control and after 1, 8 or 16 d of *in vitro* culture is shown in Table 2.

Table 2 Percentages (mean ± SD) of primordial and growing follicles in non-cultured tissues (fresh control) and in tissues cultured for 1, 8 or 16 days in α-MEM$^+$ alone or α-MEM$^+$ containing GH and/or FSH.

Treatments	Primordial follicles (%)				Growing follicles (%)			
	Day 0	Day 1	Day 8	Day 16	Day 0	Day 1	Day 8	Day 16
Control	94.5 ± 2.1	–	–	–	5.5 ± 2.1	–	–	–
α-MEM$^+$		97.7 ± 2.7^{aA}	78.7 ± 6.1*^{aB}	51.1 ± 5.1*^{aC}		2.4 ± 2.7 ^{aA}	21.1 ± 6.1*^{aB}	49.0 ± 5.1*^{aC}
FSH/FSH§		93.1 ± 2.1^{aA}	44.9 ± 1.1*^{cB}	26.7 ± 5.0*^{cC}		6.8 ± 2.1 ^{aA}	55.1 ± 1.1*^{cB}	73.2 ± 5.0*^{cC}
FSH/GH				14.0 ± 1.9*^{dC}				84.9 ± 1.9*^{dC}
FSH/FSH+GH				28.1 ± 3.5*^{cC}				72.0 ± 3.5*^{cC}
GH/GH§		93.9 ± 2.6^{aA}	60.2±12.2*^{bB}	43.9 ± 6.3*^{bC}		6.1 ± 2.6 ^{aA}	40.9±12.2*^{bB}	56.2 ± 6.3*^{bC}
GH/FSH				35.0 ± 2.0*^{cC}				65.0 ± 2.0*^{cC}
GH/FSH+GH				30.8 ± 0.9*^{cC}				69.2 ± 0.9*^{cC}
FSH+GH/FSH+GH§		88.6 ± 2.7*^{bA}	47.7 ± 3.2*^{bB}	33.6 ± 5.2*^{cC}		11.4± 2.7*^{bA}	52.2 ± 3.2*^{bB}	66.2 ± 5.2*^{cC}
FSH+GH/FSH				30.2 ± 4.7*^{cC}				69.7 ± 4.7*^{cC}
FSH+GH/GH				27.6 ± 6.6*^{cC}				72.2 ± 6.6*^{cC}

^{a,b,c,d}Different superscripts within the same day of culture indicate significant difference ($P < 0.05$).

^{A,B,C}Different superscripts within the same treatment indicate significant difference ($P < 0.05$).

§ Groups initially cultured with the same substances (FSH or GH or FSH+GH) have a common value on days 1 and 8.

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

As early as day 1 of culture, when compared to the fresh control, a reduction ($P < 0.05$) in the percentage of primordial follicles concomitant with an increase ($P < 0.05$) of growing follicles was observed only in follicles cultured with FSH+GH in the first half of culture period (FSH+GH/FSH+GH, FSH+GH/FSH and FSH+GH/GH). However, after 8 days of culture, an increase ($P < 0.05$) of growing follicles was observed in all treatments when compared to the fresh control. In addition, in all treatments, the percentage of growing follicles progressively increased ($P < 0.05$) throughout the culture period. Moreover, at day 16 of culture, the FSH/GH treatment showed the highest ($P < 0.05$) rate of growing follicles and produced more ($P < 0.05$) secondary follicles (27%) when compared to all other treatments (range, 5-12%).

3.3. In vitro growth of caprine preantral follicles

An increase in follicular diameter was observed in all treatments compared to the fresh control from day 1 of culture onwards ($P < 0.05$; Table 3). On day 1 of culture, the treatments with FSH+GH had a larger ($P < 0.05$) follicular diameter than those cultured with only α-MEM^{+}, but were similar to those cultured with FSH or GH. Furthermore, when the treatments were compared to each other on day 8, follicles cultured with FSH or FSH+GH in the first half of the culture had a larger ($P < 0.05$) follicular diameter than those initially cultured only with GH and MEM^{+} alone. After 16 days of culture, the FSH/GH treatment increased ($P < 0.05$) follicular diameter when compared to the other treatments. From days 1 to 16 of culture, all treatments increased ($P < 0.05$) follicular diameter, except FSH+GH/FSH+GH and FSH+GH/FSH ($P > 0.05$). Nevertheless, from day 8 to 16 of culture, only FSH/GH promoted an increase ($P < 0.05$) in follicular diameter.

Table 3 Follicle diameters (mean ± SD) in non-cultured tissues (fresh control) and in tissues cultured for 1, 8 or 16 days in α-MEM+ alone or α-MEM+ containing GH and/or FSH.

Treatments	Follicle diameter (µm)			
	Day 0	Day 1	Day 8	Day 16
Control	34.2 ± 3.6			
α-MEM^{+}		32.6 ± 3.1*^{bA}	34.0 ± 3.1*^{cB}	34.8 ± 1.8*^{dB}
FSH/FSH§		36.4 ± 2.9*^{abA}	40.2 ± 3.0*^{aB}	40.9 ± 2.9*^{cB}
FSH/GH				46.9 ± 5.9*^{aC}
FSH/FSH+GH				41.8 ± 1.9*^{cB}
GH/GH§		37.8 ± 3.4*^{abA}	38.0 ± 3.6*^{bAB}	39.9 ± 2.7*^{cB}
GH/FSH				41.0 ± 1.0*^{cB}
GH/FSH+GH				40.6 ± 1.9*^{cB}
FSH+GH/FSH+GH§		38.6 ± 1.8*^{aA}	39.9 ± 2.5*^{aAB}	40.8 ± 1.1*^{cAB}
FSH+GH/FSH				41.0 ± 2.3*^{cAB}
FSH+GH/GH				43.2 ± 2.8*^{bB}

^{a,b,c,d}No common superscripts within the same day of culture indicate significant difference (P < 0.05).

^{A,B,C}No common superscripts within the same treatment indicate significant difference (P < 0.05).

§ Groups initially cultured with the same substances (FSH or GH or FSH+GH) have a common value on days 1 and 8.

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

After day 1 of culture onwards there was an increase (P < 0.05) in oocyte diameter in all treatments compared to the fresh control (Table 4). On day 8, there were no differences (P > 0.05) in oocyte diameter among the treatments. However, after 16 days of culture, the FSH/GH treatment showed a higher (P < 0.05) oocyte diameter, except when compared to FSH/FSH. With the progression of the culture period from 8 to 16 days, there was an increase (P < 0.05) in oocyte diameter only in the culture performed with FSH/GH.

Table 4 Oocyte diameters (mean ± SD) in non-cultured tissues (fresh control) and in tissues cultured for 1, 8 or 16 days in α-MEM$^+$ alone or α-MEM$^+$ containing GH and/or FSH.

Treatments	Oocyte diameter (µm)			
	Day 0	Day 1	Day 8	Day 16
Control	21.7 ± 1.5			
α-MEM$^+$		22.7 ± 1.8*^{bA}	23.2 ± 1.4*^{aA}	23.4 ± 1.5*^{cA}
FSH/FSH§		22.8 ± 1.1*^{bA}	24.0 ± 1.6*^{aAB}	25.0 ± 1.0*^{abB}
FSH/GH				26.6 ± 3.0*^{aC}
FSH/FSH+GH				23.4 ± 2.4*^{cB}
GH/GH§		23.2 ± 1.1*^{abA}	23.5 ± 1.3*^{aA}	23.6 ± 0.8*^{cA}
GH/FSH				23.8 ± 1.0*^{cA}
GH/FSH+GH				23.6 ± 1.3*^{cA}
FSH+GH/FSH+GH§		23.7 ± 0.8*^{aA}	23.7 ± 1.2*^{aA}	23.7 ± 0.9*^{cA}
FSH+GH/FSH				23.8 ± 1.3*^{cA}
FSH+GH/GH				24.3 ± 2.5*^{bA}

^{a,b,c}No common superscripts within the same day of culture indicate significant difference (P < 0.05).

^{A,B,C}No common superscripts within the same treatment indicate significant difference (P < 0.05).

§ Groups initially cultured with the same substances (FSH or GH or FSH+GH) have a common value on days 1 and 8.

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

3.4. Ultrastructural features of caprine preantral follicles

For a better evaluation of follicular integrity, ultrastructural analysis of preantral follicles was performed in fresh control samples, cultured control samples (16 days in α-MEM+), as well as in samples cultured with FSH for 8 days and FSH/GH for 16 days These last two treatments were selected for generating the best overall results in the parameters evaluated by histological analysis.

Ultrastructural features of follicles evaluated in the fresh control, treatments containing FSH alone (day 8) and FSH/GH treatment (day 16) were normal and showed preserved oocytes with intact plasma and nuclear membranes, and large oocyte nuclei (Fig. 3A, B, and C, respectively). A slight increase in cytoplasmic vacuolization was observed from days 0 to 16 of culture. However, the organelles were uniformly distributed in the ooplasm, especially the mitochondria and endoplasmic reticulum. Ultrastructurally, granulosa cells were normal and well organized around the oocytes, showing large nuclei with irregular shapes and a high proportion of nuclei-to-cytoplasm area. Nevertheless, follicles cultured in α-MEM^{+} for 16 days (cultured control) showed signs of degeneration, such as high levels of cytoplasmic vacuolization, low-densities of organelles and loss of cytoplasm as atresia progressed (Fig. 3D).

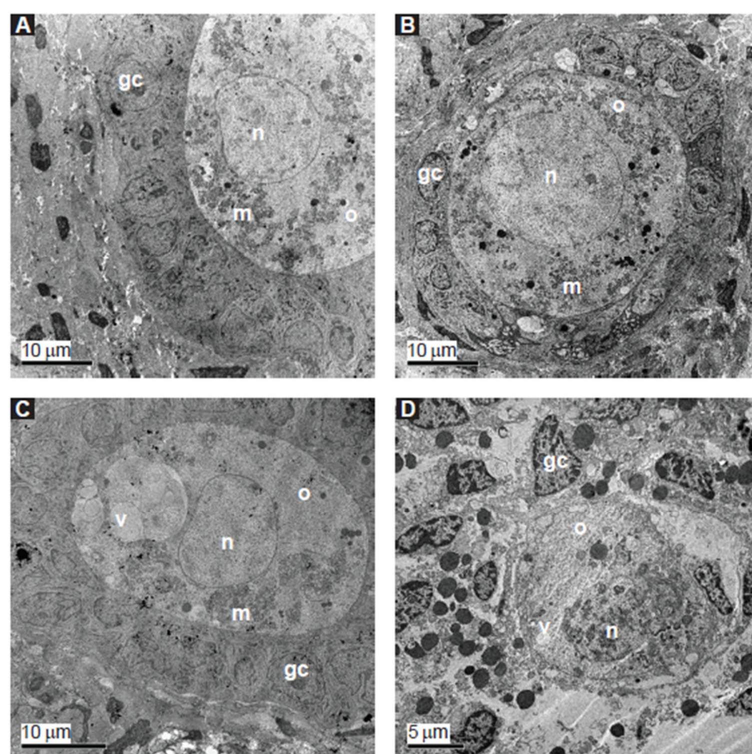

Fig. 3. Electron micrographs of follicles before and after 8 or 16 days of culture. (A) Normal preantral follicle from noncultured ovarian tissue (fresh control). (B) Normal preantral follicle after 8 days of *in vitro* culture in the FSH treatment. (C) Normal preantral follicle after 16 days of *in vitro* culture in the FSH/GH treatment. (D) Degenerated follicle after *in vitro* culture in α - MEM+ alone for 16 days. o = oocyte; n = oocyte nucleus; m = mitochondria; v = vacuole; gc = granulosa cell.

3.5. Assessment of preantral follicle viability

For the viability analysis, 90 caprine preantral follicles were analyzed by fluorescence microscopy before (fresh control) and after 16 days of culture in α-MEM^{+} alone (cultured control; Fig. 4B) and in the FSH/GH treatment (Fig. 4A). A follicle after culture in FSH/GH labeled with calcein-AM (green fluorescence), and a follicle marked with ethidium homodimer-1 (red fluorescence) after culture in α-MEM^{+} alone are shown (Fig. 4C and D, respectively).

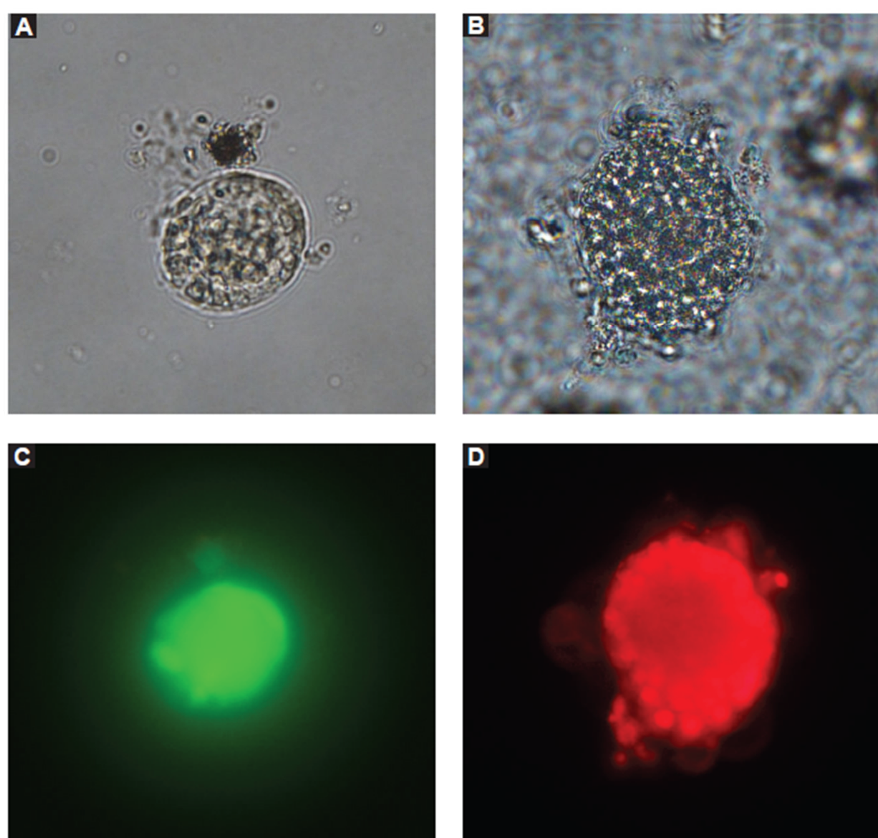

Fig. 4. Isolated (A) normal preantral follicle cultured in the FSH/GH treatment and (B) degenerated follicle cultured in α-MEM^{+} alone for 16 days. The viability assessment of follicles (A) and (B) by fluorescence analyses shows (C) viable follicle stained with calcein-AM (green fluorescence) and (D) nonviable follicle labeled with ethidium homodimer-1 (red fluorescence).

Based on quantitative analysis, follicles cultured in FSH/GH had the highest ($P < 0.05$) follicular viability (70%) among treated groups, however the viability was lower ($P < 0.05$) when

compared to the fresh control (93%). Furthermore, follicles cultured in the absence of FSH and GH (α-MEM^{+} alone) showed a reduced ($P < 0.05$) viability (20%) compared to the fresh control and FSH/GH.

4. Discussion

The present study demonstrated the importance of a sequential medium on early *in vitro* folliculogenesis in goats. Herein we showed, for the first time, the positive effects of a sequential medium containing FSH followed by GH on the survival, activation and growth of caprine preantral follicles during long-term *in vitro* culture. A recent study on ovine cortical tissue demonstrated that growth factors added sequentially on different days of culture is crucial for a successful *in vitro* culture system (Peng et al., 2010).

In this study, after 1 day of culture, the treatments with FSH alone or FSH with GH showed higher percentages of follicular survival than other treatments. Additionally, only the treatments with FSH alone were similar to the fresh control. Moreover, the highest percentage of normal follicles after 16 days was found in the treatment with FSH in the first half of culture and GH in the second half of the culture (FSH/GH). This treatment was the only one that maintained follicular survival from days 8 to 16 of culture. This result was confirmed by fluorescence analysis which showed that follicles cultured in FSH/GH had the highest follicular viability among treated groups. In general, the percentage of follicular survival (histological analysis) was higher than the percentage of viability (fluorescence analysis). This finding was expected once that the fluorescence analysis is more accurate and allows the detection of functional aspects of the cell, such as esterase activity. It has been shown that FSH maintains the survival of caprine preantral follicles (Saha et al., 2000; Matos et al., 2007; Magalhães et al., 2009). Indeed, this hormone has an inhibitory effect on apoptosis in preantral follicles cultured *in vitro* in several species (mouse: Baker and Spears, 1997; human: Wright et al., 1999; pig: Mao et al., 2002). In mice, the omission of FSH from the culture medium resulted in follicular cell death and a reduction in granulosa cell vitality (Cortvrindt et al., 1997). Although FSH receptors do not seem to be expressed in granulosa cells of primordial follicles, this hormone may act indirectly through paracrine factors secreted by larger follicles or stroma cells. FSH promotes proliferation of granulosa cells by paracrine factors, such as insulin-like growth factor 1 (IGF-1) and activin A (Van den Hurk and Zhao, 2005). The

positive effect of FSH in the first half of *in vitro* culture when compared with the other treatments might be related to the fact that FSH can regulate the expression of several genes, such as KL, GDF-9 and BMP-15 in ovarian follicles (Joyce et al., 1999, Thomas et al., 2005), which have been involved in activation of primordial follicles (Van den Hurk and Zhao, 2005).

With respect to GH, this hormone was able to maintain follicular survival in the second half of the culture. The results of a study performed by Hull and Harvey (2001) suggest that GH stimulates growth and prevents atresia in small follicles. *In vitro* studies have shown that GH has a direct inhibitory effect on apoptosis in early bovine (Sirotkin and Makarevich, 1999) and rat (Eisenhauer et al., 1995; Chun and Hsueh, 1998) follicles. The mechanism by which GH regulates survival in ovarian follicles is not exactly known yet. It is quite possible that GH may act through direct stimulation of other regulatory factors, such as insulin and IGF-I, which can play an important role in follicular survival.

In our study, after 8 days of culture, the treatments initially cultured with FSH showed the highest follicular activation when compared to α-MEM^{+} alone and the other treatments. Recent studies demonstrated that FSH improved the activation of caprine preantral follicles after 7 days of culture (Matos et al., 2007; Magalhães et al., 2009). Oktay et al. (1997) reported that the expression of FSH receptors developed progressively during the transition from primordial to primary and secondary follicles in human ovaries. The presence of FSH receptors in granulosa cells of preantral follicles suggests that FSH can promote early follicular development and growth.

Furthermore, after 16 days of culture, the treatment that received GH from day 8 to day 16 (FSH/GH) promoted the highest rate of follicular activation and the highest percentage of secondary follicles. The local action of GH may have been through an autocrine or paracrine mechanism, although the systemic actions (i.e., endocrine mechanism) of GH have been more reported. The GH receptor and its mRNA have been located in the ovaries of various species: rat (Ranke et al., 1976; Carlsson et al., 1993); human (Sharara and Nieman, 1994); sheep (Eckery et al., 1997); and cow (Kolle et al., 1998; Izadyar et al., 1999). Using recombinant human GH, Liu et al. (1998) demonstrated that earlier phases of follicular development in mice are controlled by GH, which stimulates secondary follicle formation *in vitro.* Furthermore, Kobayashi et al. (2000) showed a stimulatory effect of GH on the proliferation of granulosa cells in murine preantral follicles. The rate of follicular activation in control medium (α-MEM^{+} alone) found in this study was lower than previous studies from the same research group. This fact can be explained once in

the current experiment the intermediate follicles (one layer of flattened and cuboidal granulosa cells) were included in the primordial follicle category.

Our results on follicle morphology, based on classical histology, were confirmed by fluorescence and ultrastructural analyses. These methodologies are considered to be important tools for detecting damage to cellular membranes and organelles, respectively. Transmission electron microscopy was used as a qualitative and supplementary technique to evaluate follicular integrity after *in vitro* culture (Lucci et al., 2001). In the present study, preantral follicles from the fresh control and those cultured in FSH for 8 days and FSH/GH for 16 days showed intact oocytes and granulosa cells, which displayed a preserved ultrastructure, with intact mitochondria and endoplasmic reticula, as well as the basement, plasma and nuclear membranes.

In conclusion, the present study provides the first evidence of a positive effect of a sequential medium supplemented with FSH and GH on early *in vitro* folliculogenesis in goats. The treatment with FSH followed by GH maintained follicular survival and ultrastructural integrity and promoted the activation of primordial follicles and subsequent progression to secondary follicles. This finding shall be very useful in the future to simulate the *in vivo* ovarian environment, optimizing culture systems to allow complete follicular development *in vitro*.

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Declaration of interest

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

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9 CAPÍTULO 4 (Fase III)

Efeito do interval de troca de meio na viabilidade, crescimento e maturação *in vitro* de folículos pré-antrais isolados ovinos e caprinos

(Effect of the medium replacement interval on the viability, growth and in vitro maturation of isolated caprine and ovine pre-antral follicles)

Periódico: *Reproduction in Domestic Animal* 46: 134-140, 2011

Resumo

O objetivo do presente estudo foi avaliar o efeito de diferentes intervalos de troca de meio na viabilidade, formação de antro, crescimento e maturação *in vitro* de oócitos oriundos de folículos pré-antrais caprinos e ovinos. Folículos pré-antrais ($\geq$ 150 µm) foram isolados a partir do cortex ovariano de cabras e ovelhas e individualmente cultivados por 24 dias utilizando dois intervalos de troca de meio (dois dias (T_1) ou seis dias (T_2)). O desenvolvimento folicular foi avaliado com base na formação da cavidade antral, aumento no diâmetro folicular, presença de complexo cumulus-oócito morfologicamente normais e oócitos crescidos *in vitro*. Para a espécie caprina, os resultados mostraram um maior ($P<0,05$) percentual de folículos viáveis em T_1 quando comparado à T_2 a partir do dia 6 até o final do período de cultivo. Além disso, quando comparado ambos os tratamentos, após a mesma duração de cultivo, a taxa de formação de antro foi significativamente maior em T_1 do que em T_2 a partir do dia 12. Entretanto, em ovinos, quando os tratamentos foram comparados no dia 24 de cultivo, um maior percentual de folículos viáveis foi observado em T_2 quando comparado à T_1 ($P<0,05$). Na espécie caprina, o percentual de oócitos crescidos *in vitro* ($\geq$110 µm) destinados à maturação *in vitro* (MIV) após 24 dias de cultivo foi significativamente maior em folículos cultivados em T_1 (30,0%) do que em T_2 (6,7%; $P<0,05$). Por outro lado, em ovinos, ao final do cultivo, o percentual de oócitos destinados à MIV foi maior em T_2 do que em T_1 (23,5% vs. 2,9%; $P<0,05$). Em conclusão, sob as mesmas condições, a frequência da troca de meio afeta significativamente o desenvolvimento *in vitro* de folículos pré-antrais caprino e ovino. Para uma melhor eficiência no sistema de cultivo, o meio deve ser trocado a cada dois dias para folículos pré-antrais caprinos e a cada seis dias para a espécie ovina.

Palavras-chave: Cabra. Ovelha. Folículos pré-antrais. Cultivo. Intervalo de troca de meio.

Effect of the medium replacement interval on the viability, growth and *in vitro* maturation of isolated caprine and ovine pre-antral follicles

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Abstract

The aim of the present study was to evaluate the effects of different medium replacement intervals on the viability, antral cavity formation, growth and *in vitro* maturation (IVM) of oocytes from caprine and ovine pre-antral follicles. Pre-antral ovarian follicles ($\geq$ 150 µm) were isolated from the ovarian cortex of goats and sheep and were individually cultured for 24 days using two different medium replacement intervals [2 days (T_1) or six days (T_2)]. Follicle development was evaluated on the basis of antral cavity formation, increases in follicular diameter and the presence of healthy cumulus oocyte complexes and fully grown oocytes. For caprine species, results showed a higher percentage ($p < 0.05$) of viable follicles in T_1 than T_2 from day 6 until the end of the culture. In addition, when comparing both treatments after the same culture duration, the rate of antrum formation was significantly higher in T_1 than in T_2 from day 12 onward. Yet, in ovines, when both treatments were compared at day 24 of the culture, there were more viable follicles in T_2 than in T_1 ($p < 0.05$). In the caprine species, percentages of fully grown oocytes ($\geq$ 110 µm) acceptable for IVM after 24 days of culture were significantly higher in normal follicles cultured in T_1 (30.0%) than in T_2 (6.7%; $p < 0.05$). On the other hand, in ovines, at the end of the culture, the percentage of oocytes destined for IVM was higher in T_2 than in T_1 (23.5% vs 2.9%; $p < 0.05$). In conclusion, under the same conditions, the frequency of medium replacement significantly affected the *in vitro* development of caprine and ovine pre-antral follicles. To improve the efficiency of the culture system, the medium must be replaced every 2 and 6 days for goat and sheep pre-antral follicles, respectively.

Keywords: Goat, Sheep, Pre-antral follicles, Culture, Medium replacement

Introduction

There is a great deal of economic interest in the reproductive biotechnique of culturing pre-antral follicles *in vitro*, because the thousands of oocytes enclosed in these follicles have the potential to produce large numbers of embryos. Some authors have demonstrated that after pre-antral follicles are cultured, viable offspring can be produced in mice (O'Brien et al. 2003), while embryos can be produced in swines (Wu et al. 2001), in bubalines (Gupta et al. 2008) and rats (Daniel et al. 1989), nuclear maturation can be achieved in ovines (Tamilmani et al. 2005), and antrum formation (Huanmin e Yong 2000) and oocyte meiotic resumption (Silva et al. 2010) can be produced in caprines. Yet, the rate of embryo production from pre-antral follicles cultured *in vitro* is still very low, especially in ruminants, and requires more effort to improve conditions culture.

Several factors influence pre-antral follicle development during *in vitro* culture, such as the medium composition (hormones (Matos et al. 2007), growth factors (Martins et al. 2008), peptides (Hulshof et al. 1994), the gas atmosphere (Silva et al. 2010), the reproductive state of the ovary donor (Figueiredo et al. 1994), the culture period (Matos et al. 2007) and, likely, the frequency of medium replacement during the culture period. Interestingly, in most of the *in vitro* culture systems, the medium is totally or partially replaced by fresh medium every other day, independent of the culture period (O'Brien et al. 2003; Muruvi et al. 2005; Matos et al. 2007). In addition, in the studies performed with cultures of pre-antral follicles, the medium is sometimes changed at different intervals in the same species (ovine: Ceconni et al. 2004; Tamilmani et al. 2005). Yet, it is unclear whether frequent intervals of medium replacement could impair follicular development. Furthermore, to our knowledge, there are no reports comparing the effects of different intervals of medium replacement on the *in vitro* development of both isolated caprine and ovine pre-antral follicles. Although both species are small ruminants, they have some particularities including reproductive aspects *in vivo*, such as oestrous cycle duration and maintenance of pregnancies (Chemineau 1983; Skinner et al. 2002). Futhermore, studies performed in our laboratory with chilled (Costa el al. 2002; Matos et al. 2004) and cultured (Andrade et al. 2005; Matos el at. 2006) pre-antral follicles demonstrated that ovine pre-antral follicles are more resistant than caprine follicles. Therefore, the present study aims at evaluating the effect of different intervals of medium replacement on the viability, antral cavity formation,

growth and *in vitro* maturation (IVM) of oocytes from isolated caprine and ovine pre-antral follicles cultured *in vitro*.

Materials and Methods

Source of chemicals and ovaries

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, USA). Ovaries were collected at a local slaughterhouse from sixteen adult (ages 1-3 years) cross-breed goats (n = 16) and ewes (n = 16), for a total of four replicates/species (four animals/replicate). Immediately after 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.

Isolation and selection of caprine and ovine pre-antral follicles

In the laboratory, the fat tissue and ligaments surrounding the ovaries were stripped off. Caprine and ovine 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. Caprine and ovine pre-antral follicles that were approximately ≥ 150 µm in diameter were visualized under a stereomicroscope (SMZ 645; Nikon, Tokyo, Japan) and manually dissected from strips of the ovarian cortex using 25-gauge (25 G) needles. After isolation, follicles were transferred to 100-µl drops containing a fresh medium under mineral oil to further evaluate the follicular quality. Follicles with a visible oocyte that were surrounded by granulosa cells and had an intact basement membrane and no antral cavity were selected for culture.

Culture of caprine and ovine pre-antral follicles

After selection, follicles were individually cultured in 25 µl-drops of culture medium in Petri dishes (60 x 15 mm; Corning Incorporated, NY, USA). The culture medium consisted of α-MEM (pH 7.2-7.4) supplemented with 1.25 mg/ml bovine serum albumin (BSA), ITS (insulin 6.25 ng/ml, transferrin 6.25 ng/ml and selenium 6.25 ng/ml), 2 mM glutamine, 2 mM hypoxantine, 1000 ng/ml recombinant follicle stimulating hormone (rFSH) and 50 µg/ml ascorbic acid under mineral oil. Incubation was carried out at 39°C, in 5% CO_2 in air for 24 days. Fresh media were prepared immediately before use and incubated for 1 h prior to use. In both species, two intervals of medium replacement were tested: replacement every 2 (treatment 1, T_1) or 6 (treatment 2, T_2) days of culture. The medium was changed by replacing 15 µl of the culture medium for the same volume of fresh medium. The culture was replicated four times, and at least 34 follicles were used for ovine and 30 for caprine in each treatment.

Morphological evaluation of follicle development

Follicles were classified according to their morphological aspect, and those showing morphological signs of degeneration, such as darkness of the oocytes and the surrounding cumulus cells, or misshapen oocytes were classified as degenerated. Follicular diameter was measured only in healthy follicles in the *x* and *y* dimensions (90°) using an ocular micrometer (100 x magnification) inserted into a stereomicroscope every 6 days of culture (at days 0, 6, 12 and 18 of culture). Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers.

***In vitro* maturation of caprine and ovine oocytes from *in vitro* cultured follicles**

At the end of the 24-day culture period, all of the healthy follicles were carefully and mechanically opened with 26-G needles under a stereomicroscope, for oocyte recovery. Only oocytes ≥110 µm, with homogeneous cytoplasm that were surrounded by at least one compact layer of cumulus cells were selected for IVM. The recovery rate was calculated by dividing the number of oocytes ≥110 µm by the number of viable follicles at day 24 of culture and

multiplying this value by 100. The selected cumulus oocyte complexes (COC) were washed three times in a maturation medium composed of TCM 199 supplemented with 10% foetal calf serum, 100 µg/ml luteinizing hormone and 5 µg/ml rFSH. After washing, the oocytes were transferred to 50-µl drops of maturation medium under mineral oil and then incubated for 26 h at 39ºC with 5% CO_2 in the air. At the end of the maturation period, oocytes were stained with Hoescht 33342 and then analyzed by chromatin configuration.

Assessment of pre-antral follicle viability by fluorescence

For a more precise evaluation of follicular quality after 24 days of culture, live/dead fluorescent staining was performed on isolated cultured goat and sheep pre-antral follicles. Briefly, follicles were incubated in 100-µL droplets of MEM containing 4 µM 146 calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes; Invitrogen, Karlsruhe, Germany) at 37° C for 15 min. Afterwards, follicles were washed three times in MEM and examined under a DM LB fluorescence microscope (Leica, Wetzlar, Germany). The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. Oocytes and granulosa cells were considered to be alive if the cytoplasm was stained positively with calcein-AM (green) and if chromatin was not labelled with ethidium homodimer-1 (red).

Statistical analysis

Goats yielded different number of follicles, which were then taken as a pool for experimental procedures. Follicles were then considered as the experimental unit, following the same approach of Silva et al. (2010). Data from follicular survival, fully grown oocytes, antrum formation and meiotic resumption after *in vitro* culture in each species were compared using chi-square test, with the results expressed as percentages. Data from follicular diameters were submitted to Kolmogorov-Smirnov and Bartlett tests to confirm normal distribution and homoscedasticity, respectively. ANOVA was then carried out and treatments were compared using Student-Newman-Keuls test. Because of the heterogeneity of variances, days of culture were compared using Kruskal-Wallis non-parametric test (SAS/STAT, 1999). Results were

expressed as mean ± standard deviation (SD) and differences were considered to be significant when $p < 0.05$.

Results

Effect of the medium replacement interval on follicular viability and antrum formation

Caprine and ovine pre-antral follicles selected for culture had a centrally located oocyte and normal granulosa cells, which were enclosed by an intact basement membrane (Fig. 1a).

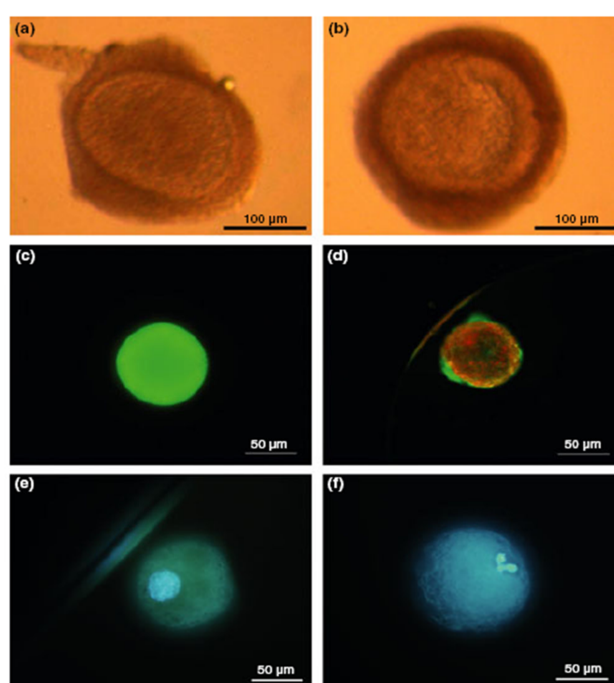

Fig. 1. Ovine (a) pre-antral at day 0 and (b) antral follicle after 6 days of *in vitro* culture with a medium change every 6 days (50x). Viability of goat follicles cultured *in vitro* under different types of medium change (c) A viable caprine oocyte cultured with medium change every 2 days labelled by calcein-AM (green fluorescence); and (d) an ovine oocyte cultured with a medium change every 2 days, which was considered non-viable as cells were marked with ethidium homodimer-1 (red fluorescence). Fully grown oocytes from ovine pre-antral follicles cultured with a medium change every 6 days. Note that the chromatin configuration shows intact germinal vesicle (e) and anaphase I (f) after Hoechst 33342 staining

The percentages of follicular viability and antral cavity formation after 0, 6, 12, 18 and 24 days of culture are shown in Figs 2 and 3 for caprine and ovine species, respectively. In caprine species, after comparing each treatment in the same culture period, there was a progressive and significant reduction in the percentage of viable follicles in both treatments (T_1 and T_2) up to day 12 of the culture ($p < 0.05$; Fig. 2a). When the comparison was made between the treatments, a significantly ($p < 0.05$) higher percentage of viable follicles was observed in T_1 than in T_2 from day 6 up to the end of the culture. The first antral follicles were observed as early as day 6 of the culture in both treatments (Figs 1b and 2b). Yet, there was no significant difference in the rate of antrum formation among days of culture in both treatments from day 6 onward (both T_1 and T_2). Nevertheless, after comparing both treatments at the same culture period, the rate of antrum formation was significantly higher in T_1 than in T_2 from day 12 onward ($p < 0.05$; Fig. 2b).

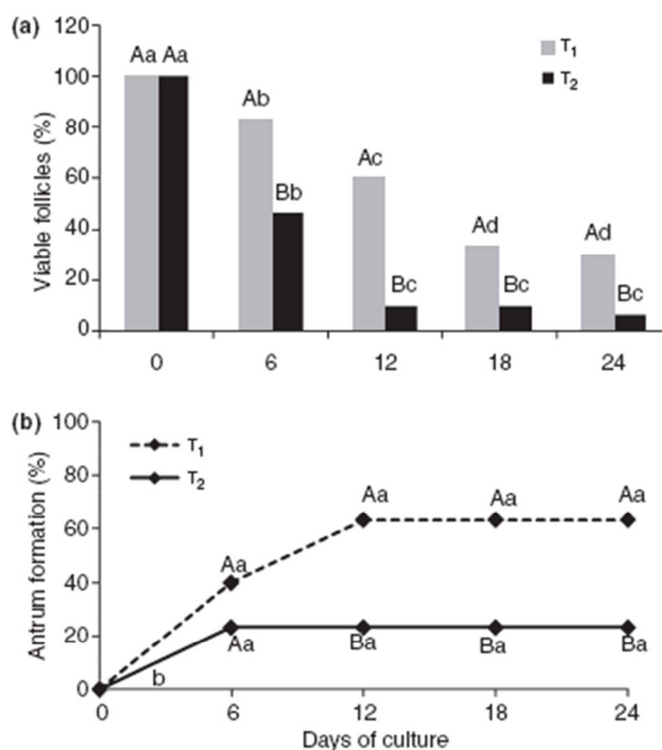

Fig. 2. Caprine pre-antral follicles cultured for 24 days. (a) Percentage of viable follicles; (b) antrum formation under the following treatments: T_1, with medium changes every 2 days, and T_2, with medium changes every 6 days. ^{A,B} indicates significant differences between treatments in the same culture period. ^{a,b} indicates significant differences among days of culture in the same treatment

For ovines, in treatment 1, there was a significant reduction of follicular viability after the progression of the culture from day 6 to day 12 and from day 18 to day 24 ($p < 0.05$; Fig. 3a). In addition, there was a progressive and significant decrease in the percentage of follicular viability in T_2 from day 0 to day 6 and from day 6 to day 18 of culture ($p < 0.05$). Yet, unlike the results for caprines, after comparing both treatments at day 24 of culture, there were more viable follicles in T_2 than in T_1 ($p < 0.05$). Similar to the observations of the goat follicles, the first ovine antral follicles were observed as early as day 6 of culture in both treatments (Fig. 3b). Furthermore, a significant increase in the rate of antral cavity formation was observed with the progression of the culture period from day 6 to day 12 in T_1. Yet, unlike the observations for the caprine species, there was no differences between T_1 and T_2 regarding the antrum formation at the various days of culture ($p > 0.05$). Figures 1c,d show viable and non-viable follicles after being cultured with medium replacement every 2 days for caprines and ovines, respectively.

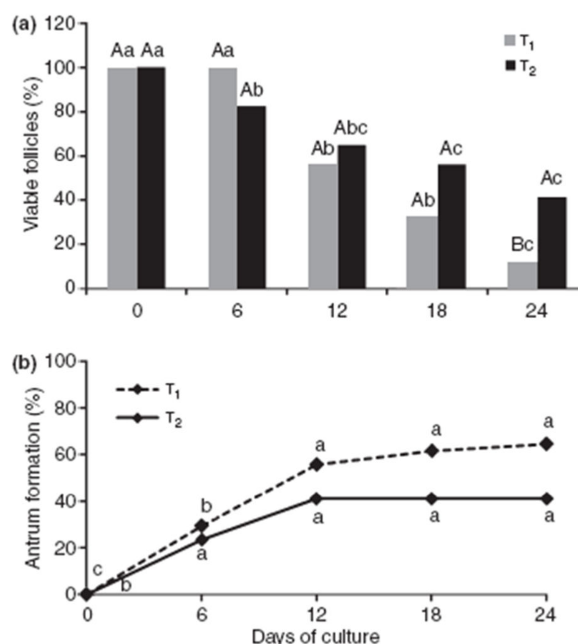

Fig. 3. Ovine pre-antral follicles cultured for 24 days. (a) Percentage of viable follicles; (b) antrum formation under the following treatments: T_1, in which the medium was changed every 2 days, and T_2, in which the medium was changed every 6 days. ^{A,B} indicates significant differences between treatments in the same culture period. ^{a,b} indicates significant differences among days of culture in the same treatment

Follicular growth after *in vitro* culture with different intervals of medium replacement

Values related to follicular growth during *in vitro* culture of isolated caprine and ovine pre-antral follicles are described in Table 1. In both species, there was a significant increase in follicular diameter up to day 12 of culture independent of the treatment used ($p < 0.05$). Yet, in both caprine and ovine follicles there were no significant changes in follicular diameter after this period or when the treatments were compared at the same day of culture ($p > 0.05$).

Table 1. Follicular diameter (mean ± SD) of caprine and ovine pre-antral follicles at different days of culture (0, 6, 12, 18 and 24) for media changed every 2 (T_1) and 6 days (T_2)

Day of culture	Caprine follicular diameter (µm)		Ovine follicular diameter (µm)	
	(T_1)	(T_2)	(T_1)	(T_2)
D0	233.33±43.52 c	234.01±43.78 c	267.41±57.55 c	275.51±58.91 c
D6	370.61±109.69 b	405.97±138.66 b	378.15±77.42 b	408.89±80.53 b
D12	505.67±117.06 a	676.87±77.93 a	461.87±95.47 a	517.62±110.67 a
D18	461.73±106.49 ab	741.29±94.82 a	538.96±121.84 a	561.76±117.55 a
D24	506.73±87.16 a	722.56±77.98 a	588.44±235.43 a	584.55±119.50 a

a,b: indicates significant differences among days of the culture into the same treatment and species. There was no significant difference between treatments.

Ability of oocytes grown *in vitro* to undergo meiotic resumption

In the caprine species, percentages of oocytes acceptable for IVM (fully grown oocytes ≥ 110 µm) after 24 days of culture were significantly higher in normal follicles cultured in T_1 (with medium replacement every 2 days, the percentage was 30.0%) than in T_2 (with medium replacement every 6 days, the percentage was 6.7%; $p < 0.05$; Table 2). On the other hand, for ovines, the T_2 treatment at the end of the culture had a higher percentage of oocytes destined to

IVM than did T_1 (23.5% vs. 2.9%; $p < 0.05$). Oocytes had a homogeneous cytoplasm and were surrounded by at least one compact layer of cumulus cells, characterizing the COC. At the end of the maturation period, expanded cumulus cells were observed in some COC. With respect to the chromatin configuration, grown oocytes from goat and ewe follicles generally had an intact germinal vesicle (Fig 1e) after culture. Exceptions were 12.5% of the goat oocytes from follicles cultured in T_1 group and 25.0% of sheep oocytes from follicles cultured in T_2 group, which resumed meiosis, reaching the anaphase I stage, as shown in Fig. 1f.

Table 2. Meiotic stages of caprine and ovine oocytes from pre-antral follicles cultured for 24 days in treatments with mediums changed every 2 (T_1) and 6 (T_2) days

Specie	Medium change	Number of follicles(%)		Number of oocytes (%)		
		Cultured	Survived	Fully grown*	GV	AN I
Caprine	Each 2 days (T_1)	30	9 (30.0)	9/30 (30.0) A	8/9 (87.5)	1/9(12.5)
	Each 6 days (T_2)	30	2 (6.7)	2/30 (6.7) B	2/2 (100.0)	0/2 (0.0)
Ovine	Each 2 days (T_1)	34	4 (11.8)	1/34 (2.9) B	1/1 (100.0)	0/1 (0.0)
	Each 6 days (T_2)	34	14 (41.2)	8/34 (23.5) A	6/8 (75.0)	2/8(25.0)

GV: germinal vesicle; AN I: Anaphase I.

*Only oocytes (≥ 110 µm) were selected for the *in vitro* maturation procedure.

A,B indicates significant differences between treatments in the same species.

Discussion

The present study showed, for the first time, that the medium replacement interval affects the *in vitro* development of isolated caprine and ovine pre-antral follicles differently. In caprine species, follicular viability and antrum development was significantly higher when the medium was replaced every 2 days than when the medium was replaced every 6 days. Zhou and Zhang (2005a,b) also observed the maintenance of follicular survival and oocyte growth after *in vitro* culturing of isolated caprine pre-antral follicles when the medium was changed every 2 days. In addition, Rajarajan et al. (2006) found satisfactory results with medium replacement every 3 days

during the *in vitro* culture of caprine pre-antral follicles. Furthermore, recent studies with cultures of pre-antral follicles enclosed in the goat ovarian cortex have shown high rates of follicular survival and growth when the medium is changed every 2 days (Matos et al. 2007; Martins et al. 2008; Celestino et al. 2009). The results of the goats in the present investigation are in accordance with these ideas, as most of the amino acids that are used between medium changes are used in the first 24 h of culture. The amino acid uptake is typically high in the first 24 h of the culture as this is the lag phase during which the cells actively establish themselves and come into equilibrium with the environment (Griffiths and Pirt, 1967).

Regarding the ovine species, the best results were obtained when the medium was replaced every 6 days. Studies with chilled (Costa et al. 2002; Matos et al. 2004) and cultured (Andrade et al. 2005; Matos et al. 2006) pre-antral follicles suggested that ovine pre-antral follicles are more resistant than caprine follicles. In these studies, authors showed that a richer medium is more suitable to maintain morphologically normal goat follicles. Therefore, in the current study, it is likely that an interval of 6 days without refreshing the culture medium may not provide enough nutrients to caprine follicles and/or could cause nutrient exhaustion. Nevertheless, ovine pre-antral follicles can be sustained and can maintain follicular viability for a longer time period without renewal of the medium. In addition to amino acids, it is likely that the presence of other components in the culture medium (for example, ITS, FSH and ascorbic acid) could help to maintain the viability of the follicles when the medium was changed every 6 days. Furthermore, replacing the medium every 6 days may have promoted an increased viability because there was a reduction in the time for which follicles were exposed to the external environment (from outside the incubator). Consequently, the stress of follicular cells and the release of reactive oxygen species would be reduced, as would cell degeneration (Correa et al. 2007). Arunakumari et al. (2007) reached the stage of metaphase II in *in vitro* culture of ovine pre-antral follicles after 6 days of culture without medium replacement during this period. In addition, Gutierrez et al. (2000) reported that medium replacement every 6 days led to an increase in bovine follicular growth and antrum formation. On the other hand, after culturing sheep pre-antral follicles, Cecconi et al. (1999, 2004) observed good rates of follicular survival and antrum formation when the medium was renewed every 2 days. In addition, medium replacement every 3 days also promoted satisfactory results in the culture of ovine pre-antral follicles (Tamilmani et

al. 2005; Tsuribe et al. 2008). These contradictory results may be because of the differences between methodologies employed, including the culture medium composition.

As the culture period progressed, an increase in follicular diameter was observed in both caprine and ovine species in both T_1 and T_2 up to day 12 of culture. The medium composition is an important factor for success during *in vitro* culture of pre-antral follicles The αMEM used in our study is one of the richest formulation and is composed of 21 essential amino acids, B-vitamin complexes, vitamins C and D, inorganic salts and pyruvate. In addition, this medium was also supplemented with glutamine, hypoxanthine, BSA, ITS, ascorbic acid and FSH. In our study, pre-antral follicles of both caprine and ovine species increased in size only up to the 12th day of culture. It is likely that follicle growth was followed by an increase in metabolism, and consequently, an increased need for nutrients in the medium. Thus, an alteration in the medium components (i.e., a sequential medium) would be necessary to promote follicular growth up to day 24 of culture.

In previous studies, oocytes from goats smaller than 100 µm were not able to resume meiosis (Crozet et al., 2000). Therefore, in the present work, only oocytes larger than 110 µm from viable follicles were considered optimal for use in IVM procedures. In our study, *in vitro* culture of goat pre-antral follicles with medium replacement every 2 days increased the number of oocytes destined for IVM compared with the group receiving a medium change every 6 days (30.0% and 6.7%, respectively). Yet, for ovines, medium replacement every 6 days produced a larger number of fully grown oocytes in comparison to the treatment with a medium change every 2 days. In addition, 25.0% of oocytes from sheep follicles cultured with a medium change every 6 days and 12.5% of oocytes from goat follicles resumed meiosis and reached the anaphase I stage. These results are interesting; although the rate of meiotic resumption reported in the present study was less than desirable, few studies in sheep have achieved meiotically competent oocytes from the *in vitro* culture of pre-antral follicles (Tamilmani et al. 2005; Arunakumari et al. 2007). In addition, after *in vitro* culture of goat pre-antral follicles, Silva et al. (2010) verified that only 16.7% of oocytes from goats resumed meiosis and achieved the metaphase I stage of development. The mechanisms necessary for pre-antral follicles to develop to maturity are complex and little known. Some hormones and growth factors can act synergically to control folliculogenesis (Hemamaline et al. 2003). Therefore, supplementation of the maturation medium with these substances may increase the rate of meiotic resumption. Thus, the rate of meiosis

resumption observed in ovine follicles cultured with medium replacement every 6 days could be caused by paracrine factors produced by the follicles, which may have stimulated oocyte development (Van Den Hurk and Zhao 2005). Nevertheless, in both species, the small percentage of *in vitro*-grown oocytes with meiotic resumption was probably because of the culture conditions, which were insufficient to promote *in vitro* development of pre-antral follicles and produce meiotically competent oocytes.

The caprine and ovine pre-antral follicles showed some different behavior under the same *in vitro* culture conditions. Although both species are small ruminants, they have some particularities, including reproductive aspects *in vitro*, such as oestrous cycle duration and maintenance of pregnancies. Oestrus cycle in caprines and ovines lasts approximately 21 and 18 days, respectively (Chemineau, 1983; Skinner et al. 2002). In addition, the superovulation protocols used also differ between the two species (Dutta et al. 1993; Dias et al. 2001).

In conclusion, under the same conditions, the frequency of medium replacement significantly affects the *in vitro* development of caprine and ovine pre-antral follicles. We recommend medium replacement intervals of 2 and 6 days for isolated caprine and ovine pre-antral follicles, respectively. The direct relationship between follicular development and the medium replacement intervals establishes a new concept in pre-antral follicle culture and must be considered in further studies aiming to obtain mature goat and sheep oocytes. Yet, because of low rates of follicle survival and oocyte maturation were obtained using the culture medium descript in this article, further experiments must be carried out to improve the culture system, including the composition of the medium.

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Conflict of interest

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

Author contributions

In this study, the co-authors DM Magalhães, DD Fernandes, MBS Mororó, CMG Silva, GQ Rodrigues, JB Bruno contributed to designed study and drafted paper. CC Campelo contributed to analysed data. Finally, MHT Matos and JR Figueiredo contributed to drafted paper.

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10 CAPÍTULO 5 (Fase IV)

Nível de RNAm para receptor do fator de crescimento semelhante à insulina-I (IGF-I) e o efeito do IGF-I no cultivo *in vitro* de folículos pré-antrais caprinos

(Steady-state level of Insulin-like growth factor-I (IGF-I) receptor mRNA and the effect of IGF-I on the in vitro culture of caprine preantral follicles)

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Resumo

O objetivo do presente estudo foi quantificar RNAm para o receptor do fator de crescimento semelhante à insulina I (IGFR-1) em folículos pré-antrais nos Dias 0 e 18 do cultivo *in vitro* na presença ou ausência de FSH e avaliar os efeitos do IGF-I na taxa de folículos normais, formação da cavidade antral, crescimento e maturação *in vitro* de oócitos caprinos nos Dias 0, 6, 12 e 18 de cultivo. A expressão de IGFR-1 foi analisada utilizando RT-PCR em tempo real antes e depois do cultivo follicular. Folículos pré-antrais foram isolados a partir do cortex de ovário caprino e individualmente cultivados por 18 dias na presença ou ausência de IGF-I bovino nas concentrações de 50 ou 100 ng/mL. Ao final do período de cultivo, os oócitos foram submetidos à MIV. A expressão de RNAm para IGFR-1 em folículos pré-antrais cultivados *in vitro* teve uma tendência a ser significativamente superior em folículos suplementados com FSH quando comparados à folículos imediatamente após a coleta ($P < 0,06$) e cultivados sem FSH ($P < 0,1$). Um maior ($P < 0,05$) percentual de folículos normais nos Dias 6, 12 e 18 de cultivo nos grupos IGF-I 50 (97, 92, 67%, respectively) e IGF-I 100 (100, 90, 80%) versus o controle (90, 64, 36%) foi observado. Além disso, a taxa de formação de antro nos dias 6 e 12 de cultivo foi maior ($P < 0,05$) nos grupos tratados com IGF-I (IGF-I 50: 72 e 90% e IGF-I 100: 69 e 85%) do que no grupo controle (41 e 59%). Após 18 dias de cultivo, o percentual de oócitos crescidos aceitáveis para a MIV foi superior ($P < 0,05$) em folículos cultivados na presença de IGF-I (82 vs. 49%). Além disso, folículos cultivados na presença de IGF-I 50 e IGF-I 100 tiveram uma maior ($P < 0,05$) taxa de retomada de meiose (63 e 66%, respectivamente) quando comparado com o grupo controle (11%). Em conclusão, o tratamento com FSH tendeu aumentar os níveis de expressão de RNAm para IGFR-I durante o cultivo *in vitro* de folículos pré-antrais e a adição de IGF-I ao meio de cultivo claramente aumentou o desenvolvimento *in vitro* de folículos pré-antrais caprinos.

Palavras-chave: Folículos pré-antrais. Desenvolvimento *in vitro*. IGF-I. IGFR. Ovário. Cabra.

Steady-state level of Insulin-like growth factor-I (IGF-I) receptor mRNA and the effect of IGF-I on the *in vitro* culture of caprine preantral follicles

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Abstract

The objectives were to quantify insulin-like growth factor receptor-1 (IGFR-1) mRNA in preantral follicles on Days 0 and 18 of *in vitro* culture in the presence or absence of FSH and to evaluate the effects of IGF-I on the rate of normal follicles, antral cavity formation, and *in vitro* growth and maturation of caprine oocytes on Days 0, 6, 12, and 18 of culture. The expression of IGFR-1 was analyzed using real-time RT-PCR before and after follicle culture. Preantral follicles were isolated from the cortex of caprine ovaries and individually cultured for 18 d in the presence or absence of bovine IGF-I (50 or 100 ng/mL). At the end of the culture period, the oocytes were submitted to IVM. The expression of IGFR-1 mRNA in preantral follicles cultured *in vitro* only approached being significantly higher in follicles supplemented with FSH when compared to follicles immediately after recovery ($P < 0.06$) and cultured without FSH ($P < 0.1$). There was a higher ($P < 0.05$) percentage of normal follicles on Days 6, 12, and 18 of culture in IGF-I 50 (97, 92, 67%, respectively) and IGF-I 100 (100, 90, 80%) groups versus the control (90, 64, 36%). In addition, the rate of antrum formation at 6 and 12 d of culture was higher ($P < 0.05$) in IGF-I groups (IGF-I 50: 72 and 90% and IGF-I 100: 69 and 85%) than the control group (41 and 59%). After 18 d of culture, the percentages of grown oocytes acceptable for IVM were higher ($P < 0.05$) in follicles cultured in the presence of IGF-I (82 vs 49%). Furthermore, follicles cultured in the presence of IGF-I 50 and IGF-I 100 had higher ($P < 0.05$) meiotic resumption rates (63 and 66%, respectively) than the control group (11%). In conclusion, treatment with FSH tended to increase IGFR-I mRNA expression during the *in vitro* culture of preantral follicles and the addition of IGF-I to the culture medium clearly improved the *in vitro* development of caprine preantral follicles.

Keywords: preantral follicle; *in vitro* development; IGF-I; IGFR; Ovary; Goat.

1. Introduction

In recent years, *in vitro* production of embryos from preantral follicles has evoked the interest of several scientific and commercial areas, due to its potential to produce thousands of viable oocytes for IVF from a single ovary. Using this reproductive biotechnology, the birth of viable offspring has only been reported in mice [1]. However, in domestic animals, low rates of *in vitro* embryo production have been obtained from preantral follicles cultured *in vitro* [2-6]. Therefore, many efforts are still required to improve the process of *in vitro* maturation (IVM) of preantral follicles.

The mechanisms behind folliculogenesis involve a complex interaction among endocrine, paracrine, and autocrine factors [7]. In this regard, an ideal culture system should contain adequate nutrients (e.g., antioxidants, hormones, growth factors) with the aim of avoiding atresia and also promoting follicular development. One of the substances related to the regulation of folliculogenesis that has great potential for *in vitro* follicular culture is insulin-like growth factor-I (IGF-I).

The IGF-I is a member of the IGF family system, which is composed of several substances, including IGF-I and IGF-II, two types of receptors (IGFR-1 and IGFR-2) and six binding proteins (insulin-like growth factor binding proteins: IGFBP-1, -2, -3, -4, -5, and -6) [8]. The IGFBPs are present in biological fluids and act by inhibiting or potentializing the action of two types of IGFs (IGF-I and IGF-II) in target cells [8]. The involvement of IGF-I in the early stages of folliculogenesis was evidenced by studies in which IGF-I induced the development of mouse preantral and antral follicles in IGF-I gene knockout animals [9]. Furthermore, the addition of IGF-I in synergy with FSH in the *in vitro* culture medium of preantral follicles stimulated follicular growth in humans [10], cows [11], rats [12], and mice [13].

Although the importance of IGF-I on folliculogenesis is well known in several species, the effect of this growth factor on the *in vitro* culture of isolated caprine preantral follicles, as well as the effect of FSH on mRNA expression of IGF-1 receptors (IGFR-1) during *in vitro* culture, has not been studied in detail during a long-term culture period (more than 2 wk). Thus, the aims of the present study were to: 1) test the hypothesis that FSH treatment would increase the expression of mRNA of IGFR-1 during *in vitro* culture of preantral follicles (Experiment 1); and 2) evaluate the effects of IGF-I on caprine preantral follicle development *in vitro* and further achievement of meiotically competent oocytes (Experiment 2).

2. Materials and methods

2.1. Collection of ovaries, isolation, selection and in vitro culture of preantral follicles

Ovary pairs were collected at a local slaughterhouse from 30 adult (ages 1 to 3 y) crossbred goats. Fourteen goats were used for Experiment 1 and 16 for Experiment 2. A total of four replicates per culture (four animals/replicate) were used in Experiment 2. Immediately postmortem, ovaries were washed in 70% ethanol for 10 s, followed by two rinses in Minimum Essential Medium (MEM) supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, and 25 mM HEPES. The ovaries were transported within 1 h to the laboratory at 4 °C [14]. The culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

In the laboratory, fat and connective tissue surrounding the ovaries were removed. Caprine ovarian cortical slices (1–2 mm in thick) were cut from the ovarian surface using a surgical blade under sterile conditions. Then, the ovarian cortex slices were placed in medium consisting of HEPES-buffered MEM. Secondary follicles ≥150 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of the ovarian cortex using 26-gauge needles. After isolation, follicles were transferred to 100 µL droplets containing fresh medium under mineral oil to further evaluate follicular quality. Follicles with a visible oocyte that were surrounded internally by granulosa cells and had an intact basement membrane and no antral cavity were selected for PCR analysis and *in vitro* culture.

For the *in vitro* culture, follicles were individually cultured in 100 µL drops of culture medium in Petri dishes (60 x 15 mm, Corning, NY, USA). Incubation was carried out at 39 °C, in 5% CO_2 in air for 18 d. The basic culture medium (α-MEM$^+$) consisted of α-MEM (pH 7.2-7.4) supplemented with 3 mg/mL bovine serum albumin (BSA), ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, and 50 µg/mL ascorbic acid, under mineral oil. Fresh medium was prepared and incubated for 2 h prior to use. Every other day, 60 µL of fresh medium was replenished in each droplet until Day 18, and, at Days 6 and 12, all medium (100 µL) was replenished.

2.2. Experiment 1. Expression of IGF-1 receptor (IGFR-1) mRNA in cultured follicles

For this procedure, 120 isolated secondary follicles were randomized into three groups: nonculture control (n = 40), α-MEM$^+$ alone (n = 40), and α-MEM$^+$ supplemented with recombinant bovine FSH (rFSH®, Nanocore, Campinas, SP, Brazil; n = 40). Increasing concentrations of FSH were used (e.g., 100 ng/mL until Day 6, 500 ng/mL until Day 12, and 1000 ng/mL until Day 18) as previously described [6,15]. None of the treatments for this experiment were supplemented with IGF-I. After 18 d of culture, 10 normal follicles from each group were frozen at -80 ^oC to further evaluate the mRNA expression for IGFR-1. The classification of normal or degenerated follicles was based on their morphological aspects, and those showing morphological signs of degeneration, such as darkness of the oocytes and surrounding cumulus cells, or misshapen oocytes, were classified as degenerated.

The mRNA isolation was performed using a Trizol plus purification kit (Invitrogen, São Paulo, SP, Brazil) as previously described in our laboratory [15]. Quantification of the mRNA for IGFR-1 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 IGFR-1. β-actin and Ubiquitin (Table 1) were used as endogenous controls for normalization of steady-state levels of mRNA of genes. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 15 min at 94 ^oC, followed by 40 cycles of 15 s at 94 ^oC, 30 s at 60 ^oC, and 45 s at 72 ^oC. The final extension was for 10 min at 72° C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Hamburg, Germany). The delta-delta-CT method was used to transform CT values into normalized relative steady-state levels of mRNA.

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

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
β-ACTIN	ACCACTGGCATTGTCATGGACTCT	S	188-211	GI:28628620
	TCCTTGATGTCACGGACGATTTCC	As	363-386	
UBQ	GAAGATGGCCGCACTCTTCTGAT	S	607-631	GI:57163956
	ATCCTGGATCTTGGCCTTCACGTT	As	756-780	
IGFR-1	TCTGTTGATACTGGGAGGCTTGGT	S	6-30	GI:110347755
	AATACTCCGGGTTCACAGACGCAT	As	103-127	

S, sense; AS, antisense

2.3. Experiment 2. Effect of IGF-I on the in vitro culture of preantral follicles

2.3.1. Experimental design

Secondary follicles were isolated and cultured from Days 0 to 18 in three treatments: α-MEM$^+$ with only FSH (control group) or with FSH plus supplementation of IGF-I at concentrations of 50 or 100 ng/mL (IGF-I 50 group and IGF-I 100 group, respectively). Furthermore, FSH was used in increasing concentrations as described in Experiment 1. The culture was replicated four times and a total of at least 39 follicles were used in each treatment. The concentrations of IGF-I were chosen based on a previous study performed in our laboratory [16].

2.3.2. Morphological evaluation of follicle development

The classification of normal or degenerated follicles was similar as performed in Experiment 1. Therefore, the rate of normal follicles was based upon the morphological analysis performed at Days 6, 12, and 18 of culture. Follicle diameter was measured only in normal follicles every 6 d of culture by an ocular micrometer (100× magnification). The average of two measurements (height and length) of the follicle was used as a measure of follicle diameter. Daily growth rate was calculated based on the diameter changes over the culture period (18 d). Antral

cavity formation was defined by the occurrence of a visible translucent cavity surrounded by the granulosa cell layer.

2.3.3. In vitro maturation (IVM) of caprine oocytes from in vitro cultured follicles

At the end of the culture period, all normal follicles were carefully and mechanically opened with 26-gauge needles under a stereomicroscope for oocyte recovery. The oocyte recovery rate was calculated based on the number of oocytes ≥ 110 µm, divided by the number of normal follicles at Day 18 of culture, multiplied by 100. Only oocytes ≥ 110 µm, with a homogeneous cytoplasm surrounded by at least one compact layer of cumulus cells, were selected for IVM [17]. The selected cumulus- oocyte complexes were washed three times in IVM medium composed of Tissue Culture Medium 199 (TCM 199) supplemented with 1 µg/mL 17β-estradiol, 5 µg/mL LH, 0.5 µg/mL rFSH, 10 ng/mL EGF, 1 mg/mL BSA, 22 µg/mL pyruvate, 50 ng/mL IGF-I, and 100 µmol/L cysteamine. After washing, the oocytes were placed in 100 µL droplets of maturation medium (10 COCs/droplet) under mineral oil and then incubated for 32 h at 39 ºC in 5% CO_2 in air. At the end of the maturation period, oocytes were labeled with Hoechst 33342 and then analyzed with regard to their chromatin configuration. They were classified as germinal vesicle (GV) or germinal vesicle breakdown (GVBD). The GVBD stage included the nucleus at metaphase I and II (MI and MII).

2.3.4. Assessment of oocyte viability by fluorescence microscopy

After IVM, all oocytes from each group were evaluated for viability by fluorescence microscopy. Oocytes were incubated in 100 µL droplets of TCM 199 containing 4 µM calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) for 15 min at 37 °C. Thereafter, oocytes were examined under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan) for evaluation of live/dead fluorescent staining. The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. Oocytes were considered alive if the cytoplasm was stained positively with calcein-AM (green color), and dead if chromatin was labeled with ethidium homodimer-1 (red color).

2.4. Statistical analyses

In the present study, follicles were considered the experimental unit, as previously reported [18]. The data for IGFR-1 mRNA expression for preantral follicles cultured *in vitro* were analyzed by unpaired Student's *t*-test. Chi-square analyses were used to examine differences in frequency for the following: normal follicle rate, antrum formation, growth of oocytes, and meiotic resumption after *in vitro* culture, with the results being expressed as percentages. Data from follicle diameters were submitted to Kolmogorov-Smirnov and Bartlett tests to confirm normal distribution and homoscedasticity, respectively. Then, ANOVA was carried out and treatments were compared using Student-Newman-Keuls (SNK) test. A probability of $P < 0.05$ indicated that a difference was significant, and probabilities between $P > 0.05$ and $P < 0.1$ indicated that the difference approached significance. Data are given as the mean ± SD, unless otherwise stated.

3. Results

3.1. Experiment 1. Expression of IGF-1 receptor mRNA in cultured follicles

Quantification of the level of IGFR-1 mRNA expression from cultured preantral follicles only approached being significantly higher in the group supplemented with FSH when compared to the follicles immediately after recovery ($P < 0.06$) and the follicles cultured in α-MEM$^+$ without FSH ($P < 0.1$). Data for IGFR-1 mRNA expression were converted to percentage change from the concentrations on Day 0, and the percentage change was used to confirm the stimulatory effect of FSH on IGFR-1 mRNA expression in goat preantral follicles cultured *in vitro*.

3.2. Experiment 2. Effect of IGF-I on the in vitro culture of preantral follicles

3.2.1. Effect of different concentrations of IGF-I on the rates of normal follicles and antrum formation

Caprine preantral follicles selected for culture presented a centrally located oocyte and

normal granulosa cells, which were enclosed by an intact basement membrane (Fig. 1A). The percentages of normal follicles and antral cavity formation at 0, 6, 12, and 18 d of culture have been shown (Fig. 2A, B, respectively). During the culture period, the control group showed a progressive reduction ($P < 0.05$) in the percentage of normal follicles. Follicles cultured in medium containing IGF-I 50 maintained the rate of normal follicles between Days 0 and 12 of culture, but this percentage was reduced ($P < 0.05$) by Day 18. The medium supplemented with IGF-I 100 maintained the percentage of normal follicles from Days 0 to 6 of culture, however, this percentage decreased ($P < 0.05$) between Days 6 and 12. When treatments were compared, higher ($P < 0.05$) percentages of normal follicles were observed in the two groups treated with IGF-I than in the control group on Days 12 and 18. No difference on the percentage of normal follicles was detected between IGF-I 50 and IGF-I 100 on Day 18 of culture.

The first antral follicles were observed on Day 6 of culture in all treatments (Fig. 1B). Moreover, a significant increase in the rate of antral cavity formation was observed with the progression of the culture period in all groups (Fig. 2B). The rate of antrum formation (Fig. 2B) was higher ($P < 0.05$) in IGF-I-treated groups than in the control group on Days 6 and 12 of culture ($P < 0.05$), whereas on Day 18 only the IGF-I 50 group was higher ($P < 0.05$) than the control group. However, no differences were detected between the two concentrations of IGF-I tested during the culture period ($P < 0.05$).

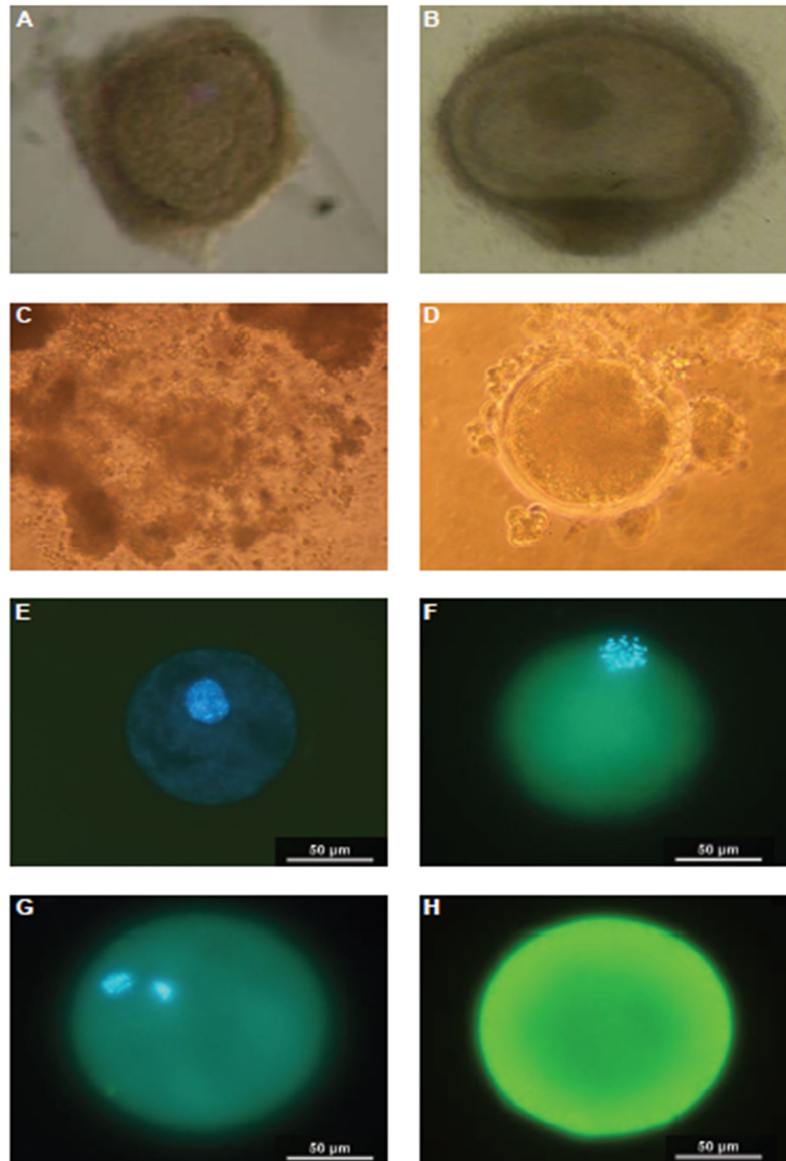

Fig. 1. (A) Caprine preantral follicle isolated for culture with a centrally located oocyte and normal granulosa cells, enclosed by an intact basement membrane; (B) Antral follicle after 12 d of in vitro culture in IGF-I 50 (magnification, 50×). (C) Good and (D) bad quality COCs after IVM of preantral follicle cultured in the presence of IGF-I 50 and control group, respectively. (E) Grown oocytes from preantral follicles cultured without or (F, G) in presence of IGF-I 50. (G) Note that the chromatin configuration shows an intact germinal vesicle, metaphase I and metaphase II after Hoechst 33342 staining, respectively. (H) Viable oocyte stained positively with calcein-AM (green color).

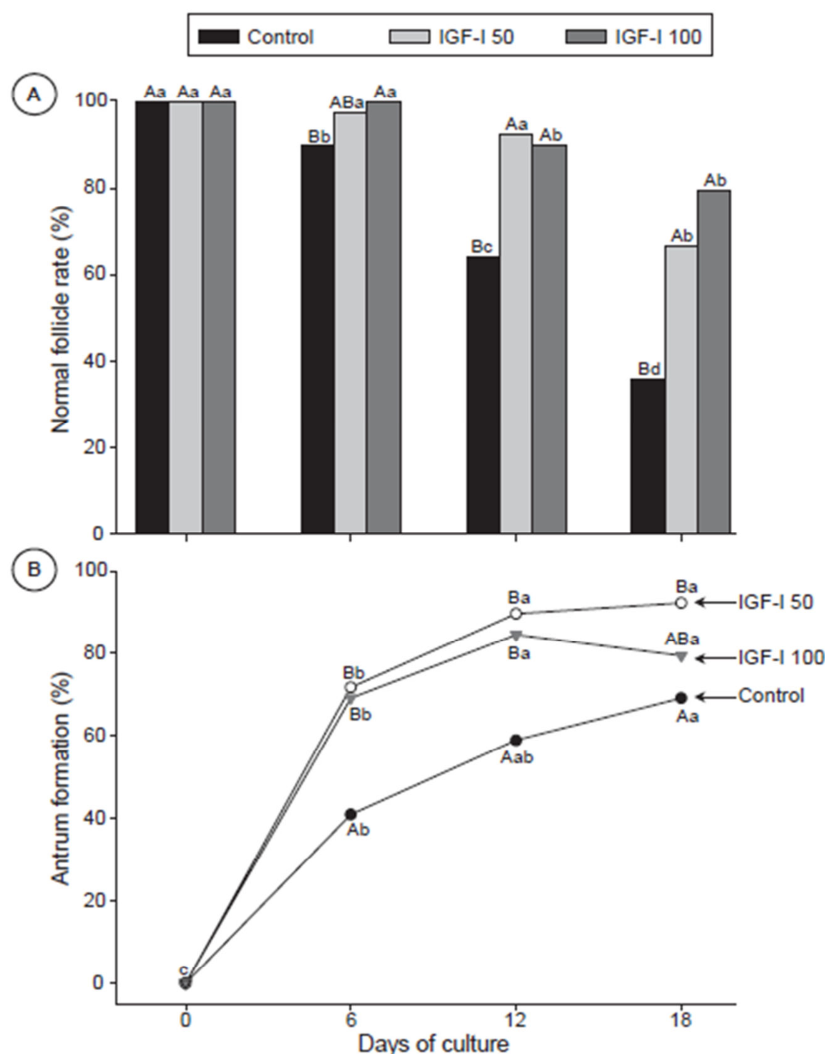

Fig. 2. Rates (%) of (A) normal follicles and (B) antrum formation of caprine preantral follicles cultured for 18 d in control, IGF-I 50 and IGF-I 100 groups.

[A,B]Within a day of culture, means without a common superscript differed ($P < 0.05$).

[a-c]Within a treatment, means without a common superscript differed ($P < 0.05$).

3.2.2. Follicular growth after in vitro culture

Mean initial diameters of follicles before culture (Day 0) did not differ among groups (Table 2). A progressive increase ($P < 0.05$) in follicle diameter was observed for all groups from Day 0 to 18. After 6 d of culture, IGF-I treated follicles showed a larger ($P < 0.05$) diameter than

the control group. However, at Days 12 and 18, follicle diameter was similar among groups. Contrarily, the daily growth rate was greater ($P < 0.05$) in both treatments with IGF-I (25.2 ± 13.6 µm and 27.1 ± 12.6 µm for IGF-I 50 and IGF-I 100 groups, respectively) than the control (18.1 ± 13.9 µm).

Table 2. Mean (± SD) follicular diameter (µm) of caprine preantral follicles at various days of culture (0, 6, 12, and 18) in the control and IGF-I (IGF-I 50 and IGF-I 100) treatments.

Days of culture	n	Control	n	IGF-I50	n	IGF-I100	CV (%)
0	39	244.1 ± 51.9 ^{a}	39	251.4 ± 47.8^{a}	39	248.8 ± 50.9^{a}	20.2
6	35	337.3 ± 105.5^{Ab}	38	410.0 ± 97.0^{Bb}	39	425.2 ± 127.7^{Bb}	28.3
12	25	508.6 ± 194.4^{c}	36	580.5 ± 190.6^{c}	35	572.9 ± 165.4^{c}	32.7
18	14	686.6 ± 227.8^{d}	26	703.3 ± 243.9^{d}	31	708.0 ± 174.0^{d}	30.3

CV, coefficient of variation.

^{A,B} Within a day, groups without a common superscript differed ($P < 0.05$).

$^{a\text{-}d}$ Within groups, days without a common superscript differed ($P < 0.05$).

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3.2.3. Viability and ability of in vitro grown oocytes to undergo meiotic resumption

All oocytes examined with live/dead fluorescent staining were considered alive if the cytoplasm stained positively with calcein-AM (green color; Fig. 1H). The percentages of oocytes acceptable for IVM (grown oocytes ≥ 110 µm) after 18 d of culture were higher ($P < 0.05$) in morphologically normal follicles cultured with IGF-I (50 or 100 ng/mL) than in the control group (Table 3). At the end of the maturation period, COCs cultured in medium containing IGF-I showed a great cumulus cell expansion (Fig. 1C, D). Regarding the chromatin configuration after

IVM, oocytes grown from goat follicles cultured without IGF-I had a higher (P < 0.05) percentage of intact germinal vesicles after 18 d of culture (Fig. 1E; Table 3). Furthermore, follicles cultured in IGF-I had more (P < 0.05) oocytes which resumed meiosis (Table 3; Fig. 1F) when compared to the control group. Only two oocytes in metaphase II stage were observed in the entire study after culturing in medium supplemented with IGF-I 50 (Fig. 1G).

Table 3. Meiotic stages of caprine oocytes from preantral follicles cultured *in vitro* in the absence or presence of IGF-I after 32 h of IVM.

		Oocytes		
Groups	Cultured n	Selected for IVM* n (%)	GV n (%)	GVBD n (%)
Control	39	19/39 (48.7) ^{A}	17/19 (89.5) ^{A}	2/19 (10.5) ^{A}
IGF-I 50	39	32/39 (82.0) ^{B}	12/32 (37.5)^{B}	20/32(62.5) ^{B}
IGF-I 100	39	32/39 (82.0) ^{B}	11/32 (34.4) ^{B}	21/32(65.6) ^{B}

GV, germinal vesicle; GVBD, germinal vesicle breakdown.

* Only oocytes ≥ 110 µm were selected for the *in vitro* maturation procedure.

^{A,B} Within a column, groups without a common superscript differed (P < 0.05).

4. Discussion

The present study documented, apparently for the first time, the IGFR-1 mRNA expression of preantral follicles during long-term *in vitro* culture in the presence or absence of FSH and the effects of IGF-I on follicular development and oocyte meiotic resumption after *in vitro* culture of isolated caprine preantral follicles. Based on the tendency for a higher IGFR-1 mRNA expression from preantral follicles cultured *in vitro* with FSH, we inferred that FSH is a potential modulator of IGF-I on *in vitro* culture of preantral follicles. Regarding the role of IGF-I on *in vitro* culture of preantral follicles, Zhao et al [19] demonstrated the presence of IGF-I and II, as well as IGF-I receptors in the oocyte and granulosa cells of ovine preantral follicles; this

finding along with our results support the participation of IGF-I as a local modulator of early follicular survival and development.

The beneficial effect of IGF-I, characterized by higher rates of normal follicles and antrum development of caprine preantral follicles cultured *in vitro* in the present study, has also been associated with maintenance of survival of caprine oocytes [7], swine preantral follicles [20], and small bovine antral follicles [21] cultured *in vitro*. Similarly, the addition of IGF-I to the culture medium promoted granulosa cell survival [22] and the maintenance of functional integrity of preantral follicles in rats [12]. In Experiment 2, the control group contained FSH, which could have interacted with its receptor and promoted intracellular signaling, such as stimulating the expression of IGF-I receptors, as showed by the PCR results in Experiment 1. This could have contributed to the maintenance of normal follicles and the greater antral cavity formation observed in follicles cultured in medium containing IGF-I. The effect of IGF-I on antrum formation may have occurred because of its action on steroidogenesis. The effects of IGF-I on steroidogenesis have been demonstrated in mouse preantral follicles cultured *in vitro* for 6, 10, and 12 d [23].

The presence of IGF-I in the medium promoted a higher daily follicular growth rate than in the control group. This finding could be associated with the effect of this factor on cellular proliferation. The synergic action of FSH and IGF-I to stimulate the growth of preantral follicles has been observed in several species [10-13]. In a study with prepubertal caprine ovaries, IGF-I at 100 mg/L promoted the growth of oocytes enclosed in preantral follicles after culture for 9 d [7]. Moreover, in swine, 50 ng/mL of IGF-I stimulated the proliferation of granulosa cells of preantral follicles cultured for 4 d in the presence of serum [20]. Furthermore, after the addition of 50 ng/mL of IGF-I during 6 d of culture, Thomas et al. [24] observed an increase in follicular diameter over the control group. However, IGF-I (in concentrations of 1, 10, and 100 ng/mL) did not stimulate the transition of primordial to primary follicles during the culture of bovine ovarian cortices [23]. These contrasting findings might have been due to the use of different species and follicular stages.

The number of oocytes selected for IVM was higher in medium supplemented with IGF-I compared to the control group. Two oocytes from the IGF-I 50 group that underwent IVM showed the extrusion of the first polar body with the chromosomes in metaphase II, which characterizes nuclear maturation. In addition, the follicles cultured with IGF-I had several layers

of cumulus cells and an accentuated expansion of these cells, which could be associated with controlling the process of oocyte maturation [25]. Although the rate of oocyte maturation reported herein (6.3%, 2/32; IGF-I 50 group) was less than desirable, this is still a valuable finding, since only a few studies in goats have achieved meiotically competent oocytes from *in vitro* cultured preantral follicles [6]. The mechanisms necessary for preantral follicles to develop to maturity are complex and unclear. However, the synergic role of some hormones and growth factors on the control of folliculogenesis are well known [26]. Therefore, supplementation of the maturation medium with these substances may increase the rate of meiotic resumption.

In conclusion, the high IGFR-I mRNA expression in medium supplemented with FSH during a long-term *in vitro* culture of preantral follicles may be associated with the positive effect of IGF-I, characterized here by improvement in the development of caprine preantral follicles and the production of grown oocytes able to resume meiosis.

Acknowledgments

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11 CAPÍTULO 6 (Fase V)

Produção in vitro de embrião caprino a partir de folículos pré-antrais cultivados em meio suplementado com hormônio do crescimento

(In vitro production of caprine embryo from preantral follicle cultured in media supplemented with growth hormone)

Periódico: *Theriogenology* 75: 182-188, 2011

Resumo

O objetivo deste estudo foi avaliar o efeito do hormônio do crecimento (GH) na sobrevivência, crescimento, maturação e fecundação de oócitos derivados de folículos pré-antrais de cabras cultivados *in vitro*. Folículos pré-antrais foram isolados do cortex ovariano de cabras e cultivados individualmente por 18 dias na ausência (controle) ou presença de GH bovino nas concentrações de 10 ou 50 ng/mL (GH10 e GH50, respectivamente). O desenvolvimento folicular foi avaliado com base na sobrevivência, formação da cavidade antral, aumento do diâmetro, presença de complexos cumulus-oócitos morfologicamente normais e maturação oocitária. Ao final do cultivo, os oócitos foram submetidos a maturação (MIV) e fecundação *in vitro* (FIV). A taxa de formação de antro após o dia 6 de cultivo foi superior em grupos tratados com GH quando comparado ao grupo controle (81,0, 92,7 e 47,6%, respectively, $P < 0.05$). Os percentuais de oócitos crescidos aceitáveis para a MIV foram superiores ($P < 0,05$) nos grupos tratados com GH (GH-10: 54,76%; GH-50: 48,78%) do que no controle (11,90%). Um maior percentual de oócitos no grupo GH50 retomaram a meiose, produziram mais oócitos e foi capaz de produzir um embrião após a FIV quando comparado ao controle (0,0%; $P < 0,05$). Em conclusão, o GH promoveu o crescimento e maturação *in vitro* de oócitos oriundos de folículos pré-antrais, sendo capaz de produzir um embrião. Esse foi o primeiro estudo a demonstrar que embrião caprino pode ser produzido por meio de fecundação *in vitro* de oócitos derivados do crescimento *in vitro* de folículos pré-antrais.

Palavras-chave: Folículos pré-antrais caprinos. GH. Embrião. Cultivo *in vitro*. Ovário.

In vitro production of caprine embryo from preantral follicle cultured in media supplemented with growth hormone

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Abstract

The objective was to evaluate the effects of growth hormone (GH) on the survival, growth, maturation and fertilization of oocytes derived from caprine preantral ovarian follicles cultured *in vitro*. Preantral follicles were isolated from the cortex of caprine ovaries and individually cultured for 18 d in the absence (control) or presence of bovine GH at concentrations of 10 or 50 ng/mL (GH10 and GH50, respectively). Follicle development was evaluated on the basis of survival, antral cavity formation, diameter increase, and the presence of healthy cumulus-oocyte complexes and mature oocytes. After culture, oocytes were subjected to *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). The rate of antrum formation after Day 6 of culture was higher in both GH10 and GH50 than in the control (81.0, 92.7, and 47.6%, respectively, $P < 0.05$). Percentages of grown oocytes that were acceptable for IVM were also higher ($P < 0.05$) in GH-treated groups than in the control (54.8, 48.8, and 11.9% for GH10, GH50, and Control). A higher percentage of oocytes in the GH50 treatment underwent meiotic resumption (50.0%), produced mature oocytes, and enabled the production of an embryo after IVF than in the control group (0.0%; $P < 0.05$). In conclusion, GH promoted *in vitro* growth and maturation of goat preantral follicle oocytes and enabled production of an embryo. Furthermore, this study was apparently the first to produce a caprine embryo by *in vitro* fertilization of oocytes derived from preantral follicles grown *in vitro*.

Keywords: Caprine preantral follicle; GH; Embryo; In vitro culture; Ovary

1. Introduction

Ovarian folliculogenesis is controlled by complex interactions among endocrine, paracrine, and autocrine factors [1]. Consequently, the outcome of follicular development is dependent on a fine balance between stimulatory and inhibitory factors within the ovary. Growth hormone (GH) is one factor involved in regulation of folliculogenesis, with potential applications for *in vitro* culture.

Based on *in vivo* studies, GH promoted development of bovine ovarian follicles [2] and increase peripheral concentrations of insulin and/or insulin-like growth factor-1 (IGF-1) in heifers [3]. Although the role of GH in the control of folliculogenesis has been well established, the effects of this hormone on the *in vitro* culture of isolated goat preantral follicles have not been reported. Thus, the objective of the present study was evaluate the effects of GH on the development of caprine oocytes (retrieved from preantral follicles) to a stage competent for *in vitro* production of embryos.

2. Material and Methods

2.1. Collection of ovaries, isolation, selection and culture of preantral follicles

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

Ovaries were collected at a local abbatoir from 16 adult (ages 1 to 3 years) cross-breed goats, for a total of four replicates (four animals/replicate). Immediately after death, ovaries were recovered, washed in 70% ethanol for 10 s, followed by two rinses in Minimum Essential Medium (MEM) supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, and 25 mM HEPES. The ovaries were transported (at 4°C) to the laboratory within 1 h after they were recovered [4].

In the laboratory, fat and connective tissue surrounding the ovaries were removed. Cortical slices (1 to 2 mm in thick) were cut with a surgical blade (under sterile conditions) and placed in a fragmentation medium consisting of HEPES-buffered MEM. Preantral follicles that were approximately at least 200 µm in diameter were visualized under a stereomicroscope (SMZ

645 Nikon, Tokyo, Japan) and manually dissected from strips of ovarian cortex using 26-gauge (26 G) needles. After isolation, follicles were transferred to 100 µL drops containing fresh culture medium under mineral oil to further evaluate follicular quality. Follicles with a visible oocyte that were surrounded by granulosa cells and had an intact basement membrane and no antral cavity, were selected for culture.

After selection, follicles were individually cultured in 100 µL drops of culture medium in Petri dishes (60 x 15 mm, Corning Incorporated, Corning, NY, USA). The basic culture medium consisted of α-MEM (pH 7.2-7.4) supplemented with 3 mg/mL bovine serum albumin (BSA), ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, 50 µg/mL ascorbic acid, and recombinant bovine FSH (rFSH, Nanocore, Campinas, São Paulo, Brazil) at appropriate growing concentrations (100 ng/mL until Day 6, 500 ng/mL until Day 12, and 1000 ng/mL until Day 18 of culture) under mineral oil. The caprine preantral follicles were allocated into three treatments: basic medium only (control) or supplemented with GH (Growth Hormone bovine from Bovine Pituitary Gland) at concentrations of 10 ng/mL (GH10) or 50 ng/mL (GH50). Incubation was carried out at 39°C, in 5% CO_2 in air for 18 d. Fresh media were prepared and incubated for 2 h prior to use. Every other day, 60 µL of medium were replenished in each drop, and, at Days 6 and 12 of culture, all the medium (100 µL) was replenished with fresh medium. The culture was replicated four times, and at least 41 follicles were used in each treatment. The concentrations of rFSH, ascorbic acid and GH were chosen based on previous studies performed in our laboratory (unpublished data).

Following culture, all healthy follicles were carefully and mechanically opened with 26 G needles under a stereomicroscope for oocyte retrieval. Only oocytes ≥ 110 µm, with homogeneous cytoplasm that were surrounded by at least one compact layer of cumulus cells, were selected for IVM [5]. The recovery rate was calculated by dividing the number of oocytes ≥ 110 µm by the number of viable follicles at Day 18 of culture, and multiplying this value by 100 (11.9, 54.8, and 48.8% in the control, GH10, and GH50 groups). Cumulus oocyte complexes (COCs) selected for use in this study were washed three times in IVM medium composed of TCM 199 supplemented with 1 µg/mL 17β-estradiol, 5 µg/mL LH, 0.5 µg/mL rFSH, 10 ng/mL EGF, 1 mg/mL BSA, 22 µg/mL pyruvate, 50 ng/mL IGF-I, and 100 µmol/L cysteamine. After washing, oocytes were placed in 100 µL drops of maturation medium under mineral oil and

incubated for 32 h at 39ºC with 5% CO_2 in the air. As a positive control (*in vivo*), cumulus-oocyte complexes derived from antral follicles (~ 2 mm) were aspirated from caprine ovaries, transported at 4ºC, and then placed in drops of 100 mL maturation medium (as used for *in vitro* cultured oocytes) for 32 h. Following maturation, all oocytes were labeled with Hoechst 33342 and their chromatin configuration was assessed as germinal vesicle (GV) or meiotic resumption. Meiotic resumption was defined when a GV was absent or the nucleus was in metaphase II (MII).

2.2. Assessment of follicle development and oocyte viability

Follicles were classified according to their morphology; those with morphological signs of degeneration, e.g. darkness of the oocyte and surrounding cumulus cells, or misshapen oocytes, were classified as degenerated. Every 6 d of culture, follicular diameter and antral cavity formation were evaluated only in healthy follicles. The follicular diameter was determined as the mean of two perpendicular measures of each follicle, using an ocular micrometer (100× magnification) inserted into a stereomicroscope (SMZ 645, Nikon, Tokyo, Japan). Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers.

For assessment of oocyte viability by fluorescence microscopy, oocytes were incubated in 100 µL droplets of Tissue Culture Medium 199 (TCM 199) containing 4 µM calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37°C for 15 min. Thereafter, oocytes were examined under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan) for evaluation of live/dead fluorescent staining. The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. Oocytes were considered alive if the cytoplasm was stained positively with calcein-AM (green) and if chromatin was not labeled with ethidium homodimer-1 (red).

2.3. In vitro fertilization of oocytes from preantral follicles

Considering that the GH50 was the only treatment that differed from control in the proportion of oocytes at GV, it was chosen for this step of study (*in vitro* fertilization trials). Fresh ovaries were harvested and transported as described previously and the preantral follicles were isolated (n=45) and grown in GH50 media, and then matured and fertilized *in vitro* as

described previously [6]. Following *in vitro* maturation (IVM), oocytes were washed three times with pre-equilibrated *in vitro* fertilization (IVF) medium, consisting of TCM 199 supplemented with 2% estrous sheep serum and 10 µg/mL heparin. The oocytes were then placed in 100 µL drops of IVF medium and covered with mineral oil. The dishes were incubated at 39°C in 5% CO_2 before insemination. Frozen semen from an Anglo-Nubian buck (Alta Genetics, Uberaba, Minas Gerais, Brazil) from one straw (0.25 mL, 50 x 10^6 sperm) was washed in TALP medium and centrifuged once at 700 g for 6 min. Motile sperm were selected using the swim-up technique in TALP medium. After a concentration adjustment to 200 x 10^6/mL, sperm were added to the 100 µL droplets of IVF medium containing the oocytes. Following 18 h of insemination, presumptive zygotes were washed and cultured in 100 µL droplets of embryo culture medium consisting of SOF supplemented with 10% fetal bovine serum and then covered with mineral oil. The dishes were placed in a 5% CO_2 incubator maintained at 39°C for 7 d. Partial (60 µL) media replacement was performed every 2 d.

2.4. Statistical analysis

Follicles were considered the experimental unit, as described [7]. Data regarding follicular survival, retrieval of grown oocytes for IVM, antrum formation, and meiotic resumption after *in vitro* culture were compared using Chi square test (or Fisher's Exact test for oocytes in MII, with expected values less than five units), with the results expressed as percentages. Data for follicular diameters were submitted to Kolmogorov-Smirnov and Bartlett tests to confirm normal distribution and homogeneity of variances, respectively; thereafter, ANOVA was done, and treatments were compared using Student-Newman-Keuls (SNK) test. Due to heterogeneity of variances, for comparisons between values recorded on different days of culture, Kruskal-Wallis non-parametric test was used (SAS, 1999) [8]. Results were expressed as mean ± SD and differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Effect of different concentrations of GH on follicular survival and antrum formation

Follicular survival and antral cavity formation after 0, 6, 12, and 18 days of culture are shown (Fig. 1).

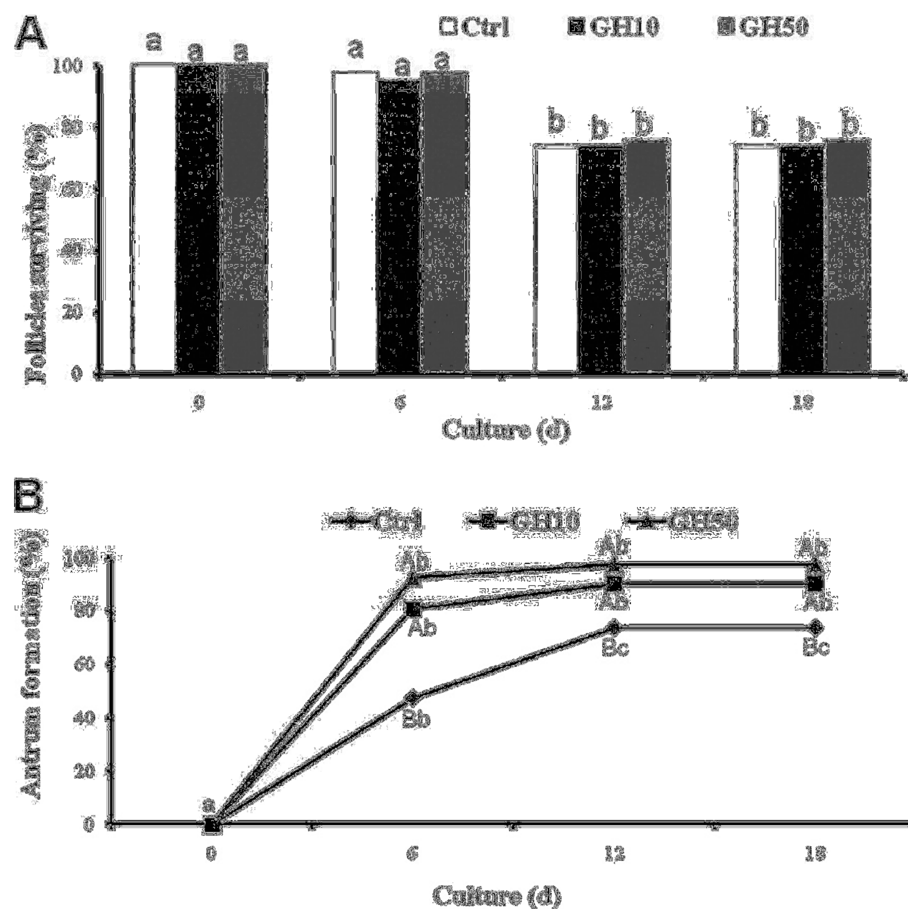

Figure 1. Caprine preantral follicles cultured for 18 d. A) Percentage of viable follicles; B) Antrum formation, after culture in medium supplemented with 0, 10, or 50 ng/mL of growth hormone (Control, GH10, and GH50, respectively).

[a-c]Within a treatment, days without a common superscript differ ($P < 0.05$).

[AB]Within a day, treatments without a common superscript differ ($P < 0.05$).

Survival rates were not significantly different from Days 0 to 6 of culture, decreased significantly from day 6 to 12, but did not change from Days 12 to 18 ($P < 0.05$). Furthermore, survival rates were no significantly different among groups. The rate of antrum formation increased ($P < 0.05$) in all treatments from Days 0 to 6 and in the control group only from Days 6 to 12, with no other significant increases thereafter (Fig. 1B). The rate of antrum formation was higher ($P < 0.05$) in GH-treated groups than in the control group after Day 6), with no significant difference between the two concentrations of GH. A viable follicle after 18 d of culture with GH50 is shown (Fig. 2A)

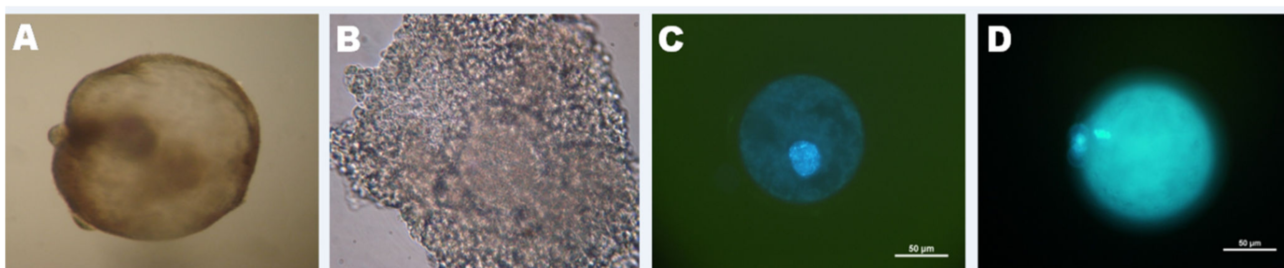

Figure 2. (A) Caprine antral follicle after 18 d of in vitro culture in presence of 50 ng/mL growth hormone (GH50; magnification, 50×). (B) Good quality cumulus oocyte complexes (COCs) after IVM of caprine follicles cultured *in vitro* in the presence of 50 ng/mL of GH. Grown oocyte from caprine preantral follicles cultured without (C) or in presence of GH50 (D). Chromatin configuration of intact germinal vesicle and metaphase II after Hoechst 33342 staining, respectively.

3.2. Follicular growth after in vitro culture

Follicular diameter increased significantly in all treatments between Days 0 and 6 and between Days 6 and 12 of culture; this increase was maintained ($P < 0.05$) until Day 18 (Table 1). Follicular diameter on Day 6 was greatest ($P < 0.05$) in GH50, followed by the GH10 and control groups (in that order). In addition, on Day 12, follicular diameter in both GH-treated groups was greater than in the control group, but they were not significantly different from each other. By the end of the culture (Day 18), there was no significant difference among groups in follicular diameter.

Table 1. Mean (± SD) diameter (µm) of caprine preantral follicles on various days of culture in media supplemented with 0, 10, or 50 ng/mL of growth hormone (Control, GH 10 and GH 50, respectively).

	n	Control	n	GH 10	n	GH 50	CV (%)
D0	42	217.44 ± 20.96 ^{Aa}	47	219.44 ± 16.29 ^{Aa}	43	220.01 ± 16.77 ^{Ac}	8.28
D6	41	379.54 ± 119.31 ^{Ab}	40	456.89 ± 128.17 ^{Bb}	40	545.15 ± 97.19 ^{Cb}	25.15
D12	31	582.62 ± 141.08 ^{Ac}	30	704.42 ± 149.33 ^{Bc}	31	695.52 ± 136.70 ^{Bc}	21.56
D18	26	669.15 ± 181.90 ^{Ac}	26	735.48 ± 178.61 ^{Ac}	27	726.00 ± 149.86 ^{Ac}	24.00

CV, coefficient of variation.

$^{A-C}$Within a row, means without a common superscript differ ($P < 0.05$).

$^{a-c}$Within a collumn, means without a common superscript differ ($P < 0.05$).

3.3. Assessment of in vitro grown oocytes viability and ability to undergo meiotic resumption

All oocytes examined for an evaluation of live/dead fluorescent staining were considered alive, since the cytoplasm stained positively with calcein-AM (green). The percentage of oocytes that were acceptable for IVM (diameter ≥110 µm) after 18 d in culture was significantly higher in the follicles that had been cultured with GH than in the control group (Table 2). At the end of the maturation period, some of the cumulus-oocyte complexes contained more cumulus cells (Fig. 2B). All grown oocytes in the control group had an intact germinal vesicle chromatin configuration (Fig. 2C) after the maturation period. Follicles from the GH50 group had a significantly lower percentage of oocytes in germinal vesicle (GV) compared to the control group ($P < 0.05$; Table 2). However, the two GH groups were not significantly different from each other. In the GH50, three oocytes (15%) completed nuclear maturation and were at metaphase II

(MII) (Fig. 2D). However, in the *in vivo* positive control, both the rate of oocytes resuming meiosis and the rate of oocytes at MII were higher than in the other groups ($P < 0.05$; Table 2).

Table 2. Meiotic stages of caprine oocytes, either derived *in vivo*, or from preantral follicles cultured *in vitro* for 32 h, supplemented with 0, 10, or 50 ng/mL of growth hormone (Control, GH 10, and GH 50, respectively).

Treatments	Number of follicles	Number of oocytes (%)			
	Cultured	Fully grown*	GV	Meiotic resumption	MII†
In vivo	-	37	-	37/37 (100.0) ^{A}	20/37 (54.05)^{A}
Control	42	5/42 (11.90) ^{A}	5/5 (100.00) ^{A}	-	-
GH 10	42	23/42 (54.76) ^{B}	14/23(60.87) ^{AB}	9/23(39.13) ^{B}	1/23 (4.34) ^{B}
GH 50	41	20/41 (48.78) ^{B}	10/20(50.00) ^{B}	10/20(50.0) ^{B}	3/20 (15.00) ^{B}

GV, Germinal Vesicle; MII, Metaphase II.

* Only oocytes (≥110 µm) were selected for the *in vitro* maturation procedure.

†The percentage of oocytes in MII was included in meiotic resumption.

^{A,B} Within a column, means without a common superscript differ ($P < 0.05$).

3.4. Embryo production using oocytes from in vitro grown preantral follicles

Since GH50 was the only treatment that differed from control in the proportion of oocytes at GV, it was chosen for this step of study (*in vitro* fertilization trials). Of the 45 follicles cultured, 35 produced oocytes with the minimum diameter (110 µm) to be allocated to IVM and five degenerated. Thus, 35 oocytes were used for IVF. After IVF of oocytes derived from follicles cultured with GH50, the rate of cleavage was evaluated approximately 48 h after IVF by the presence of a 2-cell embryo. The embryo evaluated reached the compact morula stage after 7 d in culture (Fig. 3).

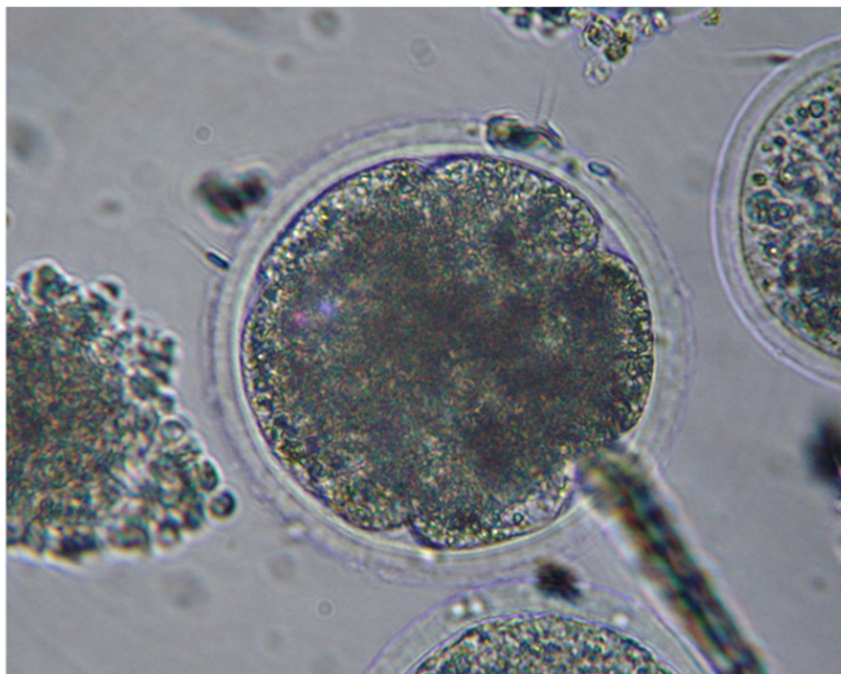

Figure 3. Morula produced from oocytes recovered from an *in vitro*-grown caprine preantral follicle.

4. Discussion

This was apparently the report of the production of a caprine embryo from preantral follicles grown *in vitro*. Although rat, pig and buffalo embryos have been produced using *in vitro* grown preantral follicles [9-11], production of viable offspring derived from preantral follicles has only been reported for mice [12]. In other domestic species, such as sheep [13,14], cattle [15] and goats [16], development of the antrum has been achieved by culturing large secondary follicles. In the current study, adding GH to *in vitro* cultures of caprine preantral follicles yielded oocytes capable of maturation, which enabled production of a morula. Notwithstanding the production of only a single live offspring, these results provided proof of concept for future large-scale production of caprine embryos derived from preantral follicles grown and matured *in vitro*.

In the present study, there were high rates of follicular survival at the end of culture (Day 18), regardless of whether GH was added to the media. It was noteworthy that α-MEM, a medium with a rich formulation, was used. Furthermore, it was supplemented with glutamine, hypoxanthine, BSA, ITS, ascorbic acid and FSH, which are considered essential for the survival

of caprine preantral follicles [17-19]. Although survival rates were not significantly affected by the addition of GH, antrum development was significantly higher when the medium was supplemented with this hormone. Therefore, we inferred that GH acted as a regulator of follicular development during the initial antral phase. In that regard, GH can affect cell fate both directly by binding to receptors on ovary cells, as well as indirectly by stimulating ovarian expression of IGF-1 [20]. It was reported that bovine GH increased the population of antral follicles *in vivo* in mature heifers [2]. Moreover, in *in vitro* studies, GH augmented FSH-stimulated formation of LH receptor, progesterone synthesis and cAMP production in both porcine and rat granulosa cells [21]. Maturation of denuded oocytes in the presence of GH caused retardation in the process of GV breakdown, perhaps due to an effect of the hormone on denuded oocytes, similar to the effect of dibutyryl-cAMP [21], suggesting signal transduction of GH via the cAMP pathway.

The role of GH in follicular growth may be associated with its reported effects on cellular proliferation and steroidogenesis, considering that steroid hormones other than progesterone and estradiol secreted from COCs are essential for meiotic maturation and cumulus expansion [22]. Addition of GH to *in vitro* cultures of murine preantral follicles stimulated stimulated production of estradiol, secretion of inhibin and proliferation of granulosa and theca cells [23]. Furthermore, GH promoted an increase in the diameter of mice preantral follicles cultured *in vitro* [24, 25]. In the cattle, GH with insulin increased progesterone synthesis and proliferation of granulosa cells after culture for 4 d [26].

In our study, *in vitro* cultures of goat preantral follicles in a medium supplemented with GH yielded more ($P < 0.05$) oocytes suitable for IVM than did the control group. Furthermore, after IVM, oocytes cultured with GH50 had a lower percentage of oocytes at the germinal vesicle stage. This treatment was capable of promoting nuclear maturation (defined as achievement of the MII phase) of caprine oocytes from *in vitro* grown preantral follicles that were subsequently able to produce a morula stage embryo. Although the rate of meiotic resumption reported in the present study was suboptimal, to our knowledge, no other studies with goats have been able to generate meiotically competent oocytes for embryo production from the *in vitro* culture of preantral follicles. The mechanisms involved in preantral follicle maturation are complex and not well understood. Some hormones and growth factors can act synergistically to control folliculogenesis [27]. In this study, the addition of GH to the culture generated oocytes capable of

completing nuclear maturation. We inferred that GH interacted synergistically with the other factors in the basal medium (e.g. insulin and FSH) to promote oocyte maturation.

In conclusion, GH played an important role in the *in vitro* growth and maturation of oocytes that are derived from caprine preantral follicles and enabled the production of an embryo. This is apparently the first report of a caprine embryo generated *in vitro* from preantral follicles. Further studies are needed to address low maturation rates, which may increase the efficiency of this promising system and consequently increase the success rate of embryo production.

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Conflict of interest

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12 CAPÍTULO 7 (Fase VI)

Expressão gênica durante a foliculogênese inicial em cabras usando a análise de microarranjo

(Gene expression during early folliculogenesis in goats using microarray analysis)

Periódico: Aceito na *Biology of Reproduction*

Resumo

A compreensão acerca da expressão gênica e das vias metabólica, biológica e fisiológica no desenvolvimento folicular ovariano pode ter um significante impacto na dinâmica de sobrevivência ou atresia folicular. De fato, uma grande perda oocitária ocorre durante a transição de folículos secundários para terciários. O objetivo desse experimento foi estudar, por meio da técnica de microarranjo de DNA, as mudanças no perfil transcricional de folículos secundários e antrais iniciais (terciários) em ovários caprinos. Folículos ovarianos (secundários e terciários) foram isolados por microdissecção e armazenados em pool para extração de RNA. O RNA teve hibridização cruzada com chip bovino. Dentre os 23.987 genes bovino, um total de 14.323 foram hibridizados com RNAm caprino enquanto 9,664 não foram hibridizados ou não foram expressos. Dos genes hibridizados, 2.466 foram estádio-específico, up- e down-regulados na transição de folículos secundários para terciários. O perfil de expressão gênica mostrou que três vias de sinalização (metabolismo lipídico, morte celular e sistema hematológico) foram significativamente diferenciadas entre os dois estádios foliculares. A análise de PCR quantitativa em tempo real (PCRq) foi utilizada para confirmar os resultados de microarranjo de DNA para alguns genes. SLIT3, GTPBP1, AKR1C4 e PIK3R6 foram significativamente diferentes entre os estádios foliculares e, entre os 10 genes testados na PCRq, apenas o ABLIM não foi expresso em folículos secundários assim como em terciários. Em conclusão, esse estudo identificou importantes genes e vias de sinalização envolvidos na transição de folículos secundários para terciários em caprinos.

Palavras-chave: Bovine genome array. Folliculogenesis. Gene expression profiling. Goat. Ovarian follicle development.

Gene Expression during Early Folliculogenesis in Goats Using Microarray Analysis[1]

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Running head: Gene expression during folliculogenesis.
Summary: Gene expression profile differs between secondary and tertiary ovarian follicles and microarray analysis is an important tool to identify genes and pathways involved.

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ABSTRACT

Understanding of gene expression and metabolic, biological and physiological pathways in ovarian follicular development can have a significant impact on the dynamics of follicular atresia or survival. In fact, the majority of oocyte loss occurs during the transition from secondary to tertiary follicles. This study aimed understand, by microarray analysis, the temporal changes in transcriptional profiles of secondary and early antral (tertiary) follicles in caprine ovaries. Ovarian follicles were microdissected and pooled to extract total RNA. The RNA was cross hybridized with the bovine array. Among 23,987 bovine genes, a total of 14,323 genes were hybridized with goat mRNAs while 9,664 genes were not. Of all the hybridized genes, 2,466 were stage-specific, up- and down-regulated in the transition from secondary to tertiary follicles. Gene expression profiles showed that three major metabolic pathways (lipid metabolism, cell death and hematological system) were significantly differentiated between the two follicle stages. Quantitative real-time PCR was used to confirm microarray findings for the expression of several genes. SLIT3, GTPBP1, AKR1C4 and PIK3R6 were significantly different between follicle stages and among the 10 genes tested only ABLIM was not expressed in both stages. In conclusion, this study has identified important genes and pathways involved in the transition from secondary to tertiary follicles in goats.

Keywords: *bovine genome array, folliculogenesis, gene expression profiling, goat, ovarian follicle development*

INTRODUCTION

The ultimate goal of in vitro follicle culture is to successfully produce offspring from oocytes grown, matured and fertilized in vitro. However, this goal has been achieved only in mice until recently [1]. In large animals as pig, goat, ewe and buffalo, the best results reported so far were the production of a few and variable number of embryos from in vitro culture of secondary follicles [2-5]. The development of an efficient *in vitro* culture system for large animals requires the knowledge of many factors involved in the regulation of folliculogenesis. Our research group has been evaluating the effect of several substances on the in vitro development of preantral follicles in goats [6-8], which are an important farm animals for many countries [9]. The in vitro culture system should be able to mimic the physiological environment (e.g. hormonal concentrations) when a few follicles in the ovary escape from atresia and continue growth and differentiation. Thus, it is critical to understand the gene expression that renders the right physiological environment during folliculogenesis.

Recent progress has been geared to find the mechanisms controlling early folliculogenesis in goats. Quantitative Polymerase Chain Reaction (qPCR) has been used to study ovarian mRNA expression of hormone receptor genes such as FSHR, LHR and GHR [10] and growth factor genes as IGF-I [6] and GDF-9 [11]. Although qPCR analysis is able to provide information regarding few genes, it cannot provide an overview of all possible genes up- and down-regulated in the developmental changes of folliculogenesis. Thus, such genomic tools as DNA microarrays and genome sequence are necessary in goat studies. Recently the goat genome has been sequenced and assembled but the data are not available (http://www.ncbi.nlm.nih.gov/projects/genome/guide/goat/). It needs yet to be fully annotated and released by the International Goat Genome Consortium to be useful for the research community.

Microarrays have been used to determine gene expression profiling in model eukaryote organisms since 1997 [12]. In the studies of follicular and oocyte development, microarray analysis simultaneously present expression of thousands of genes and elucidate the complex relationship among the numerous interactors. For example, microarray analysis has been used to observe over 11 generations in swine on the dynamic changes between follicular growth and oocyte maturation of genes involved in the synthesis of steroids, ovarian tissue remodeling and

apoptosis in swine selected for their high ovulation rate and embryo survival [13]. A recent study in mouse and human ovaries demonstrated that gonadotropins regulated natriuretic peptide precursor type C (*NPPC*) / C-type natriuretic peptide (*CNP*) expression, and confirmed the role of CNP as a potent paracrine oocyte maturation inhibitor [14]. In a study with the Nobox gene (expressed in oocytes and essential to the oogenesis process) in newborn knockout mice, there was increased expression of genes related to male sex determination, demonstrating that the oocyte suppressed the expression of these genes [15]. In addition, the glial cell line-derived neurotropic factor (GDNF) is predominantly produced by germ cells (oocytes) rather than somatic cells and promoted the development of primordial follicles in rat and mediated paracrine and autocrine interactions of follicular cells during early folliculogenesis [16]. In rats, the microarray analysis has demonstrated that the expression of several growth factors changed during primordial follicle development, including vascular endothelial growth factor and insulin-like growth factor II [17].

The unavailability of a goat genome sequence and genome arrays may have stalled the research progress in early goat folliculogenesis. However, cross-species microarray hybridization using available genome tools of a close related model organism, e.g. bovine genome arrays, has enabled new findings in goats. The success of applying bovine genome arrays to goats [18, 19] provides a new alternative to study gene expression in a whole genome scale. Currently there is no gene expression study during the development of ovarian follicles in goats. The objective of this study was to fill this void, evaluating, by microarray analysis, the temporal changes of transcriptional profile in secondary and early antral (tertiary) ovarian follicles.

MATERIALS AND METHODS

Collection of Ovaries and Follicle Harvest

Ovaries of adult (ages 1–3 yr) non-pregnant crossbred goats (n = 30) were obtained from a local slaughterhouse. Immediately *postmortem*, the pair of ovaries was washed in 70% ethanol for 10 s followed by several rinses in α–Minimum Essential Medium (α–MEM) supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin and 25 mM HEPES. The ovaries were transported to the laboratory at 4°C within 1 h [20].

Before follicle isolation, fat tissue and ligaments surrounding the ovaries were stripped off using 26-ga needles. Cortical slices (1–2 mm in thickness) were cut off from the ovarian surface using a surgical blade under sterile conditions in a medium consisting of HEPES-buffered α–MEM. Secondary (100–350 μm) and tertiary (350–600 μm) follicles were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of ovarian cortex using 26-ga needles. A total of 300 secondary and 300 early tertiary follicles were individually isolated (Fig. 1B, C; respectively). Follicles were stored in Eppendorf tubes and kept frozen at –80°C until RNA extraction. Follicles in Eppendorf tubes were not pooled until RNA extraction to prevent RNA degradation.

Goat Follicle RNA Extraction

Secondary and tertiary follicles were pooled to have approximately 100 follicles per follicular stage per extraction, in triplicate. Total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Maryland, USA) for animal tissue. The elutes of total follicle RNA was treated with 1 μl DNase (Fisher Scientific, 1u/μl) for 10 min at 37°C to remove genomic DNA and 2 min at 65°C to terminate DNase reaction. Quality of the extracted RNA was checked by gel electrophoresis while quantity by ND 1000 (NanoDrop 1000 spectrophotometer). The entire total mRNA was intact with high quality, i.e. optical density (O.D.) 260/280 and 260/230 ratios were between 1.8 to 2.0 and 1.8 to ≥1.8, respectively. The elutes were aliquoted into 40 μl to be used to hybridize with bovine genome array (microarray analysis) and 5 μl for qPCR. The RNA samples were shipped out on dry ice for microarray service at the Genomics Facility of the Kansas State University (http://www.ksre.ksu.edu/igenomics/).

Affymetrix Bovine Genome Array Hybridization

RNA quality was assessed using Agilent 2100 Bioanalyzer, prior to hybridization. First strand cDNA was synthesized, followed by second strand cDNA synthesis. cDNA was used in in vitro transcription reaction (IVT) to generate biotinylated cRNA. The quality of the cRNA was assessed prior to its fragmentation. After fragmented, the cRNA was evaluated and hybridized

with the Affymetrix GeneChip Bovine Genome Array (#900562) using the Affymetrix GeneChip System. This system can study gene expression of approximately 24,000 bovine transcripts, based on the International Goat Genome Consortium. Raw data of goat samples were normalized by MAS 5.0 software [21] at the Genomics Facility of the Kansas State University and normalized array data were provided (Supplemental Table SI).

Bioinformatics and Microarray Statistics

Three replicates of the normalized array data from secondary and tertiary follicles were first averaged with standard deviation and standard errors. The replicate data were tested with Students' *t*-test to find the *P*-values for two developmental stages per Affymetrix gene ID (Supplemental Table SII, column F). A *P*-value <0.01 was deemed significant. Then an *M*-value ($\log_2 S/T$; *S*, secondary follicle average; *T*, tertiary follicle average) was calculated (Supplemental Table SII, column G).

Since this was cross-species hybridization, presence or absence of signal for each Affymetrix gene ID was important and recorded, depending on the *P*-value for each gene expression signal from a *t*-test against background noise. When *P*-value <0.05, the signal was present, recorded as P. In cases of *P*-value >0.1, the signal was defined absent and recorded as A. If the *P*-value was between 0.05 and 0.1, the signal was considered marginal and recorded as M (Supplemental Table SII, columns I and J). Gene ontology (GO) terms were downloaded from Ensembl (www.ensembl.org/, release 64 in September 2011). If there was no GO term present in Ensembl, the source sequence of an Affymetrix gene ID (i.e. cDNA or expressed sequence tag - EST) was blasted with blastn, i.e. "nucleotide blast" [22] in NCBI (www.ncbi.nlm.nih.gov/) to search for gene symbol and gene name. As a third source to get GO terms, gene symbols and gene descriptions from the Ingenuity Pathway Analysis were used (see below).

Pathway Analysis

The genes from both secondary and tertiary follicles with *M*-value and *P*-value from *t*-test of the three replicates per follicle stage, between two follicle stages were uploaded to the new core analysis package of Ingenuity Pathway Analysis (IPA) V7.5 software server

(www.ingenuity.com) in order to analyze and map into most significantly differentiated pathways and functional groups. IPA can automatically find potentially connected interactions from its own database with uploaded gene IDs. Then a P-value between significantly regulated (either up- or down-regulated) genes and neutral genes were calculated and assigned to each allocated pathway network. The filter mechanisms in the core analysis were (1) P-value <0.01, (2) M-value >0.05, (3) two chosen data from both experimental data and high confidence predicted data (from IPA databases), and (4) Affymetrix bovine genome chip identifiers.

Cluster Analysis

Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used for genome-wide expression analysis [23] and the results were visualized and displayed by Java TreeView (http://jtreeview.sourceforge.net/) [24]. Six sets of raw data (three from secondary follicles and other three from tertiary follicles; Supplemental Table SI) were uploaded to Cluster 3.0. The filter parameter for genes was 80% cutoff. Hierarchical method was used to cluster both genes and arrays; centered correlation was applied and average linkage was employed. Three heat maps were selected based on their genes being expressed significantly more ($P < 0.001$) in one stage than the other and the importance in folliculogenesis.

Gene Ontology Analysis

AgriGO (http://bioinfo.cau.edu.cn/agriGO/) was used for gene ontology analysis [25] of all the expressed genes from secondary and tertiary follicle stages. Singular Enrichment Analysis (SEA) in AgriGO for *Bos Taurus* species and Bovine Affymetrix Genome Array were used as reference to perform the analysis. The results were generated and displayed (Supplemental Table SII). GO terms are represented as boxes containing detailed description, organized and connected based on their relationship. Further information such as gene description and protein domain annotation was also available for all the expressed genes in AgriGO (Supplemental Table SIII-SX). In addition, font and rank direction of the tree were customized. Elaborate graphical output was generated to facilitate us to explore molecular function in an intuitive way (Fig. 9).

Quantitative PCR of selected genes

To confirm the results in the detection threshold of the microarray analysis, the same pools of follicle mRNA used in the microarray hybridizations were tested and evaluated with qPCR. Although Affymetrix microarray data are reproducible and need no qPCR confirmation as previously suggested [26], ten genes were chosen for qPCR confirmation. The primers for those ten genes (Table 1) were designed by Gene Runner software version 3.05 (Hastings Software, Inc.), and ordered from Eurofins MWG Operon Huntsville, AL (http://www.eurofinsdna.com/).

The mRNA was reversed transcribed into first-strand cDNA using iScriptcDNA synthesis kit (Bio-Rad, Hercules, California, USA) with random primers. The relative expression of mRNA was determined by qPCR. Aliquots of 2 µl of cDNA as a template were used in 12.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µl of ultra-pure water and 2 µM of each primer. Ubiquitin was used as endogenous controls for normalization of steady-state levels of mRNA of tested genes. The amplifications were carried out by one initial denaturation and activation of the polymerase for 10 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 60 or 58°C (according to each primer), 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 (Bio-Rad, Hercules, California, USA). The delta-delta-CT method [27] was used to transform CT values into normalized relative steady-state levels of mRNA. The statistical analysis was performed by *t*-test and differences were considered to be significant when $P < 0.05$.

Table 1. Oligonucleotide primers used for PCR analysis of goat cells (secondary and tertiary follicles).

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
ABLIM1	CAAGGTCAAGGCGATTTAC	S	1026-1104	GI:XM_002698530.1
	GTCATCATAGCCCGAAGAGTAG	As		
SLIT3	ACTATTTGATGGGCTGGTGTC	S	975-1024	GI:NM_001191450.1
	TGTTGGCATTGAGGAGGAG	As		
TYMS	TTTGGAGGAGTTGCTGTGG	S	756-780	GI:NM_001037816.1
	GTTCCTTAGCGTTGGTGG	As		
ARHGEF12	GCTCCTCCAAGAAGACAAAG	S	261-310	GI:NM_001123033.1
	ACACAACGCTGAACAAGAC	As		
CLEC6A	AACACAGAAACAGAGCAGG	S	146-218	GI:NM_001034479.1
	AGTTGCCATTCCCTTGTG	As		
AKR1C4	TTGCCTATGGTGCTCTGGG	S	241-334	GI:NM_181027.2
	TAGCGAAGGGCAACCAGAG	As		
CYTL1	AGAGATTTGGTGTTCCTGAC	S	641-776	GI:XM_002688425.1
	TGGGTATTCTAACGCATTG	As		
PIK3R6	TGGAGAAGGCAGAAAGTG	S	182-231	GI:NM_001102028.1
	CAGCAAAGGAATGATGATG	As		
MAOB	CCATACAAAGGGCAAATC	S	270-319	GI:NM_177944.1
	ACACAGACGGGAATGAGC	As		
GTPBP1	AGATTGAGTCGGGTCGCAC	S	605-654	GI:BC151310.1
	AAAGCCCAGAATGTCGTTG	As		
UBQ	GAAGATGGCCGCACTCTTCTGAT	S	607-631	GI:57163956
	ATCCTGGATCTTGGCCTTCACGTT	As		

S, sense; AS, antisense

RESULTS

Cross-species Hybridization between Bovine Genome Chips and Goat Follicle RNA of Two Developmental Stages

Similarly as used for mammary tissue in previous studies by Ollier et al. [19, 28], Affymetrix bovine genome arrays could be applied to secondary and tertiary goat ovarian follicles (Supplemental Table SI for the normalized data for both follicle stages with three replicates each). The transition from secondary (Fig. 1B) to tertiary (Fig. 1C) follicles was evidenced by gene expression and morphological changes such as the antrum formation. The majority of expressed genes were plotted within a linear model with few outliers (Fig. 1A) indicating unique expression differences in the development of the two follicle stages.

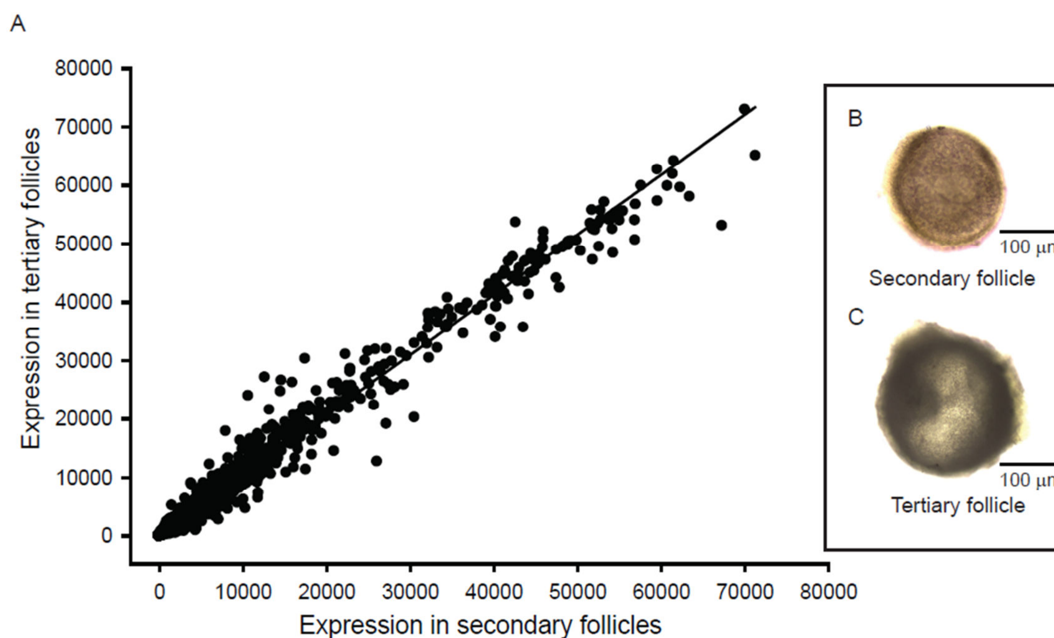

Figure 1. Expression and morphology of secondary and tertiary follicles. (A) Array data profiled based on gene expression. The overall expression of two developmental stages was similar, plotted in a linear model, but with unique outliers. These outliers were subjected to further analyses. Morphology of (B) secondary and (C) tertiary follicles. Scale bars = 100 µm.

Expression Profiling

Three lines of refinements were applied in the expression profiling to prevent false positives and false negatives. First, based on *t*-test analyses for each gene expression, 516 expressed genes were different ($P < 0.01$) between secondary vs. tertiary follicles. Second, *M*-values were presented as log2 ratio of secondary follicle divided by tertiary follicle expression to highlight up- and down-regulated genes (Supplemental Table SII). Third, signals between the two developmental stages and among three replicates, i.e. absence (A), presence (P) and marginal (M), were recorded for comparison (Supplemental Table SII, columns I and J). Only the expressed genes with at least two same letter indicators, i.e. AA, AAA, PP or PPP, were considered along with their *P*- and *M*-values.

Among 23,987 bovine Affymetrix gene IDs, a total of 14,323 genes were hybridized with goat RNAs and expressed while 9,664 genes were not. Therefore, approximately 60% bovine Affymetrix gene IDs were expressed in these two developmental follicle stages (secondary and tertiary) in goats (Fig. 2).

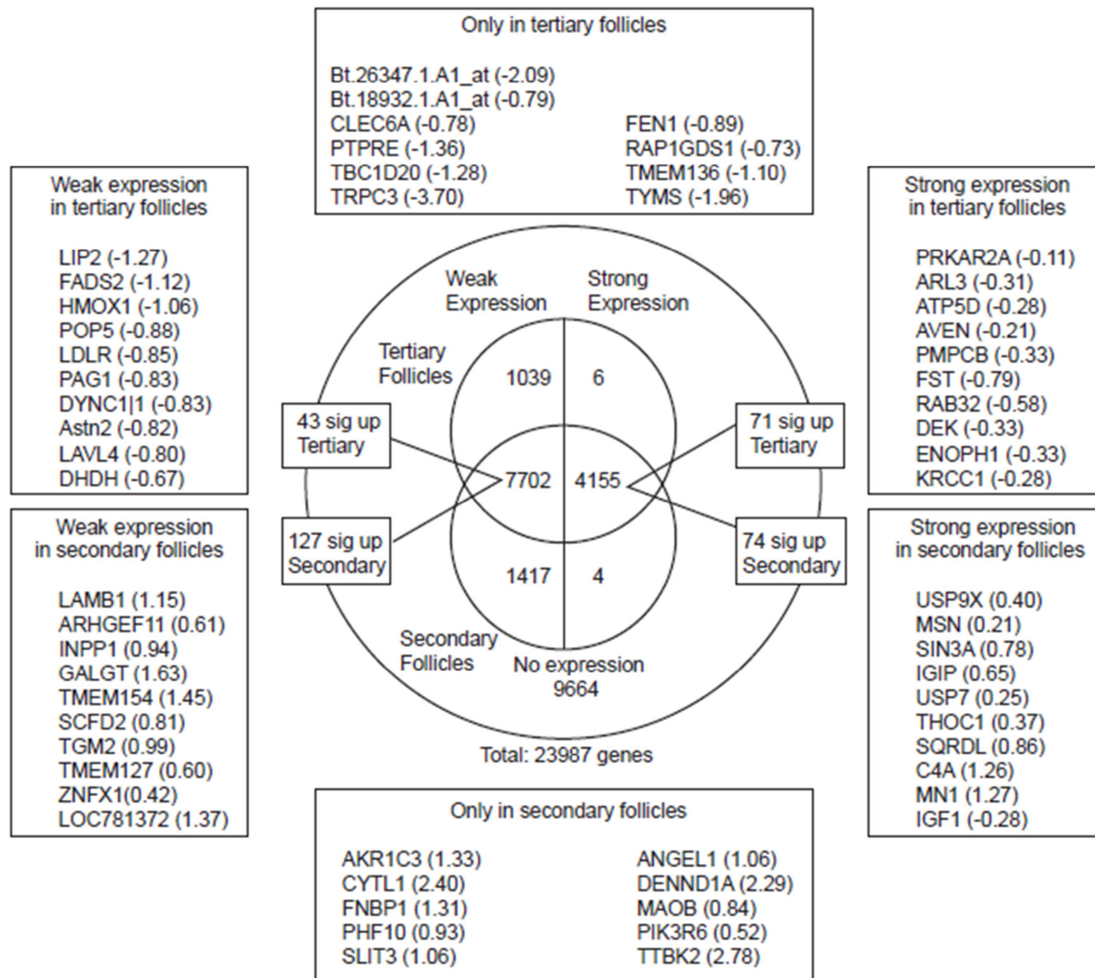

Figure 2. Overview of differential gene expression between secondary and tertiary ovarian follicles in a Venn diagram. Expressed genes were grouped into two categories weakly (left half) and strongly (right half) expressed. Strong expression means the expression level above the genome average and weak expression below the genome average. Top ten representative genes from six categories were listed in boxes with their M-value. M-value was Log2 ratio of secondary follicle/tertiary follicle expression.

These two stages shared approximately 82.3% of similarity in their expression profiling (i.e. 7702 + 4155 = 11857; 11857/14323 = 82.3%). The remaining 2,466 genes (i.e. 1039 + 1417 + 4 + 6 = 2466) were stage-specific. In order to search for hidden differentiation or similarity, expression pattern was allotted into two categories – strong or weak. The average of genome

expression was 800 normalized fluorescence units (based on all the genes expressed in all the six arrays); thus strong expression was referred to the expression level above the average genome expression, while weak expression was referred to below the average. Although the expressed genes shared by the two follicle stages were up to 59.7%, a suite of genes could be found up- and down-regulated in the transition from secondary to tertiary follicle. The top ten genes in both follicle stages in three categories are listed (Fig. 2). Among this suite of genes, some are related to lipid metabolism, signaling and transcription factors and the others related to oncogenes and cell death/apoptosis inhibition (Supplemental Table SIII-SV).

Genome-wide Coexpression Analysis

All the 14,323 expressed genes were clustered based on coexpression. A suite of genes were identified to be coexpressed with the four currently known genes – *follistatin*, *NDUFS1* (NAD dehydrogenase), *IGF1* and *DEPDC7* (DEP domain containing 7) – involved in secondary and tertiary follicle development (Fig. 3). Follistatin is an autocrine glycoprotein that is expressed in nearly all tissues of higher animals and its primary function is the binding and bioneutralization of members of the TGF-β superfamily, with a particular focus on activin [29, 30]. NDUFS1 is a NADH dehydrogenase (ubiquinone) Fe-S protein 1 and an important member of the mitochondrial inner membrane Complex I in the electron transport chain [31] (Fig. 3A). A protein of similar function, *NDUFS4*, and *MRPL43* (mitochondrial ribosomal protein L43) also were coexpressed with *NDUFS1*. Insulin-like growth factor I (IGF-I; Fig. 3B), also called Somatomedin C, is a peptide growth factor that has a high degree of structural homology with proinsulin. This factor has been proved regulating ovarian follicular growth and atresia [32, 33]. DEP domain containing protein 7 (DEPDC7) is a globular protein domain of about 80 amino acids that is found in over 50 proteins involved in G-protein signaling pathways. DEPDC7 is involved in intracellular signal transduction; when mutated, the Wnt (wingless) signaling pathway, network of proteins best known for their roles in embryogenesis, cancer and normal physiological processes in adult animals, is disrupted [34] (Fig. 3C). Interesting, in the same cluster as IGF1, *RSPO3* (R-spondin 3) activates Wnt signaling in *Xenopus* myogenesis [35] (Fig. 3B).

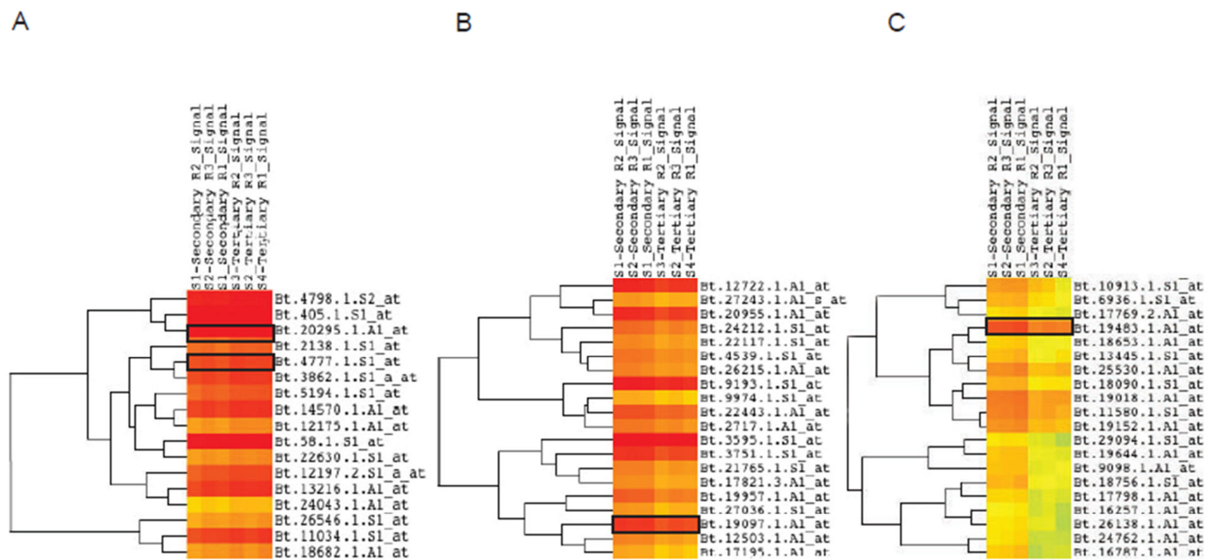

Figure 3. Hierarchical clustering analysis of the coexpressed genes. Four genes were selected for their importance in the development of secondary and tertiary follicles: A) follistatin e NAD dehydrogenase; B) IGF-I and C) DEP domain containing 7. Each line represents a single gene and the connectivity between genes is based on distance. The relative expression of individual gene is indicated as increased (red) or decreased (yellow).

Different Metabolic Pathways in the Transition from Secondary to Tertiary Follicles

Among all the expressed genes, a total of 8,165 genes were found to have known pathways, but 6,478 genes had no assigned pathways. Among known pathways, five major metabolic pathways were found significantly differentiated between secondary and tertiary follicles. They were (1) lipid metabolism, small molecule biochemistry, cell death (Fig. 4); (2) cellular movement, inflammatory response, hematological system development and function (Fig. 5); (3) cardiovascular system development and function, cell death, skeletal and muscular system development and function (Fig. 6); (4) molecular transport, cancer, cell death (Fig. 7); (5) lipid metabolism, small molecule biochemistry, molecular transport (Fig. 8). The identification of the genes related to pathways 1-5 has been supplied (Supplemental Table SIII-SV, respectively). The predominant gene expressions related with the pathways were for lipid metabolism, cell death and hematological system.

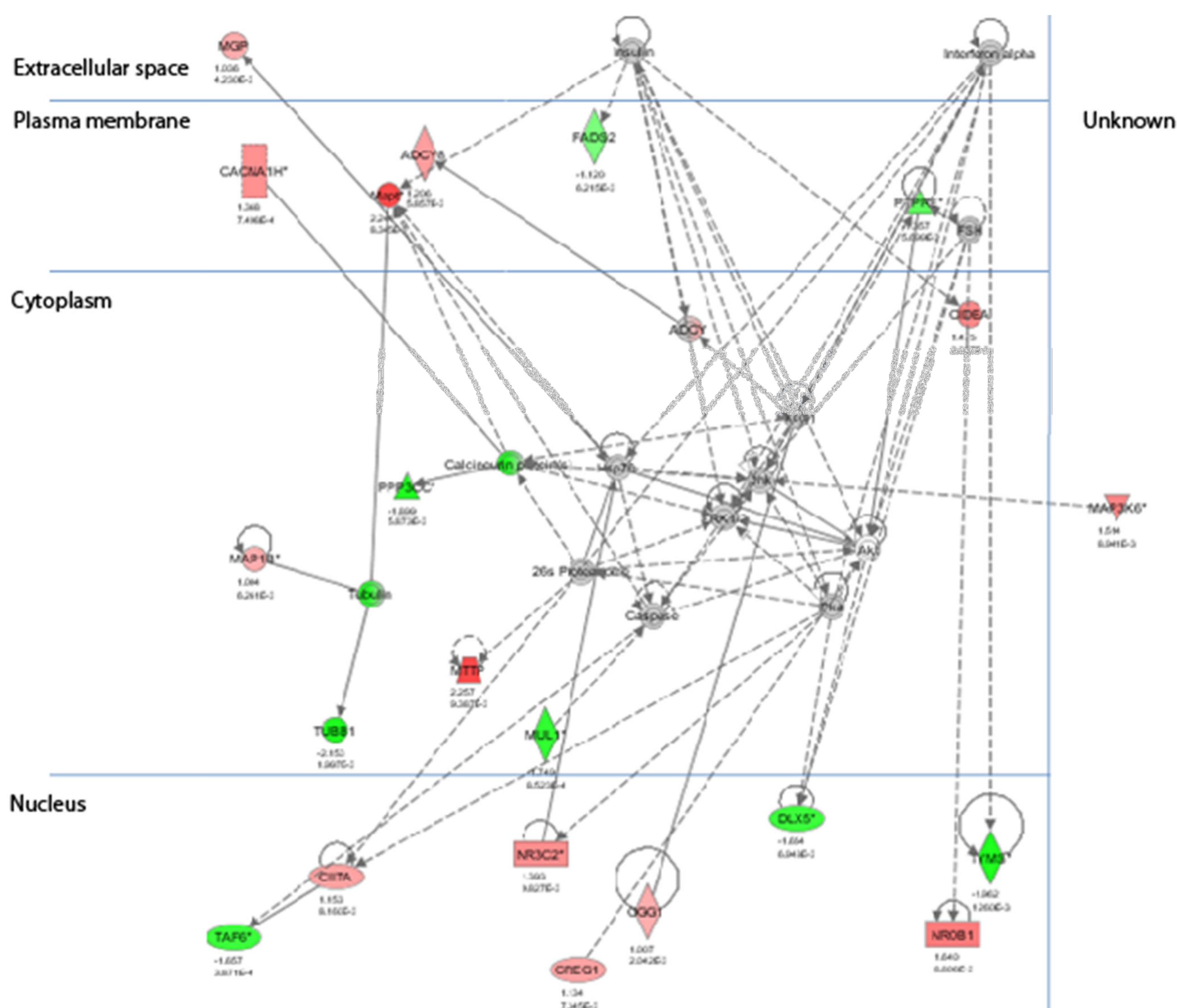

Figure 4. Pathway interaction network analyses based on Ingenuity Pathway Analysis (IPA; www.ingenuity.com). Lipid metabolism, small molecule biochemistry, and cell death pathways. Each network was displayed with nodes (i.e. genes/gene products) and edges (i.e. the biological interacting relationship between nodes). Solid lines indicate direct interactions between nodes while dash lines indirect relationship. The color intensity of the nodes refers to fold-changes of increase (red) or decrease (green). The color grey means neither up-regulated nor down-regulated in the nodes and the color white refers to the nodes which are user-specific but are added into the networks due to other associated nodes. The shapes (for path designer and network) of the nodes refer to different functions defined by IPA.

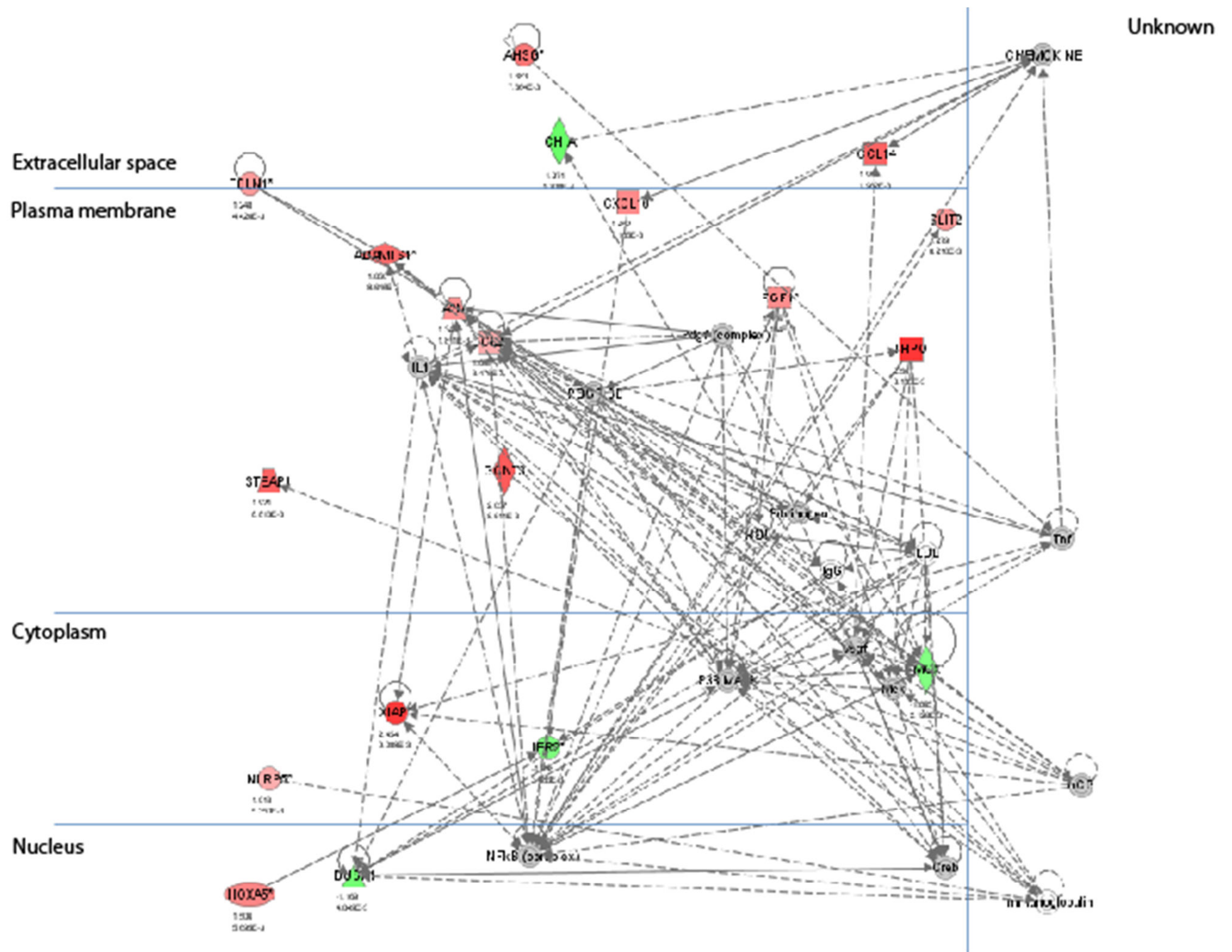

Figure 5. Cellular movement, inflammatory response, hematological system development and function pathways.

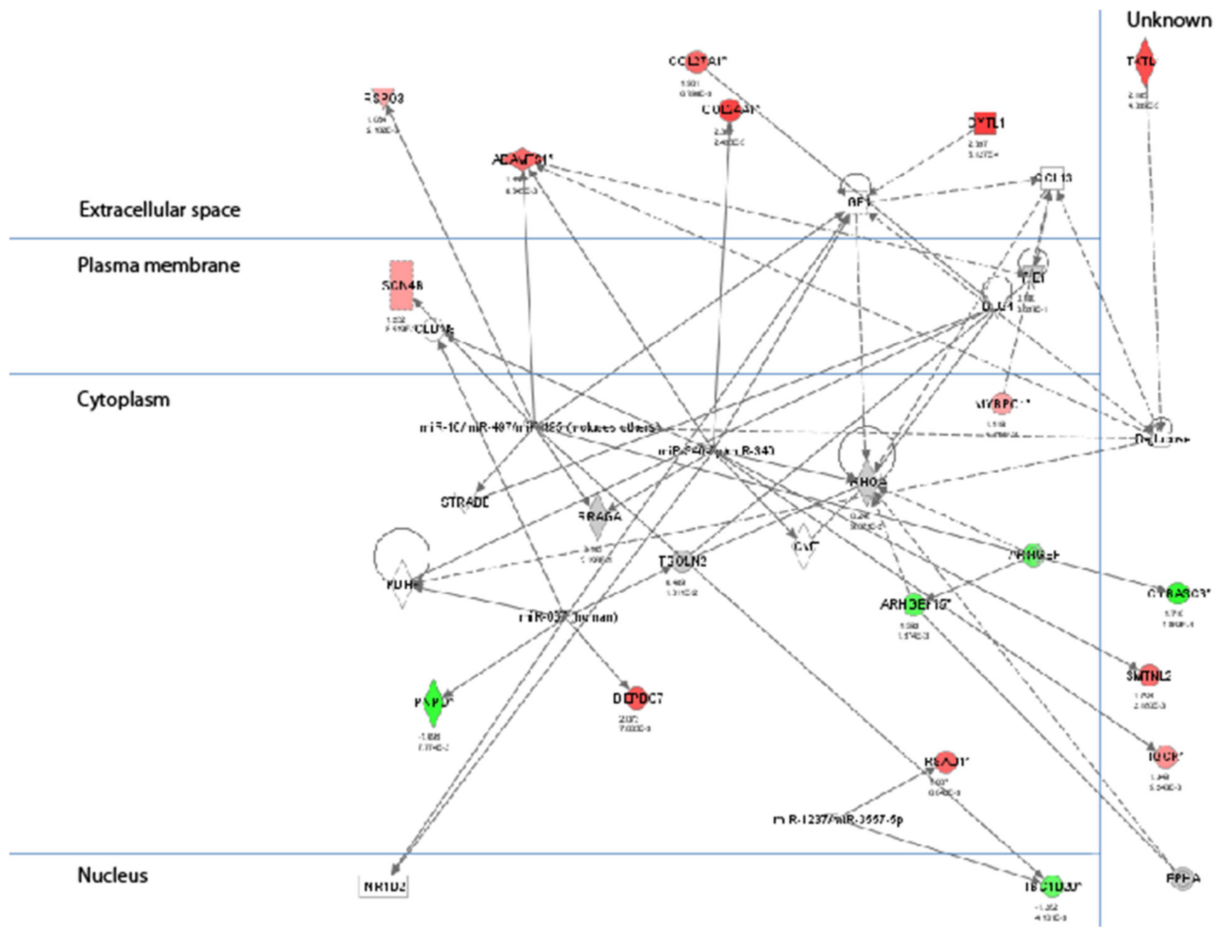

Figure 6. Cardiovascular system development and function, cell death, skeletal and muscular system development and function pathways.

Figure 7. Molecular transport, cancer, and cell death pathways.

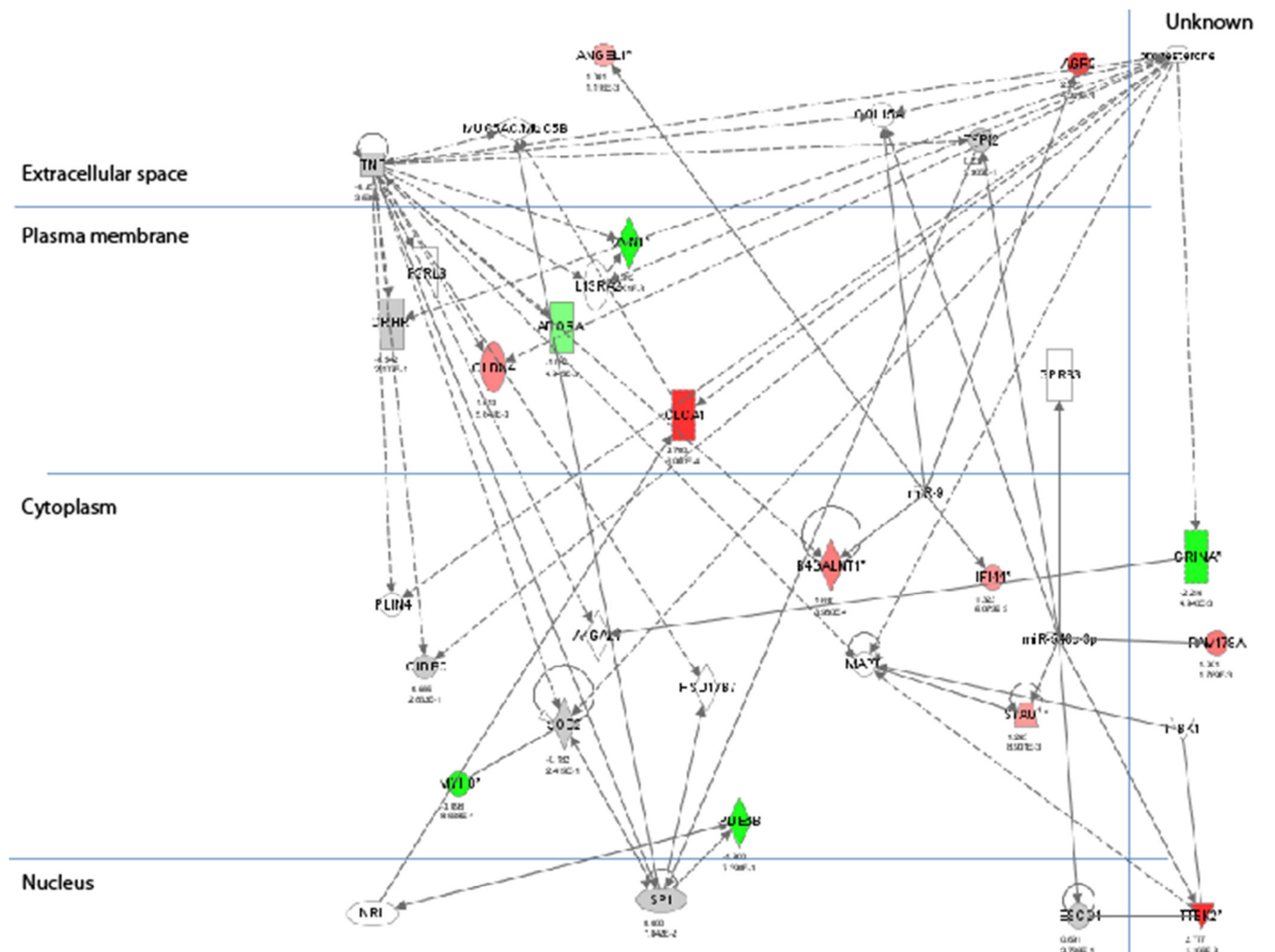

Figure 8. Lipid metabolism, small molecule biochemistry, molecular transport pathways.

Gene Ontology Analysis

Based on gene ontology (GO) analysis and in molecular function category, oxidoreductase activity was the only one found significant ($P < 0.05$) in secondary follicles (Fig. 9A). However, in tertiary follicles six other functions were significant (Fig. 9B). Four of six functions were related to transcription such as: transcription factors and suppressors ($P < 0.005$; yellow boxes in Fig. 9B), and transcription regulators and factor binding ($P < 0.05$). The remaining two of six functions were DNA binding and receptor binding which also concur the metabolic pathways in transport signals and small molecules (Fig. 4, 7 and 8).

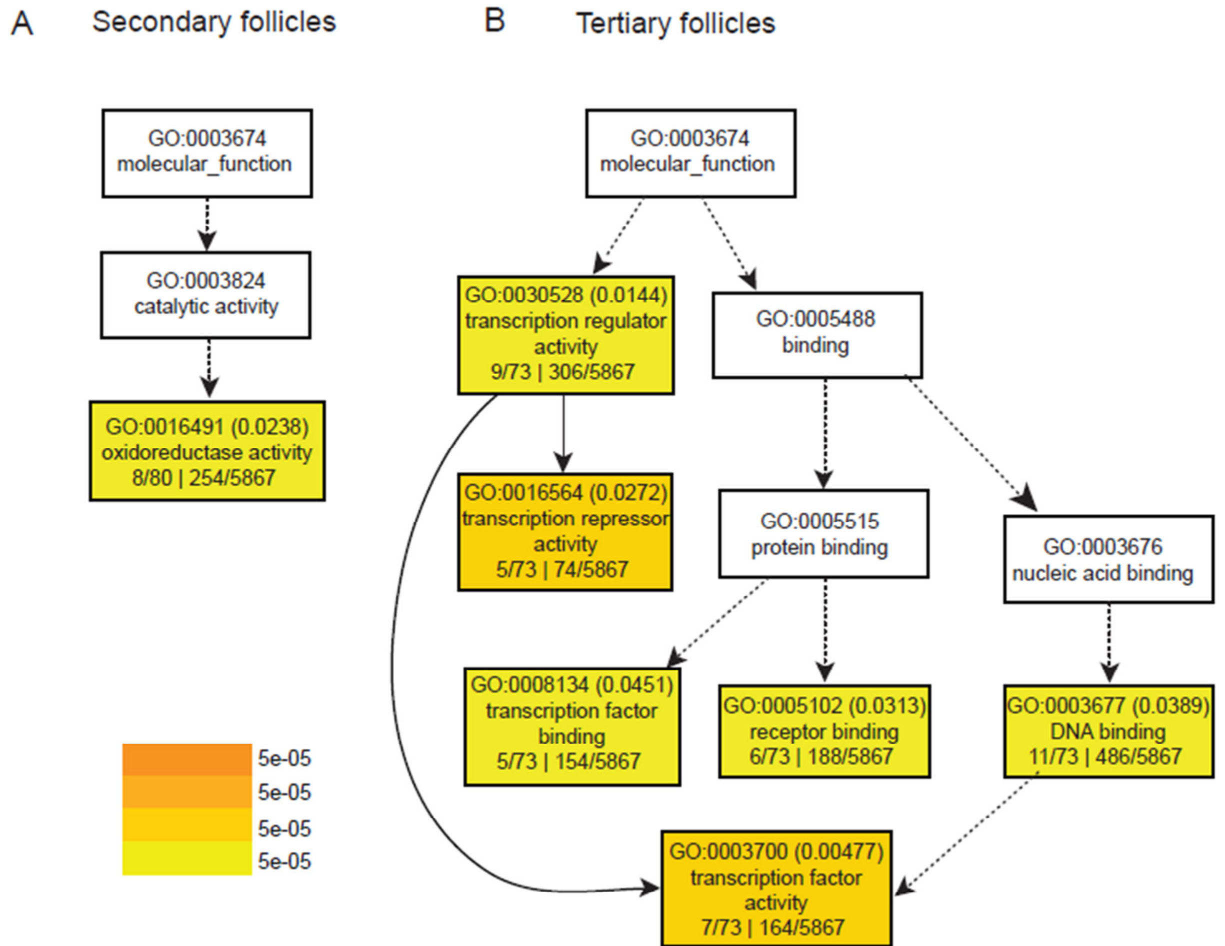

Figure 9. Hierarchical tree graph of overrepresented GO terms in molecular function category generated by SEA. Boxes in the graph represent GO terms labeled by their GO ID, term definition and statistical information. The significant term (adjusted $P < 0.05$) are marked with color, while non-significant terms are shown as white boxes. The degree of color saturation of a box is positively correlated to the enrichment level of the term.

Quantitative PCR Analysis

In order to validate the microarray findings and evaluate some genes at the detection threshold of the microarray hybridization, differences in the expression of ten genes (*ABLIM*, *SLIT3*, *TYMS*, *GTPBP1*, *AKR1C4*, *ARHGEF12*, *CLEC6A*, *CYTL1*, *PIK3R6*, and *MAOB*; Fig. 10) were confirmed by qPCR using the same RNA from those used for the microarrays. From the 10

genes tested, only one gene, *ABLIM*, was not expressed in both stages. According with *t*-test, *SLIT3*, *GTPBP1*, *AKR1C4* and *PIK3R6* genes were more expressed ($P < 0.05$) in secondary than in tertiary follicles; this finding was consistent with the results of the microarray analysis. However, there was no expression difference in the other five genes, unlike what was observed in the microarray analysis.

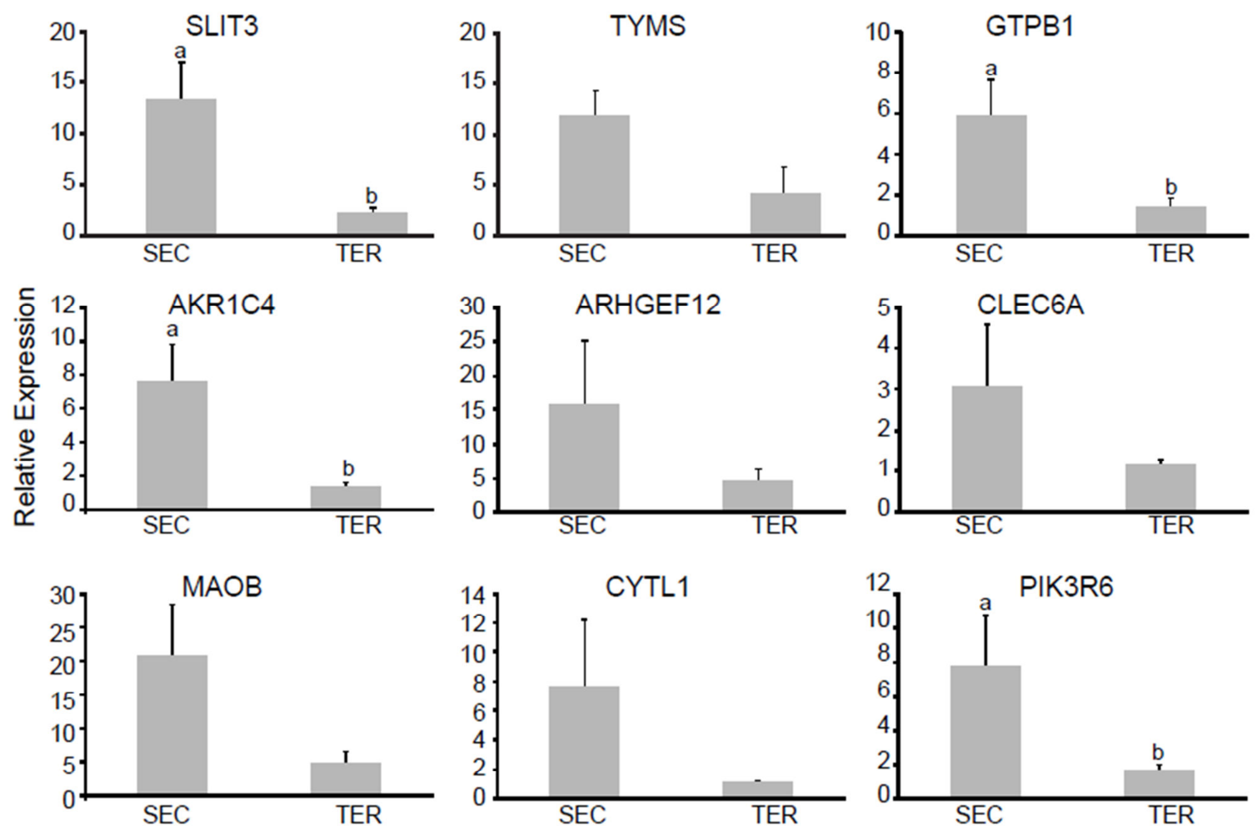

Figure 10. Quantitative real-time PCR analysis of selected genes for microarray validation. Across 18 comparisons (9 genes and 2 follicular staged, secondary and tertiary) the expression pattern of four selected genes obtained by qPCR were consistent with the results from the microarray analysis in all. For each transcript, bars with different superscripts differ ($P < 0.05$).

DISCUSSION

Due to the unavailability of goat genome arrays, bovine arrays with 8,329 unique genes have been first and successfully adapted and applied in goat mammary tissues by Ollier et al. [19, 28]. Later, two other research groups also applied bovine genome microarrays of 23,232 probes in goat mammary studies [18, 36]. The present study demonstrated the feasibility of applying bovine genome microarrays of 24,000 bovine transcripts on goat ovarian follicle studies. It showed, for the first time, detailed gene expression of the ovarian follicles in goats, specifically secondary and tertiary stages. The study demonstrated that although most of the genes expressed were common between the two follicle stages, some genes were specific for each stage. The findings of this study might help in the future to design a sequential medium appropriate for in vitro culture based on the substances required in each early follicle stage, and ultimately to improve in vitro growth, maturation and fertilization rates of oocytes from preantral follicles.

The developmental transition from secondary to tertiary follicles is marked by morphological changes, proliferation and differentiation of granulosa cells and the formation of antral cavity, characterized by the presence of the follicular fluid that contains substances secreted by oocyte and granulosa cells, and especially transudate of blood plasma [37]. The development of preantral follicle is primarily controlled by intraovarian regulators (e.g., growth factors, cytokines, and gonadal steroids) and does not require gonadotropins for growth, although it is also stimulated by the presence of FSH and LH in the in vitro culture medium [7, 8, 38].

Gene expression of three cell death related pathways were found in the transition from secondary to tertiary follicles in the current study, such as: cell death, lipid metabolism, and hematological system. According to cell death, the most expressed genes found in the present study in secondary follicles were related to: apoptosis activation (*CIDEA*), assembly and stability of cytoskeleton (*MAP1B*, *MAPT*), transcription factor to cell growth and differentiation – interaction with *IGF2R* (*CREG1*), control of oxidative stress (*OGG1*), tight junction at apical surface of epithelial cells (*CRB3*) and enzymes responsible by citrate (*CLYBL*), AMP (*ADSSL1*), ascorbic acid production (*CYB5B*) and removal of urea from the cells (*CPS1*). In tertiary follicles, the most of expressed genes in the cell death related pathways were related to: membrane trafficking, intracellular motility, organelle biogenesis (*SPG7*), microtubules formation – cell organelles organization (*TUBB1*), biosynthesis of phospholipid (*ETNK2*), NADP (*NADK*),

inductor of caspase dependent apoptosis when overexpressed (*MUL1*), microtubules (*Tubulin*) and DNA replication and repair (*TYMS*). Apoptosis is a process genetically determined and dependent of pro– (*Bax*, *Bak*, *Bok*, and *Diva*) and anti-apoptotic (*Bcl-2*, *Bcl-xL*, *Bcl-w*, *Mcl-1*, *Boo*, *surviving*) gene expression. It is characterized by DNA fragmentation in each 180-200 base pairs and apoptotic body formation. The *Bcl-2* and *Bax* expression is found in the granulosa cells of ovaries [39, 40]. In the ovary, *surviving* acts in granulosa cells regulating the cell cycle as well as inhibiting the apoptosis [41]. Intrinsic mechanisms and extrinsic factors can mediate the apoptosis process [42], 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 [43]. Caspase activation through cell death receptors (extrinsic factor) is mediated by a subset of the tumoral necrosis factor (*TNF*) [44, 45]. The intrinsic pathway involves the mitochondria with the release of cytochrome–c into the cytosol and formation of large pore channels that allow the passage of molecules [46].

Another pathway – lipid metabolism – could be essential in the transition from secondary to tertiary follicles. This transition includes differentiation and proliferation of granulosa, theca and stroma cells. This process and its regulation are still poorly understood. However, lipid oxidation products by cytochrome p450 are likely to play a critical role in preovulatory follicles [47]. Most of hormones secreted by granulosa cells, such as estradiol, progesterone and testosterone, are originated from cholesterol, a steroid lipid. Understanding cellular sterol metabolism in the ovary is a complex process further by the wide variety of cell types and steroids produced, by a changing hormonal environment during the reproductive cycle and by compartmentalization. In this context, the thecal and luteal cells are exposed to all lipoprotein classes, whereas the basement membrane restricts access by granulosa cells to the full complement of lipoproteins found in blood circulation [48]. In the lipid metabolism pathway, the most expressed genes found in the present study in secondary follicles were related to: secretion of plasma lipoproteins (apolipoprotein B) (*MTTP*), receptor mineralocorticoid (aldosterone, androgen, progesterone and others) (*NR3C2*) and transcription factor, nuclear receptor required to development of hypothalamic-pituitary-adrenal-gonadal-axis (*NROB1*). In tertiary follicles, the most of expressed genes in the lipid metabolism pathway were related to: lipid biosynthesis (*FADS2*), phosphorylation of protein and regulation of insulin action (*PTPRE*) and protein G receptor (*ADORA1*).

The hematological system pathway also was highlighted as very important in secondary and tertiary follicles. The blood vessels formation plays an essential role for the female's reproductive system. In secondary follicles, the theca cell layer contains just a few blood vessels, and the paracrine mechanism is extremely important for survival and development of this type of follicle. Subsequently, during the antrum formation, follicles become surrounded by a capillary network, which promotes the nutrition of both these cells and granulosa cells [49]. In this stage, the follicles become very dependent of endocrines factors and consequently more fragile to undergo atresia [50]. The ovarian blood supply is formed according to the estrous cycle/menstrual stage. In women, during the menstrual cycle, small primordial follicles do not have own blood supply (capillary system) and are dependent on stroma vessels [51]. With progressive growth, the primary follicles start to develop around one or two arterioles, and the acquisition of vessels is established around the theca cells. The establishment of the vascular system in the follicles coincides with the period of rapid growth and differentiation, which is correlated to increased sensitivity and/or dependence of gonadotropins [52].

In the present study, *NDUFS1* and ubiquinone cytochrome–c oxidoreductase expression profiles increased from secondary to tertiary follicles (Supplemental Table SII). These enzymatic complexes are involved in aerobic metabolism and are called complex I and III, respectively. Normally, in the electron transport chain, complex IV (cytochrome oxidase) converts molecular oxygen to water through an oxidation-reduction reaction [53]. However, under transient conditions, the electrons that pass down the electron transport chain combine with molecular oxygen at complex I and III, producing superoxide anion radical (O_2^-). In addition, an increase in the expression of superoxide dismutase (*SOD*) and lactate dehydrogenase (*LDH*) genes was observed in tertiary follicles (Supplemental Table SII). SOD constitutes the first enzymatic step, which plays a vital role in the control of cellular O_2^- production by catalyzing the dismutation of O_2^- into H_2O_2 and O_2. On the other hand, LDH converts pyruvate to lactate when the cells cannot be supplied with sufficient oxygen. Therefore, our results suggest that during the transition from secondary to tertiary follicle, the cells of the follicle produce radical oxygen species (ROS), O_2^- and H_2O_2, through the uncoupling of electron transfer from complex IV to complex I and III. The increased consumption of molecular oxygen by these complexes to produce ROS causes transient hypoxia and the follicular cells overexpress LDH, an enzyme related to anaerobic metabolism. Cellular ROS has been implicated in the initiation of developmental and differentiation events

[54, 55]. There is increasing evidence that ROS can function as second messengers in mammalian cells [56, 57] to regulate signal transduction pathways that control both gene expression and posttranslational changes of proteins [58, 59] involved in cell function, growth, differentiation, lipid oxidation and death [60]. ROS have been recognized as key molecules, which can selectively modify proteins and regulate cellular signaling including apoptosis. Cell death may be initiated intrinsically by the mitochondrial pathway or extrinsically through membrane associated death receptors in response to different stimuli [61].

The expression of the IGF-I encoding gene decreased from the secondary to tertiary follicle stage as demonstrated by the microarray analysis. This finding confirms that IGF-I regulates the early stages of oocyte and preantral follicular development and has a positive effect on antrum formation as recently demonstrated in goats [6]. In addition, the mRNA and proteins of IGF-I are expressed in different follicle compartments (i.e. cumulus-oocyte complex, mural granulosa and theca cells) at various stages (primordial, primary, secondary and antral follicles) of follicular development in goats [11]. According to the qPCR analysis, the data were consistent with microarray analysis. From the 10 genes tested only one gene (*ABLIM*) was not expressed in both stages. This finding suggests that either there is no expression of *ABLIM* (mRNA) in goat ovarian follicles (secondary and tertiary) or there is no hybridization between caprine and bovine chip for the *ABLIM* gene.

In conclusion, a detailed gene expression profile of goat secondary and early tertiary follicles has been presented for the first time. The results could provide tentative and indicative gene markers for normality of follicular growth. The gene expression findings can also help us to identify the needs of ovarian preantral follicles to establish an in vitro culture system in goats. The identification of qualitative and quantitative alterations of major biological functions and expression of individual genes in secondary and tertiary follicles seem to be associated with the survival and development of caprine preantral follicles. Finally, the microarray analysis has identified three important biological pathways involved in the transition from secondary to tertiary follicles and helped to better understand the role of ROS in this process.

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CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Supporting Information Available.

SI Table I. Three sets of normalized microarray data.

SI Table II. Dataset with *M*-value and *P*-value.

SI Table III. Top ten genes weakly expressed in secondary and tertiary follicles

SI Table IV. Top ten genes strongly expressed in secondary and tertiary follicles

SI Table V. Top ten genes only expressed in secondary or tertiary follicles.

SI Table VI. Pathway interaction network analysis of Lipid Metabolism, Small Molecule Biochemistry, Cell Death based on Ingenuity Pathway Analysis (IPA; www.ingenuity.com). The data were used to construct Fig. 4.

SI Table VII. Pathway interaction network analysis of Cellular Movement, Inflammatory Response, Hematological System Development and Function. The data were used to construct Fig. 5.

SI Table VIII. Pathway interaction network analysis of Cardiovascular System Development and Function, Cell Death, Skeletal and Muscular System Development and Function. The data were used to construct Fig. 6.

SI Table IX. Pathway interaction network analysis of Molecular Transport, Cancer, and Cell Death. The data were used to construct Fig. 7.

SI Table X. Pathway interaction network analysis of Lipid Metabolism, Small Molecule Biochemistry, and Molecular Transport. The data were used to construct Fig. 8.

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13 CONCLUSÕES

- A associação de FSH e IGF-I em um cultivo *in vitro* de longa duração de folículos inclusos em tecido ovariano caprino promoveu o desenvolvimento follicular, mantendo a expressão de RNAm para receptores de FSH (Fase I).
- O meio sequencial suplementado com FSH seguido de GH durante um cultivo de longa duração de folículos inclusos em tecido ovariano manteve a sobrevivência, a viabilidade e a ultraestrutura de folículos pré-antrais de cabras e promoveu a ativação e formação de folículos secundários (Fase II).
- Sob as mesmas condições, a frequência de troca de meio afetou o desenvolvimento *in vitro* de folículos pré-antrais caprinos e ovinos isolados, sendo recomendada a troca a cada 2 e 6 dias para folículos pré-antrais de cabras e ovelhas, respectivamente (Fase III).
- A adição de IGF-I ao meio de cultivo aumentou o desenvolvimento *in vitro* de folículos secundários caprinos isolados e o tratamento com FSH tendeu aumentar os níveis de expressão de RNAm para IGFR-I durante o cultivo *in vitro* de folículos secundários (Fase IV).
- O GH promoveu o crescimento e maturação *in vitro* de oócitos oriundos de folículos pré-antrais isolados, sendo capaz de produzir um embrião. Esse foi o primeiro estudo a demonstrar que embrião caprino pode ser produzido através da fecundação *in vitro* de oócitos derivados do crescimento *in vitro* de folículos pré-antrais (Fase V).
- A análise de microarranjo de DNA demonstrou que o perfil de expressão gênica diferiu entre folículos secundários (pré-antrais) e terciários (antrais), além de ter identificado genes e vias de sinalização cruciais na transição desses dois estádios foliculares (Fase VI).

14 PERSPECTIVAS

O cultivo *in vitro* de folículos pré-antrais, e posterior maturação e fecundação dos oócitos crescidos *in vitro*, é uma ferramenta essencial para se estudar os mecanismos envolvidos na foliculogênese, evento ainda não totalmente elucidado, e, futuramente, melhorar o potencial reprodutivo de fêmeas domésticas. Apesar dos resultados serem promissores com relação aos meios utilizados e aos sistemas de cultivo empregados, existe um grande desafio ainda para os pesquisadores em alcançar aceitáveis taxas de maturação e produção de embrião em animais domésticos. Um detalhado conhecimento acerca desse processo pode ser empregado tanto no desenvolvimento de biotecnologias reprodutivas, principalmente em mamíferos ameaçados de extinção, como para o tratamento de infertilidade humana. Conforme demonstrado neste trabalho, existem fortes evidências de que a foliculogênese inicial é regulada por uma complexa interação de hormônios e fatores de crescimento adicionados de forma sequencial, de acordo com cada fase folicular.

Uma vez que já foi confirmada em vários estudos a importância do hormônio FSH em experimentos envolvendo folículos pré-antrais, a presente tese foi baseada no teste do fator de crescimento IGF-I e do hormônio GH, em associação ao FSH, com o objetivo de se aprimorar os sistemas de cultivo *in vitro*. Além disso, os resultados obtidos nesta pesquisa foram de grande valia uma vez que além de identificar o efeito do IGF-I e GH no cultivo *in vitro* de folículos pré-antrais iniciais (primordiais e primários) e tardios (secundários avançados), ainda foi identificada a expressão dos receptores de IGF-I e FSH em folículos pré-antrais antes e após o cultivo. Além disso, a valiosa técnica de microarranjo de DNA utilizada neste trabalho foi essencial para desvendar alguns mecanismos envolvidos na transição de folículos pré-antrais para antrais, fato que poderá contribuir futuramente para a elaboração de um meio sequencial eficiente.

Diante das conclusões deste trabalho, os resultados obtidos poderão ser utilizados para elaborar um eficiente sistema de cultivo “em dois passos” que proporcione elevadas taxas de maturação oocitária e a produção de embriões a partir do desenvolvimento *in vitro* de folículos pré-antrais de fêmeas de interesse zootécnico ou em vias de extinção.

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16 APÊNDICE

CERTIFICADO

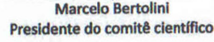
Certificamos que o trabalho intitulado Produção de embrião após o crescimento, maturação e fertilização in vitro de oócitos oriundos de folículos pré-antrais caprinos cultivados na presença do hormônio de crescimento (GH)

apresentado por Deborah de Melo Magalhães

foi premiado em 3° LUGAR na categoria " COMPETIÇÃO DE ESTUDANTES" durante a XXIV REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE TECNOLOGIA DE EMBRIÕES, realizada de 19 a 21 de Agosto de 2010 em Porto de Galinhas- PE.

Alexsandra Fernandes Pereira
Primeira secretária

Vicente José de Figueirêdo Freitas
Presidente



Marcelo Bertolini
Presidente do comitê científico

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June 21, 2012

To whom it may be concerned,

This letter is to confirm that Ms. Deborah de Melo Magalhães Padilha of Veterinary Medicine from State University of Ceará, Brazil, had been trained in the Geisler & Geisler-Lee Laboratory at the Department of Plant Biology, Southern Illinois University of Carbondale, USA, from May to December 2011. During the training, Ms. Padilha was supported by a scholarship from Conselho Nacional de Pesquisa (CNPq), Brazil.

The training Ms. Padilha had received at the Geisler & Geisler-Lee Laboratory was listed below.

1. Participation in the weekly progress meetings between Ms. Padilha and Dr. Geisler-Lee. Ms. Padilha had one meeting notebook detailing each meeting for her research progress and trouble-shooting.
2. Laboratory note keeping and data backup. Ms. Padilha had one lab notebook (#17) and produced one data DVD. Note: Ms. Padilha keeps another copy of the identical data for herself.
3. RNA extraction. Ms. Padilha successfully produced quality RNA from goat (*Capra aegagrus hircus*) ovarian follicles (both secondary and tertiary stages) and accumulated sufficient RNA for microarray analysis with three replicates per developmental stage.
4. RNA quality and quantification. Ms. Padilha was able to detect intact RNAs through horizontal gel electrophoresis and measure RNA quantity by NanoDrop.
5. Microarray analysis. Ms. Padilha had the capacity to differentiate quality of array data and verify them with RT-PCR. Note: the technique of RT-PCR was not done in the Geisler & Geisler-Lee Laboratory.
6. Gene expression analyses. Ms. Padilha was capable of working with Ingenuity IPA Software for pathway analysis and Cluster 3 program for coexpression analysis, and extracting biological meanings from her processed data.
7. Basic metadata analysis. Ms. Padilha could download and process array data from GEO (Gene Expression Omnibus) in order to compare goat expression with other model animal organisms.
8. Participation in the bi-weekly mentoring program which was to help the members of the Geisler and Geisler-Lee Laboratory (1) design and perform research, (2) present their research, and (3) abide by safety regulations.

9. Participation in the semi-annual laboratory symposium. Note: Ms. Padilha was awarded as the first prize winner in the 2011 autumn laboratory symposium.
10. Leading the regular research progress meetings for the goat follicle development group, including seven members from Plant Biology and Animal Science:
    a. Ms. Deborah de Melo Magalhães Padilha, the trainee
    b. Dr. Jane Geisler-Lee, Plant Biology
    c. Dr. Matt Geisler, Plant Biology
    d. Dr. Eduardo Gastal, Animal Science
    e. Dr. Melba Gastal, Animal Science
    f. Dr. Aurea Wischeral, Animal Science, visiting scholar from Brazil
    g. Ms. Gabriela Rios da Fonseca (undergraduate student), Animal Science, visiting student from Brazil
11. Literature search and organization. Ms. Padilha learned how to download literature and organize it in EndNote. Her manuscript was prepared in EndNote.

If you have any question(s) regarding to Ms. Padilha's training at the Geisler & Geisler-Lee Laboratory, please do not hesitate to contact me.

Sincerely Yours,

Jane Geisler-Lee