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JESÚS DE LOS REYES CADENAS MORENO

CULTIVO *IN VITRO* DE FOLÍCULOS OVARIANOS CAPRINOS ISOLADOS: EFEITO DE DIFERENTES COMBINAÇÕES DE GH E VEGF SOBRE DIFERENTES CATEGORIAS FOLICULARES E ESTUDO INDIVIDUALIZADO DA DINÂMICA DE CRESCIMENTO FOLICULAR.

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Tese apresentada ao curso de Doutorado em Ciências Veterinárias do Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial à obtenção do título de doutor em Ciências Veterinárias. Área de Concentração: Reprodução e Sanidade Animal.

Orientador: Prof. Dr. José Ricardo de Figueiredo

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Dedico,

À minha pessoa favorita

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RESUMO

Os objetivos da presente tese foram: 1) Avaliar o efeito da adição do hormônio do crescimento (GH) e do fator de crescimento endotelial vascular (VEGF) isoladamente ou em associação, bem como de forma fixa ou sequencial sobre o cultivo *in vitro* de folículos (CIVF) pré-antrais (FPs) e antrais iniciais (FAs) isolados (Fase I); 2) Analisar o efeito do sistema de maturação *in vitro* (MIV) (individual vs. em grupo) sobre a produção de oócitos maturos, além de investigar o efeito do tempo de cultivo sobre o CIVF de FAs (Fase II); 3) Verificar se a linhaça na dieta afeta a eficiência do CIVF de FAs (Fase III). Na Fase I, FPs e FAs foram cultivados em: αMEM^{+} (Controle), ou αMEM^{+} suplementado com 50 ng/mL de GH, 100 ng/mL de VEGF, a combinação de ambos (GH+VEGF), GH durante os primeiros 12 dias e VEGF a partir do dia 12 até o final do cultivo (GH/VEGF) e vice-versa (VEGF/GH). Posteriormente, os complexos *cumulus*-oócito (CCOs) recuperados foram maturados *in vitro*. Foram avaliados: a morfologia folicular, as taxas de crescimento e de formação de antro, a produção de estradiol (E2), de progesterona (P4) e de testosterona (T), a viabilidade e o estágio meiótico dos oócitos, bem como a expressão de RNAm do receptor do hormônio luteinizante (*LHR*), do hormônio anti-mülleriano (*Amh*), da hialironano sintase-2 (*HAS2*), e da prostaglandina endoperóxido sintase-2 (*PTGS2*) no *cúmulus*, e de três enzimas esteroidogênicas (*CYP17*, *CYP19A1* e *3βHSD*) na parede folicular. Na Fase II, os CCOs crescidos *in vivo* foram submetidos a MIV em grupos (10 CCOs / gota de 100 μL) ou individualmente (1 CCO / gota de 10 μL). Além disso, FAs foram cultivados no melhor tratamento da Fase I por 12 (CIVF-12) ou 18 dias (CIVF-18), seguido da MIV individual dos CCOs recuperados. Avaliou-se a morfologia e crescimento foliculares, a produção de E2, e a configuração da cromatina de oócitos antes do CIVF, bem como antes e depois da MIV. Para a Fase III, FAs procedentes de animais alimentados com duas dietas diferentes (Controle e Linhaça) foram submetidos à CIVF e MIV como descrito na Fase I. Após a MIV, todos os oócitos foram fecundados *in vitro* por 18 h, seguido do cultivo *in vitro* dos presumíveis zigotos por mais 48 h. No dia 3 pós-fecundação, todos os embriões clivados foram transferidos no oviduto de 3 receptoras. Trinta e seis dias depois, avaliou-se a possível prenhez por ultrassonografia. Os parâmetros avaliados na Fase III foram: a morfologia folicular, a produção de E2, o diâmetro oocitário, fecundação e produção embrionária. Na Fase I, os FPs e FAs se comportaram de forma diferente quando submetidos as mesmas condições experimentais. O tratamento GH para FAs apresentou o maior ($P < 0,05$) diâmetro oocitário médio (117, 7 μm), e as maiores ($P < 0,05$) taxas de maturação meiótica (42,5%).

Na Fase II, o sistema de MIV individual não afeitou negativamente a maturação oocitária. O tratamento CIVF-18 promoveu as maiores ($P < 0,05$) taxas de oócitos em metáfase II (MII) (46,2%) e o maior ($P < 0,05$) diâmetro oocitário médio. Também, observou-se que os folículos com uma taxa de crescimento diária > 7,1 μm e que atingiram pelo menos 600 μm de diâmetro, foram mais propensos ($P < 0,05$) a produzir oócitos capazes de atingir a MII. Finalmente, na Fase III a linhaça não afeitou a foliculogênese *in vitro*, mais sim aumentou ($P < 0,05$) as taxas de recuperação de oócitos ≥ 110 μm e diminuiu ($P < 0,05$) as taxas de fecundação, embora não tenha afeitado as taxas de clivagem.

Palavras-chave: Cabra. Cultivo *in vitro.* Folículo ovariano. GH. Maturação oocitária

ABSTRACT

The main goals of the present thesis were: 1) to evaluate the effect of growth hormone (GH) and vascular endothelial growth factor (VEGF) added alone, sequentially or in combination on the *in vitro* culture of two different follicular categories: preantral (PAF) and early antral (EAF) (Phase I); 2) to investigate the effect of the *in vitro* maturation (IVM) system (individual vs. in group) on the production of metaphase II (MII) oocytes, as well as the effect of the culture period (12 vs. 18 days) on EAFs development (Phase II); 3) To verify if a linseed-supplemented diet affects the *in vitro* follicle culture (IVFC) (Phase III). In the Phase I, PAFs and EAFs were cultured as follows: αMEM^{+} (Control), or αMEM^{+} supplemented with 50 ng/mL GH, 100 ng/mL VEGF, the combination of both (GH+VEGF), GH during the first 12 days and VEGF from day 12 until the end of the culture (GH/VEGF) and vice versa (VEGF/GH). Afterwards, recovered *cumulus*-oocyte complexes (COCs) were matured *in vitro*. It was evaluated: follicle morphology, levels of estradiol (E2), progesterone (P4) and testosterone (T), oocyte viability and meiotic stage, as well as mRNA expression of *LHR* (Luteinizing hormone receptor), *Amh* (Anti-müllerian hormone), *HAS2* (Hyaluronan synthase 2), and *PTGS2* (Prostaglandin endoperoxide synthase 2) in cumulus cells, and three steroidogenic enzymes (*CYP17*, *CYP19A1*, and *3βHSD*) in follicular walls. In the Phase II, *in vivo* grown COCs were submitted to IVM either individually (1 COC/10 µL-drop) or in group (10 COCs/100 µL-drop). Also, EAFs were cultured using the best treatment of the Phase I for 12 (IVFC-12) or 18 days (IVFC-18), followed by individual IVM of the recovered COCs. Follicular morphology and growth, E2 production, and oocyte chromatin configuration were evaluated before IVFC and before and after IVM. Finally, for the Phase III, EAFs from animals fed with two different diets (Control or Linseed) were submitted to IVFC and IVM as described in the Phase I. After the IVM, all oocytes were submitted to *in vitro* fertilization (IVF) for 18 h, and subsequent *in vitro embryo* culture for 48 h. On day 3-post IVF, all presumptive zygotes were surgically transferred into the oviduct of 3 recipients. Thirty-six days later, a transrectal ultrasonography was performed for pregnancy diagnosis. Follicle morphology, oocyte diameter, fertilization and embryo production were evaluated. In the Phase I, PAFs and EAFs behaved differently under the same culture conditions. The GH treatment for EAFs presented the highest ($P < 0.05$) mean oocyte diameter (117.74 µm) and meiotic maturation (42.5%). In the Phase II, individual IVM system did not negatively affect oocyte nuclear maturation. The IVFC-18 treatment produced higher ($P < 0.05$) MII oocytes (46.2%) and oocyte diameter (119 µm). Moreover, follicles with a daily growth > 7.1 µm, and

that reached at least 600 μm in diameter, were more likely ($P < 0.05$) to produce meiotically competent oocytes. Finally, in Phase III, linseed did not affect goat *in vitro* folliculogenesis, but it increased ($P < 0.05$) the recovery rate of oocytes ≥ 110 μm, and negatively affected ($P < 0.05$) the sperm penetration rate, even though with no further effect on the cleavage rate.

Key words: Caprine. GH. *In vitro* culture. Oocyte maturation. Ovarian follicles

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LISTA DE ABREVIATURAS E SIGLAS

Amh	Anti-müllerian hormone (Hormônio anti-mülleriano)
BSA	Bovine serum albumin (Albumina sérica bovina)
CA	California
Calceína-AM	Calceína acetoximetil
CCO/COC	Complexo cúmulos-oócito (*Cumulus*-oocyte complex)
cDNA	Complementary deoxyribonucleic acid (Ácido desoxirribonucleico complementar)
CE	Ceará
CI	Confidence Interval (Intervalo de confiança)
CIV	Cultivo *in vitro*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO2	Dióxido de carbono
CYP17	Cytochrome P450 17α-hydroxylase
CYP19A1	Cytochrome P450, Family 19, Subfamily A, polypeptide 1
D	Day (dia)
DEG	Degenerated (degenerado)
DNA	Deoxyribonucleic acid (Ácido desoxirribonucleico)
E2	Estradiol
EAFs	Early antral follicles
EGF	Epidermal growth fator (Fator de crescimento epidermal)
ELISA	Ensaio de imunoabsorção enzimática
Exp.	Experiment (Experimento)
FA	Folículos antrais
FAVET	Faculdade de Veterinária

Fig.	Figure (Figura)
FIV	Fecundação *in vitro*
Flt-1	Fms-like tyrosin kinase 1 (Tirosina quinase 1 semelhante a Fms)
Flt-4	Fms-like tyrosin kinase 4 (Tirosina quinase 4 semelhante a Fms)
FOPAS	Folículos ovarianos pré-antrais
FP	Folículos pré-antrais
FSH	Follicle-stimulating hormone (Hormônio folículo-estimulante)
FUNCAP	Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico
G	G-force (força G)
GADPH	Glyceraldehyde-3-phosphate-dehydrogenase
GH	Growth hormone (Hormônio do crescimento)
GHR	Growth hormone receptor (Receptor do hormônio do crescimento)
GV	Germinal vesicle (Vesícula germinal)
GVBD	Germinal vesicle break-down (Quebra da vesícula germinal)
h	Hours (horas)
HAS2	Hyaluronan synthase 2 (Hialuronano sintase-2)
i.e.	This is (isto é)
IGF-I	Insulin-like growth fator-1 (Factor de crescimento semelhante à insulina-1)
ITS	Insulina, transferrina e selênio (Insulin, transferrin and selenium)
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVFC	*In vitro* follicle culture
IVM	*In vitro* maturation
JANUS2	Janus quinase 2

KDR	Kinase domain receptor (Receptor de domínio quinase)
L	Litro
LAMOFOPA	Laboratório de Manipulaçao de Oócitos e Folículos Pré-antrais
LH	Luteinizing hormone (Hormônio luteinizante)
LHR	Luteinizing hormone receptor (Receptor do hormônio luteinizante)
M	Molar
MAPK	Mitogen-activated protein kinase (Proteínas quinases activada por mitógeno)
MEM	Meio essencial mínimo (Minimum essential medium)
MEM-HEPES	Meio essencial mínimo tamponado com HEPES
MI	Metaphase I (Metáfase I)
MII	Metaphase II (Metáfase II)
min	Minuto
MIV	Maturação *in vitro*
mL	Mililitro
mm	Milimetro
mM	Milimolar
Mo	Missouri
MOIFOPA	Manipulação de oócitos inclusos em folículos pré-antrais
ng	Nanograma
nm	Nanômetro
NY	New York
OH	Ohio
OR	Odds ratio (Razão de probabilidade)
$P < 0.05$	Probabilidade de erro menor que 5%

P > 0.05	Probabilidade de erro maior que 5%
P	Probabilidade de erro
P4	Progesterone (Progesterona)
PBS	Phosphate buffered saline (Tampão fosfato salina)
PFs	Preantral follicles
PI3K/Akt	Phosphatidylinositide 3-kinase pathway (Vía da fosfatidil inositol 3-quinase)
PIGF	Placental growth factor (Fator de crescimento placentário)
PKC	Protein kinase C (Proteina quinase C)
PLC	Phospholipase C (Fosfolipase C)
PPIA	Peptidylprolyl isomerase I
PTGS2	Prostaglandin-endoperoxide synthase 2 (Prostaglandina endoperóxido sintase-2)
PUFAs	Polyunsaturated fatty acids (Ácidos graxos poli-insaturados)
qPCR	Quantitative real-time polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
r	Pearson's correlation coeficient (Coeficiente de correlação de Pearson)
R^2	Coefficient of determination (Coeficiente de determinação)
rFSH	Recombinant follicle-stimulating hormone (Hormônio folículo-estimulante recombinante)
RIA	Raioimunoensaio
RNA	Ribonucleic acid (Ácido ribonucleico)
RNAm	Messenger ribonucleic acid (Ácido ribonucleico mensageiro)
SEM	Standard error of means (Erro padrão da média)
STAT	Signal transducters and activators of transcription (Transdutores de sinal e ativação de transcrição)

T	Testosterone (Testosterona)
TCM 199	Tissue medium culture 199 (Méio de cultivo de tecido 199)
TCM199-HEPES	Meio TCM199 tamponado com HEPES
TNF-α	Fator de necrose tumoral
UECE	Universidade Estadual do Ceará (State University of Ceara)
USA	United States of America
VEGF	Vascular endothelial growth factor (Fator de crescimento endotelial vascular)
VEGF-A165	Monômero de VEGF-A com 165 aminoácidos
VEGF-A-E	Isoformas de VEGF dos tipos A, B, C, D, e E.
VEGFR-1-3	Isoformas do receptor de VEGF dos tipos 1, 2, e 3.
αMEM	Alpha-minimum essential médium (Meio essencial mínimo alfa)
αMEM+	Supplemented minimum essential medium alpha (Meio essencial mínimo alfa suplementado)
%	Percentagem
<	Menor que
>	Maior que
≤	Menor ou igual a
≥	Maior ou igual a
°C	Grau Celsius
µg	Micrograma
µL	Microlitro
µm	Micrômetro
µM	Micromolar
26-G	Gauge 26 (calibre 26)

3βHSD	3-β-hydroxysteroid dehydrogenase

SUMÁRIO

1 INTRODUÇÃO

O ovário mamífero contém de milhares a milhões de oócitos imaturos que constituem a reserva ovariana. A grande maioria destes (~ 90%) é encontrada inclusa em folículos ovarianos pré-antrais (FOPAs: folículos primordiais, transição, primários e secundários). No entanto, uns poucos folículos atingem o estágio pré-ovulatório (~ 0,1%), sendo que o restante degenera pelo processo de atresia durante seu crescimento e maturação (CLARK et al., 2004). Por esse motivo, o desenvolvimento da biotécnica de manipulação de oócitos inclusos em FOPAs (MOIFOPA ou ovário artificial) ganhou destaque nas últimas décadas. A MOIFOPA visa resgatar e cultivar *in vitro* os FOPAs a fim de evitar sua atresia, visando obter um grande número de oócitos maduros que podem ser utilizados em outras biotécnicas reprodutivas (FIGUEIREDO et al., 2011a). No entanto, o êxito futuro da MOIFOPA depende diretamente do progresso alcançado em modelos animais. Nesse sentido, os pequenos ruminantes podem servir como um excelente modelo para grandes mamíferos (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010; MAGALHÃES et al., 2011b), e a cabra, em particular, foi descrita como um modelo adequado para o ovário humano devido às suas semelhanças tanto no tempo necessário para completar o desenvolvimento folicular (~ 6 meses) (SANTOS et al., 2009) quanto nos diâmetros folicular e oocitário (LUCCI et al., 2001).

Até o momento, os melhores resultados da MOIFOPA foram obtidos em camundongos, sendo obtidas crias vivas (O'BRIEN, 2003). No entanto, em mamíferos de grande porte, os principais resultados podem ser resumidos à produção de um pequeno e variado número de oócitos em metáfase II (MII) e embriões (WU; EMERY; CARRELL, 2001; ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010; MAGALHÃES et al., 2011a; SILVA et al., 2014a).

Uma grande variedade de fatores podem afetar os resultados da MOIFOPA, como por exemplo, o modelo animal (MAGALHÃES et al., 2011b; ROSSETTO et al., 2013a; ROCHA et al., 2014), o estado nutricional do animal (ADAMIAK et al., 2005; FOULADI-NASHTA et al., 2007), o sistema de cultivo (ARAÚJO et al., 2014, 2015; PESSOA et al., 2014), a duração do cultivo (SUN; LI, 2013; PESSOA et al., 2014), a tensão de oxigênio (GIGLI; BYRD; FORTUNE, 2006; XU et al., 2011), o meio de base (ROSSETTO et al., 2013b; CASTRO et al., 2014; ARAÚJO et al., 2015) e a suplementação utilizada (FERREIRA et al., 2016). Nesta última variável, na espécie caprina destaca-se o uso do fator de crescimento endotelial vascular (VEGF) e do hormônio do crescimento (GH), uma vez que

estes suplementos promoveram os melhores resultados em relação às taxas de maturação oocitária (29,4%) (ARAÚJO et al., 2011b) e desenvolvimento embrionário *in vitro* (Mórula) (MAGALHÃES et al., 2011a). No entanto, resultados obtidos ainda são inferiores aos obtidos a partir de folículos crescidos *in vivo*.

Para uma melhor compreensão do tema investigado na presente tese, na revisão da literatura a seguir será realizada uma breve abordagem sobre a caracterização dos ovários de mamíferos, oogênese, foliculogênese e caracterização folicular, população e atresia folicular, biotécnica da MOIFOPA, sistemas de cultivo *in vitro*, variáveis que afetam ao cultivo *in vitro*, importância da composição do meio de cultivo, com ênfase em GH e VEGF, ferramentas e parâmetros para avaliar a eficiência do cultivo *in vitro*, bem como o estado atual da biotécnica de MOIFOPA.

2 REVISÃO DE LITERATURA

2.1 CARACTERIZAÇÃO DO OVÁRIO DE MAMÍFEROS

O ovário é um importante órgão do sistema reprodutor feminino, tendo como principais funções a diferenciação e a liberação de um oócito maduro com capacidade de ser fecundado a fim de produzir descendentes (MCGEE; HSUEH, 2000). Além de produzir oócitos, o ovário também produz esteroides sexuais e fatores de crescimento que atuam na regulação de diferentes aspectos da fisiologia reprodutiva feminina (EDSON; NAGARAJA; MATZUK, 2009). O ovário é constituído por duas regiões diferenciadas: a medula, que na maioria das espécies ocupa a região interna do ovário, formada, principalmente, por tecido conjuntivo, nervos e vasos sanguíneos; e o córtex, que na maioria das espécies é a região externa do ovário, em que se situam os folículos ovarianos e corpos lúteos em diferentes estágios de desenvolvimento (REEVES, 1971).

A unidade morfológica e funcional do ovário é o folículo ovariano, constituído por um oócito circundado por células somáticas (células da granulosa e da teca), as quais proporcionam um ambiente ótimo para o desenvolvimento oocitário (CORTVRINDT; SMITZ, 2001).

2.2 OOGÊNESE, FOLICULOGÊNESE E CARACTERIZAÇÃO FOLICULAR

Nos mamíferos, o desenvolvimento do oócito começa no período fetal com a diferenciação, proliferação e subsequente migração das células germinativas primordiais para as cristas genitais, em que se diferenciam em ovogônias, entrando na sua primeira divisão meiótica, tornando-se oócitos primários (MCLAUGHLIN; MCIVER, 2009).

Os oócitos primários, que permanecem em prófase da primeira divisão meiótica, estão circundados por uma camada de células pavimentosas da pré-granulosa e uma membrana basal, formando os folículos primordiais. Estes folículos são os de menores diâmetros (30 μm, aproximadamente) (BRAW-TAL; YOSSEFI, 1997; BEZERRA et al., 1998; MCNATTY et al., 2000) e os mais numerosos dentro do ovário (90% do total de folículos), constituindo o *pool* de reserva de folículos em quiescência (MCGEE; HSUEH, 2000). Alguns desses folículos primordiais são estimulados a entrarem em fase de crescimento (MCLAUGHLIN; MCIVER, 2009) e converte-se em folículos primários (50 μm), que são caracterizados por possuírem uma camada completa de células da granulosa

cuboides entorno do oócito (BRAW-TAL; YOSSEFI, 1997; BEZERRA et al., 1998; MCNATTY et al., 2000).

As células cuboides da granulosa continuam se dividindo e formando camadas concêntricas. Quando os folículos adquirem duas ou mais camadas de células cuboides, passam a ser denominados de secundários. Ao mesmo tempo, aderidas ao exterior da membrana basal, ocorre a proliferação das células da teca, que serão substituídas pela teca interna e teca externa, proporcionando ao folículo, entre outras coisas, um suprimento independente de sangue (YOUNG; MCNEILLY, 2010).

Os pequenos folículos secundários apresentam um diâmetro de aproximadamente 80 µm. No entanto, quando alcançam o diâmetro de aproximadamente 200 µm começam a exibir pequenas cavidades preenchidas por líquido entre as camadas de células da granulosa (BRAW-TAL; YOSSEFI, 1997; BEZERRA et al., 1998; MCNATTY et al., 2000), passando a ser chamados de folículos terciários. As pequenas cavidades coalescem formando a chamada cavidade antral que está repleta de fluido folicular composto por uma grande variedade de proteínas, substratos energéticos, hormônios, citocinas/fatores de crescimento, esteroides e outros fatores não definidos (SUTTON; GILCHRIST; THOMPSON, 2003). Nessa etapa, a maioria dos folículos entram em atresia e apenas um número reduzido continuam crescendo até alcançar o estágio pré-ovulatório ou folículo de *Graaf* (MCGEE; HSUEH, 2000), que em caprino pode ter 6-7 mm de diâmetro (VAN DEN HURK; ZHAO, 2005). A formação do antro promove a diferenciação das células da granulosa em dois tipos celulares: células murais da granulosa, que se encontram em contato com a membrana basal e apresentam função endócrina; e as células do *cumulus*, que estão em contato com o oócito, colaborando no seu metabolismo e maturação. O conjunto oócito e *cumulus*, formam o complexo *cumulus*-oócito (CCO) (COLLADO-FERNANDEZ; PICTON; DUMOLLARD, 2012). Em geral, os folículos ovarianos podem ser classificados em dois grandes grupos: folículos pré-antrais (primordiais, primários e secundários) e folículos antrais (terciários e pré-ovulatórios ou de Graaf).

Paralelamente ao crescimento folicular, o oócito, que se encontra no interior do folículo, vai aumentando o diâmetro e o volume, passando de aproximadamente 30 µm na fase primordial a mais de 120 µm na fase antral (BEZERRA et al., 1998; MCNATTY et al., 2000). Este aumento de diâmetro e volume é resultado do acúmulo de água, íons, carboidratos, lipídios, proteínas e mRNA (FAIR et al., 1997). O crescimento oocitário está intimamente relacionado com a proliferação de células da granulosa e vice-versa, enquanto

que a presença do oócito promove a diferenciação das células da granulosa em células do *cumulus*, bem como a expansão dessas células (DIAZ; WIGGLESWORTH; EPPIG, 2007). As células do *cumulus* são a principal fonte de energia do oócito (MUNAKATA et al., 2016; ITAMI et al., 2017).

O oócito adquire o seu tamanho máximo antes de chegar na fase pré-ovulatória. No entanto, as maturações citoplasmática e nuclear continuam ocorrendo até o final da foliculogênese. No citoplasma, as mudanças que ocorrem estão destinadas a fornecer as condições necessárias para a penetração espermática, o bloqueio da polispermia, a descondensação da cromatina do espermatozoide, a formação do pró-núcleo e as primeiras divisões embrionárias (FERREIRA et al., 2009). O retículo endoplasmático e o complexo de Golgi são redistribuídos e sofrem mudanças estruturais como resultado da grande atividade de síntese do oócito. O número de vesículas e de ribossomos aumentam, aparecendo os grânulos corticais e glicoproteínas são exportadas para formar a zona pelúcida (FAIR et al., 1997). Estas glicoproteínas começam a ser sintetizadas simultaneamente ao crescimento oocitário (RANKIN; SOYAL; DEAN, 2000), porém a zona pelúcida não é visível até se atingir o estágio de folículo secundário (FAIR et al., 1997). Além disso, o número de mitocôndrias e o nível de glutationa (GSH) (importante para reduzir o estresse oxidativo, a descondensação do núcleo do espermatóide e a formação do pró-núcleo masculino) aumentam (LUBERDA, 2005). No núcleo, ocorrem uma série de eventos moleculares em cascata, associados a retomada da meiose I: quebra da vesícula germinal (GVBD, da sigla em inglês “germinal vesicle breakdown”), progressão da divisão meiótica, extrusão do primeiro corpúsculo polar e parada na metáfase II (M II) (TRIPATHI; KUMAR; CHAUBE, 2010).

Finalmente, este processo termina quando um CCO maduro (ou seja, um oócito em metáfase II associado ao primeiro corpúsculo polar no espaço perivitelino e com o *cumulus* expandido) é ovulado pelo folículo pré-ovulatório.

2.3 POPULAÇÃO FOLICULAR E ATRESIA

Os ovários de mamíferos contêm milhares de oócitos imaturos, formando a reserva ovariana, sendo que a grande maioria destes (aproximadamente 90%) se encontra inclusa em FOPAs, principalmente nos folículos primordiais (LIU et al., 2001). Embora seja aceito que a reserva ovariana constitua-se de um estoque finito e não renovável de oócitos que se estabelece durante o período fetal (WOODRUFF, 2008), recentemente demonstrou-se

indícios da formação de novos oócitos e folículos ovarianos no período pós nascimento, a partir de células germinativas (JOHNSON et al., 2004; WHITE et al., 2012).

Independentemente da existência da neofoliculogênese pós-natal, no momento do nascimento, a população folicular varia entre indivíduos e espécies, entre aproximadamente 1.500 em camundongos (SHAW; ORANRATNACHAI; TROUNSON, 2000) a 2.000.000 na mulher (BAKER, 1963), a qual decresce durante a vida reprodutiva. Apenas 0,1% dos oócitos presentes no ovário alcançam a ovulação, devido principalmente a dois processos: ovulação e atresia ou morte folicular. A atresia, por sua vez, pode ocorrer por duas vias: necrótica ou apoptótica (CLARK et al., 2004).

A necrose se produz como resposta a algum tipo de estresse físico-químico que ocasione a perda da homeostasia (BRAS; QUEENAN; SUSIN, 2005), como por exemplo, um choque térmico, um choque osmótico, um estresse mecânico, isquemia, ou um aumento excessivo das espécies reativas de oxigênio. Nessas circunstâncias, a morte celular se produz de um modo rápido, acidental e não controlado (KRYSKO et al., 2008). Não obstante, alguns fatores como o fator de necrose tumoral (TNF-α) também podem induzir este processo, o que sugere que este processo também pode ser controlado (VANDEN BERGHE; DECLERCQ; VANDENABEELE, 2007). Por outro lado, a apoptose ou morte celular programada é um processo desencadeado a nível genético, o qual depende do balanço na expressão de genes pró e anti-apoptóticos associado a receptores de morte celular localizados na membrana plasmática, ou a nível de sinalização intracelular (via mitocondrial) (HUSSEIN, 2005).

A atresia folicular, apesar de ser um processo natural que controla e limita o número de oócitos fertilizáveis que se liberam do ovário em cada ciclo (CLARK et al., 2004), também reduz significativamente o potencial reprodutivo do animal. Por este motivo, para tentar evitar a enorme perda folicular por atresia, vem sendo aprimorada nas últimas décadas a biotécnica de MOIFOPA.

2.4 A BIOTÉCNICA DE MOIFOPA (OVÁRIO ARTIFICIAL)

A biotécnica de manipulação de oócitos inclusos em folículos ovarianos pré-antrais (MOIFOPA) tem como objetivo reproduzir a foliculogênese *in vitro*, resgatando e cultivando folículos ovarianos pré-antrais evitando sua atresia, para assim obter um grande número de oócitos maduros que possam ser utilizados em outras biotécnicas reprodutivas (FIGUEIREDO et al., 2011).

2.4.1 Sistemas de cultivo *in vitro*

Os folículos podem ser cultivados *in vitro* principalmente de duas formas: *in situ*, ou seja, os folículos são cultivados dentro do tecido ovariano (TANG et al., 2012; ALMEIDA et al., 2015; PAES et al., 2016), ou ainda na forma isolada (SUN; LI, 2013; JORSSEN et al., 2014; FERREIRA et al., 2016). Há ainda a possibilidade de combinar ambos os métodos: cultivar os folículos *in situ* durante as primeiras etapas do desenvolvimento folicular, para em seguida isolá-los assim que alcancem o estádio de folículo secundário e cultivá-los (O'BRIEN, 2003; TELFER et al., 2008).

No sistema de cultivo *in situ*, dependendo do tamanho do ovário, os folículos podem ser cultivados dentro do ovário inteiro (FORTUNE, 2003), ou em finos fragmentos de córtex ovariano. Este sistema tem servido para estudar a ativação folicular e as primeiras etapas da foliculogênese em diferentes espécies, como a ovina (BERTOLDO et al., 2014), a caprina (MAGALHAES-PADILHA et al., 2012), a bovina (PAES et al., 2016) e a humana (ZHANG et al., 2004). O cultivo *in situ* é prático, requer pouco tempo de manipulação e mantém a estrutura tridimensional do folículo. Entretanto, a presença de tecido ovariano pode atuar como barreira física, evitando a correta perfusão de meio para os folículos (MARTINS et al., 2014), impedindo que os folículos se desenvolvam além do estádio de folículo secundário (FORTUNE, 2003). Por outra lado, o cultivo de folículos isolados tem como vantagens permitir o acompanhamento individual do folículo e seu desenvolvimento até a etapa antral (PICTON et al., 2008). Como desvantagens, deve-se considerar a dificuldade no isolamento, bem como permite a recuperação de um número limitado de folículos com a possibilidade que os folículos possam ser danificados durante seu isolamento. Neste sistema de cultivo, os folículos podem ser cultivados de forma bidimensional, sobre superfície plana do próprio plástico da placa de cultivo (APOLLONI et al., 2015), sobre uma monocamada de células somáticas (RAMESH et al., 2008), ou sobre uma matriz extracelular (SÁNCHEZ et al., 2012). Já na forma tridimensional, os folículos são inclusos em uma matriz extracelular, como por exemplo o colágeno (ITOH et al., 2002) e o alginato (BRITO et al., 2014), a fim de preservar sua arquitetura tridimensional.

2.4.2 Fatores que afetam o cultivo *in vitro*

A foliculogênese *in vivo* é um processo biológico extremamente complexo, regulado por vários fatores autócrinos, parácrinos e endócrinos (DEMEESTERE et al., 2005) o quais até o presente momento não são totalmente compreendidos. Devido a isto, reproduzir

este processo *in vitro* não é menos complexo, e durante os últimos anos descreveu-se um grande número de fatores que podem afetar o resultado do cultivo *in vitro* de folículos, tais como o modelo animal (MAGALHÃES et al., 2011b; ROSSETTO et al., 2013; ROCHA et al., 2014), o estado nutricional do animal doador dos ovários (ADAMIAK et al., 2005; FOULADI-NASHTA et al., 2007), o sistema de cultivo (ARAÚJO et al., 2014, 2015; PESSOA et al., 2014), a duração do cultivo (SUN; LI, 2013; PESSOA et al., 2014), a tensão de oxigênio (GIGLI et al., 2006; XU et al., 2011), o meio de base (ROSSETTO et al., 2013b; ARAÚJO et al., 2015) e a suplementação hormonal ou de fatores de crescimento (FERREIRA et al., 2016). Adicionalmente, uma vez que a foliculogênese é um processo dinâmico, é possível que os requerimentos foliculares mudem ao longo do desenvolvimento folicular, tendo em vista a diferença na expressão gênica entre folículos pré-antrais avançados e antrais iniciais caprinos (MAGALHÃES-PADILHA et al., 2013). Diante disso, os requerimentos foliculares de acordo com a fase do desenvolvimento deve ser uma variável a ser considerada no momento do desenvolvimento de um meio de cultivo eficiente para os folículos pré-antrais e antrais. Adicionalmente, a origem do ovário doador dos folículos também pode afetar o resultado da MOIFOPA, isto é a idade das fêmeas (SILVA et al., 2014a) e o status fisiológico (fase do ciclo estral, a condição nutricional) (SOUZA-FABJAN et al., 2014). Neste contexto, trabalhos vêm sendo realizados no sentido de se avaliar o efeito de diferentes dietas incluindo aquelas à base de lipídeos na foliculogênese (MATTOS; STAPLES; TATCHER, 2000; BILBY et al., 2006; FOULADI-NASHTA et al., 2007). A linhaça constitui uma importante fonte de ácidos graxos poli-insaturados (BERNACCHIA; PRETI; VINCI, 2014) e será utilizada na terceira fase do trabalho para avaliação do seu efeito.

2.4.3 Importância da composição do meio de cultivo

Dentre os muitos fatores que afetam o cultivo *in vitro* de folículos, o meio de cultivo é um dos fatores limitantes e mais importantes, já que é responsável pelo aporte de nutrientes, eletrólitos, substratos energéticos, antioxidantes, aminoácidos, vitaminas, hormônios e fatores de crescimento essenciais para manter a viabilidade e o crescimento dos folículos (PICTON et al., 2008).

Têm sido utilizados diferentes meios de base comerciais para o cultivo de folículos em diferentes espécies, como o meio essencial mínimo (MEM) em caprinos (ALMEIDA et al., 2015; SÁ et al., 2017), ovinos (LUZ et al., 2012); Waymouth em murinos (O'BRIEN, 2003), humanos (LARONDA et al., 2014), bovinos (GIGLI; BYRD; FORTUNE,

2006), ovinos (BERTOLDO et al., 2014); TCM 199 em bovinos (ROSSETTO et al., 2013b; ARAÚJO et al., 2015) e caprinos (AMIN et al., 2013); além do meio McCoy em bovinos (JORSSEN et al., 2014) e humanos (TELFER et al., 2008).

Normalmente estes meios de cultivo de base são enriquecidos com outros suplementos que ajudam no desenvolvimento folicular. Por exemplo, a adição de piruvato, hipoxantina, glutamina, ITS (Insulina, transferrina e selênio), além do ácido ascórbico, que ajuda a manter a morfologia folicular, e estimula o crescimento, bem como aumenta a viabilidade de folículos caprinos (SILVA et al., 2004; ROSSETTO et al., 2009) e murinos (MURRAY et al., 2001; DEMEESTERE et al., 2005). Além disso, das sustâncias citadas anteriormente, outros suplementos como hormônios e fatores de crescimento são comumente adicionados aos meios de cultivo, ainda que a efetividade destes para promover o desenvolvimento folicular dependa diretamente de sua concentração e da associação com outros suplementos, assim como do sistema de cultivo e da espécie animal. Dentre os hormônios e fatores de crescimento testados no cultivo *in vitro* de folículos caprinos, pode-se destacar o GH e o VEGF. As seções seguintes abordarão a importância destas sustâncias na foliculogênese.

2.4.3.1 Hormônio do crescimento (GH)

O GH é sintetizado majoritariamente na adenohipófise, sendo então liberado na corrente sanguínea, e transportado por todo o organismo influenciando diversos órgãos/tecidos. Entretanto, o GH pode também ser sintetizado em outros órgãos além da hipófise (pulmões, fígado e ovários) (HARVEY, 2010; CHESNOKOVA et al., 2013), atuando de forma autócrina e parácrina. Trata-se de uma proteína de cadeia simples formada por 191 aminoácidos e duas pontes dissulfeto (CHEN et al., 1991). O GH apresenta dois sítios de ligação para acoplar ao domínio extracelular de seu receptor de membrana (GHR). O acoplamento do GH ao seu receptor leva à dimerização deste, resultando na ativação de sua via de sinalização (CARTER-SU; SCHWARTZ; SMIT, 1996). O GHR pertence a superfamília de receptores de citocinas. Na via de sinalização clássica do GH, cada monômero de GHR se une e ativa uma tirosina quinase citoplasmática, a Janus quinase 2 (JAK2), que por sua vez ativa uma família de fatores de transcrição (STATs). Posteriormente, dímeros de STAT fosforilados se translocam para o núcleo para regular a transcrição gênica. A proteína JAK2 também pode ativar outras vias de sinalização, como a das MAP quinases (MAPK), a da fostatidilinositol 3-quinase (PI3K/Akt), ou a da fosfolipase C/proteína quinase C (PLC/PKC) (ZHU et al., 2001). Adicionalmente, o GHR pode dissociar-se da membrana

plasmática e translocar-se para o núcleo, para regular a transcrição gênica diretamente (SWANSON; KOPCHICK, 2007).

Nas últimas décadas é crescente o desenvolvimento de diversas investigações, as quais têm demonstrado evidências de que este hormônio desempenha um importante papel, seja direta ou indiretamente, sobre a foliculogênese. O GH estimula várias atividades ovarianas como a esteroidogênese, a resposta a gonadotrofinas e a ovulação (HULL; HARVEY, 2014). Seu receptor, GHR, localiza-se em todos os tipos de células ovarianas na maioria das espécies (KOLLE et al., 1998; ABIR et al., 2008; FROTA et al., 2011; MARTINS et al., 2014). Estudos *in vitro* têm demonstrado que o GH estimula a ativação, sobrevivência e crescimento de folículos pré-antrais de camundongos (LIU et al., 1998), ovelhas (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010) e cabras (MARTINS et al., 2010; MAGALHÃES et al., 2011a). Adicionalmente, a adição ao meio de maturação *in vitro* (MIV) promoveu a expansão das células do *cumulus* em macaco *Rhesus* (DE PRADA; VANDEVOORT, 2008), melhorou as taxas de fecundação *in vitro* em humanos (HASSAN et al., 2001), assim como as taxas de maturação oocitária e desenvolvimento embrionário em bovinos (IZADYAR et al., 1998; MTANGO et al., 2003).

Vale destacar que o estágio mais avançado de desenvolvimento embrionário obtido com a biotécnica de MOIFOPA na espécie caprina, o estágio de mórula, foi produzido por meio da suplementação de meio de cultivo com 50 ng/mL de GH (MAGALHÃES et al., 2011a).

2.4.3.2 Fator de crescimento endotelial vascular (VEGF)

A família do fator VEGF consiste em glicoproteínas homodiméricas (FERRARA; HENZEL, 1989) que atuam como citocinas, incluindo o VEGF-A, o VEGF-B, o VEGF-C, o VEGF-D, o VEGF-E e o fator de crescimento placentário (PIGF) (ARAÚJO et al., 2011a). Dentre todos, o mais estudado é o VEGF-A, que é comumente conhecido como VEGF. Seus receptores são do tipo de tirosina quinase, entre os quais estão Flt-1 (VEGFR-1), KDR / Flt-1 (VEGFR-2) e Flt-4 (VEGFR-3) (NEUFELD et al., 1999).

Este fator de crescimento, juntamente com seus receptores, foram identificados em muitos tipos de celulares, incluindo células ovarianas de diferentes mamíferos (ARAÚJO et al., 2011b) como bovinos (YANG; FORTUNE, 2007), ratos (YANG et al., 2008), caprinos (BRUNO et al., 2009), suínos (BARBONI et al., 2000), ovinos (CHOWDHURY et al., 2010) e primatas não humanos (TAYLOR; HILLIER; FRASER, 2004), bem como no fluido

folicular de humanos (FERRARI et al., 2006). No entanto, sua síntese parece ser dependente do estádio folicular durante a foliculogênese, em que seus níveis vão aumentando à medida que o folículo cresce (BARBONI et al., 2000; TAYLOR; HILLIER; FRASER, 2004), passando a ser especialmente expresso a partir do estágio de folículo secundário (TAYLOR; HILLIER; FRASER, 2004; BRUNO et al., 2009).

O VEGF é considerado o fator angiogênico mais poderoso que existe. Além disso, inibe a apoptose e promove a mitose, migração e diferenciação das células endoteliais dos vasos (DVORAK, 2000), bem como o aumento da permeabilidade vascular (REDMER; REYNOLDS, 1996). Essas respostas biológicas são mediadas principalmente pela interação do VEGF-A com seu receptor VEGFR-2, através de uma rede complexa de diferentes vias de sinalização de células interconectadas, como a via MAP quinase (MAPK/Erk), fosfatidilinositol 3-quinase (PI3K/Akt) ou fosfolipase C/proteína quinase C (PLC/PKC) (KOCH; CLAESSON-WELSH, 2012).

A regulação da angiogênese no ovário é muito importante durante a foliculogênese e o VEGF desempenha um papel fundamental neste processo (ROBINSON et al., 2009). Durante a fase pré-antral da foliculogênese, os folículos não possuem irrigação própria e as células que os formam recebem oxigênio e nutrientes através de difusão passiva, oriundos dos vasos sanguíneos do estroma (TAMANINI; DE AMBROGI, 2004). No estádio do folículo secundário, os vasos sanguíneos começam a ser formados por angiogênese nas células da teca, aumentando em número e diâmetro com o subsequente desenvolvimento folicular. Esta aquisição de um fornecimento vascular adequado parece ser o passo limitante na seleção do folículo dominante, pois facilita a entrada no folículo de oxigênio, nutrientes e outras substâncias necessárias ao desenvolvimento folicular, como fatores de crescimento e hormônios (STOUFFER et al., 2001). Nesse sentido, estudos *in vivo* em camundongos mostraram que a administração de VEGF diretamente no ovário aumenta a vascularização e promove o desenvolvimento e sobrevivência (QUINTANA et al., 2004), em contrapartida, quando um inibidor de VEGF foi injetado, as taxas de atresia folicular foram aumentadas (ABRAMOVICH; PARBORELL; TESONE, 2006)

Em estudos *in vitro*, o VEGF inibiu a apoptose das células da granulosa (KOSAKA et al., 2007), estimulou a transição do folículo primário para secundário (YANG; FORTUNE, 2007) e promoveu o crescimento folicular e aumento das taxas de formação de antro em bovinos (ARAÚJO et al., 2014). Em caprinos, esse fator de crescimento manteve a integridade estrutural e estimulou o crescimento folicular (BRUNO et al., 2009; ARAÚJO et

al., 2011b), além de gerar as maiores taxas de maturação de oócitos obtidas pela biotécnica da MOIFOPA até o momento (29,4%) (ARAÚJO et al., 2011b).

2.4.4 Ferramentas e parâmetros para avaliar a eficiência do cultivo *in vitro*

Dependendo do tipo de sistema de cultivo *in vitro* (*in situ* ou isolado) existem diferentes ferramentas e parâmetros para avaliar a sua eficiência. Uma vez que a presente tese se concentra no sistema de cultivo isolado, este item irá abordar os parâmetros de avaliação utilizados neste tipo de cultivo *in vitro*.

O sistema de cultivo isolado permite o acompanhamento individual do desenvolvimento folicular *in vitro*. Esta é uma grande vantagem, já que se utiliza apenas uma ocular micrométrica (aumento 100 X) associada a uma lupa estereoscópica ou um microscópio invertido para avaliar, de forma quantitativa, a morfologia (percentual de folículos intactos, extrusos ou degenerados), as taxas de crescimento e o diâmetro folicular, bem como a formação de antro (SUN; LI, 2013; SÁ et al., 2017). Além disso, após o cultivo folicular, é possível mensurar o diâmetro oocitário, que é um dos parâmetros de avaliação mais importantes, pois está diretamente relacionado com a capacidade do oócito em maturar (FAIR; HYTTEL; GREVE, 1995; CROZET; DAHIREL; GALL, 2000).

O potencial esteroidogênico, ou seja, a capacidade que as células da granulosa e da teca tem de secretar esteroides sexuais, pode ser usado como parâmetro qualitativo de viabilidade folicular. A detecção e a quantificação da produção de hormônios no meio de cultivo folicular, durante e ao final do cultivo, pode ser realizada através de diferentes técnicas como, por exemplo, eletroquimioluminescência (CADENAS et al., 2017), ensaio de imunoabsorção enzimática (ELISA) (BRODZKI et al., 2015) ou radioimunoensaio (RIA) (APOLLONI et al., 2015).

Outra ferramenta para avaliar a viabilidade, tanto folicular quanto oocitária, é a microscopia de fluorescência. Geralmente, os folículos são incubados com sondas fluorescentes como, por exemplo, a calceína-AM e o etídio homodímero. A calceína-AM é clivada por enzimas com atividade esterase no interior das células vivas emitindo fluorescência verde a 486 nm. O etídio homodímero liga-se ao ácido nucléico de células não viáveis com danos de membrana, emitindo fluorescência vermelha a 568 nm (LIMA et al., 2012). Este tipo de microscopia também pode ser utilizada para avaliar o *status* da maturação nuclear oocitária, que é um parâmetro crucial para avaliar a eficiência do sistema de cultivo *in vitro*. Neste caso, os oócitos são incubados com *Hoechst* 33342, o qual penetra no interior da

célula e se intercala entre as bases nitrogenadas do DNA, emitindo fruorescência azul a 483 nm. Desse modo, esta técnica permite classificar o estágio meiótico dos oócitos, segundo sua cromatina, em: vesícula germinal (GV), quebra da vesícula germinal (GVBD), metáfase I (MI) ou metáfase II (MII), em que os três primeiros estágios são considerados imaturos e o último, M II, meioticamente maduro. Também pode-se classificar os oócitos como degenerados (DEG), quando apresentam a configuração da cromatina anormal (NISHIO et al., 2014).

Uma vez que o sistema de cultivo está produzindo oócitos em MII (meioticamente maduros), outro ponto para avaliar a eficiência do sistema de cultivo é o desenvolvimento embrionário. Somente desta forma é possível assegurar que a maturação nuclear está sincronizada à maturação citoplasmática, ou seja, que o sistema de cultivo está sendo capaz de gerar oócitos competentes. Esta avaliação pode ser realizada através da ativação partenogenética (LUZ et al., 2012) ou por fecundação *in vitro* (FIV) (SILVA et al., 2014a), seguidas do cultivo *in vitro* de embriões (CIV).

Finalmente é possível quantificar a expressão relativa de alguns genes relacionados à foliculogênese através da PCR quantitativa em tempo real (qRT-PCR). Esta técnica permite avaliar, por exemplo, a expressão de diferentes enzimas esteroidogênicas nas células da granulosa e da teca (PARRISH et al., 2011; FERREIRA et al., 2016), como por exemplo a CYP17 (responsável pela produção de andrógenos) (YOUNG; MCNEILLY, 2010), a CYP19A1(enzima responsável pela sínteses de estrógenos a partir de andrógenos) (EDSON; NAGARAJA; MATZUK, 2009) e a 3βHSD (responsável pela produção de progesterona entre outros hormônios) (TING; XU; STOUFFER, 2015), bem como a expressão de genes relacionados à diferenciação das células do *cumulus*, como o *Amh* (hormônio anti-mülleriano), e o *LHR* (receptor do hormônio luteinizante) (SÁNCHEZ et al., 2012), ou a competência oocitária, como o *HAS2* (Hialuronano sintase-2) (EPPIG, 2001) e o *PTGS2* (Prostaglandina endoperóxido sintase-2) nas células do *cumulus*, ambos relacionados com a expansão das células do *cumulus* (EPPIG, 2001; XIAO et al., 2015). Esta ferramenta, embora não proporcione uma ideia definitiva sobre a eficiência do cultivo, pode ajudar a compreender os resultados obtidos.

2.4.5 Estado atual da biotécnica de MOIFOPA

Durante as últimas décadas tem se observado um avanço significativo quanto ao cultivo *in vitro* de folículos pré-antrais nas várias espécies. Indiscutivelmente, os melhores

resultados, de forma geral, têm sido obtidos em camundongos, em que foi possível reproduzir por completo a foliculogênese, desde o estágio de folículo primordial até o nascimento de prole viva (O'BRIEN, 2003). No entanto, não foi possível repetir esse resultado em outros mamíferos. A espécie bovina é a que tem apresentado maiores dificuldades, tendo como melhor resultado a obtenção de folículos no estágio antral a partir de folículos primários (SUN; LI, 2013), embora tenham sido capazes de produzir blastocistos (HUANG et al., 2013) e, inclusive, o nascimento de crias vivas a partir de complexos granulosa-oócito obtidos de folículos antrais iniciais (YAMAMOTO et al., 1999; HIRAO et al., 2004). Na espécie humana foi possível produzir oócitos meioticamente maduros a partir de folículos secundários (XIAO et al., 2015). Em ovinos (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010; LUZ et al., 2012), suínos (WU; EMERY; CARRELL, 2001), bubalinos (GUPTA et al., 2008), primatas não humanos (XU et al., 2011, 2013b) e caprinos (SARAIVA et al., 2010; MAGALHÃES et al., 2011a; SILVA et al., 2014a) foi possível produzir oócitos maduros a partir do cultivo de folículos secundários que, após FIV bem sucedida, resultou na produção de embriões *in vitro*. Embora se tenha alcançado o estágio embrionário de mórula a partir de oócitos provenientes de folículos crescidos *in vitro,* as taxas de maturação oocitária ainda são muito baixas quando comparadas com as obtidas a partir de oócitos provenientes de folículos crescidos *in vivo*. Estes resultados, embora promissores, demostram a necessidade de continuar com os estudos, a fim de melhorar a eficiência dos meios e/ou sistemas de cultivo atuais.

3 JUSTIFICATIVA

A biotécnica de MOIFOPA consiste em resgatar folículos pré-antrais dos ovários de mamíferos para cultivá-los *in vitro* com o objetivo de completar a foliculogênese, evitando a atresia, e potencializando a produção de oócitos maduros fertilizáveis. O desenvolvimento desta biotécnica pode ajudar a conhecer o processo de foliculogênese, até agora pouco compreendido, aumentando exponencialmente o potencial reprodutivo de espécies de interesse econômico (FIGUEIREDO et al., 2007), e ainda, preservar a fertilidade em mulheres acometidas com câncer (YIN et al., 2016). No entanto, atualmente o principal problema da MOIFOPA em mamíferos de médio e grande porte são as baixas taxas de maturação dos oócitos e produção de embriões.

Diferentes modelos animais têm sido utilizados para auxiliar no desenvolvimento desta biotécnica, dentre os quais destaca-se o modelo de caprino. Esta espécie tem uma grande importância econômica para a região Nordeste do Brasil, onde encontramos 93% do rebanho (IBGE, 2006), sendo uma importante fonte de carne, leite, queijo e peles. Além disso o caprino é considerado um bom modelo para o ovário humano por suas semelhanças no tempo necessário para completar a foliculogênese (~ 6 meses) (SANTOS et al., 2009) e diâmetros foliculares e oocitários (LUCCI et al., 2001). Portanto, este trabalho busca dar continuidade as diversas investigações realizadas nos últimos anos relacionadas à biotécnica da MOIFOPA na espécie caprina.

Os melhores resultados obtidos até esta data na espécie caprina são 29,4% de oócitos em MII (sendo apenas 5 de 17 oócitos ≥ 110 µm de diâmetro) (ARAÚJO et al., 2011b), e um embrião em estádio de mórula (MAGALHÃES et al., 2011a). Estes resultados foram obtidos utilizando meio de cultivo suplementado com VEGF e GH, respectivamente. Ambos os meios de cultivo foram suplementados com alta concentração de insulina (10 µg/mL) e hormônio folículo-estimulante (FSH) em concentrações crescentes (de 100 a 1000 ng/mL). No entanto, o equilíbrio adequado entre insulina e FSH no cultivo *in vitro* continua a ser um assunto de debate. Alguns autores sugerem que a presença de ambos os hormônios em altas concentrações pode afetar negativamente a competência do oócito (EPPIG et al., 1998; SANCHEZ et al., 2010). Outros autores demonstraram que as concentrações fisiológicas de insulina (10 ng/mL) são mais eficazes na promoção da retomada da meiose (CHAVES et al., 2012).

Para tanto, dentre as diversas variáveis que afetam o cultivo de folículos pré-antrais, nunca foi considerada a natureza dinâmica do processo de foliculogênese. Já foi observada uma grande variação na expressão gênica entre as fases pré-antral avançada e antral inicial (MAGALHÃES-PADILHA et al., 2013), indicando que as necessidades foliculares podem variar ao longo do seu desenvolvimento. Esta pode ser a chave para o desenvolvimento de futuros sistemas de cultivo que reproduzam a foliculogênese completa. Portanto, no presente estudo foi investigado o efeito da adição de GH e VEGF separadamente, associado, ou sequencialmente, na presença de 10 ng/mL de insulina e na ausência de FSH em duas categorias diferentes de folículos: pré-antral avançado e antral inicial.

Ao contrário de folículos pré-antrais, não se sabe qual o período de cultivo apropriado para folículos antrais iniciais. Assim, tanto um período de cultivo curto como um prolongado podem afetar negativamente a maturação oocitária (MARTEIL; RICHARD-PARPAILLON; KUBIAK, 2009). Assim, o período de cultivo *in vitro* para a categoria de folículos antrais iniciais foi estudado neste trabalho. Para esta finalidade, os oócitos foram avaliados antes e depois de diferentes períodos de cultivo folicular (antes da MIV), bem como após a MIV.

Para desenvolver um meio de cultivo eficiente é necessário compreender a dinâmica do crescimento folicular *in vitro*. Neste sentido, tem sido observada uma grande variação individual entre os folículos isolados cultivados nas mesmas condições experimentais (primatas não-humanos (XU et al., 2011), bovinos (JORSSEN et al., 2015), e caprinos (SILVA et al., 2013; APOLLONI et al., 2015). No entanto, não foi possível relacionar os diferentes padrões de crescimento folicular com a maturação dos oócitos *in vitro,* porque depois do cultivo folicular, os oócitos recuperados são normalmente maturados em grupo. Portanto, o desenvolvimento de um sistema de MIV individual permitiria rastrear os oócitos relacionando-os com seus respectivos folículos. Assim, este sistema poderia ajudar a compreender a foliculogênese *in vitro* e descrever padrões de crescimento folicular que possam predizer a maturação oocitária, o que poderia acarretar no aumento significativo da eficiência dos sistemas de cultivo atuais.

Por fim, conforme relatado anteriormente a origem do ovário doador dos folículos pode afetar o resultado da MOIFOPA, sendo o nível nutricional do animal um fator de grande relevância. A importância da suplementação lipídica na dieta é um assunto de grande controversa (ADAMIAK et al., 2005; FOULADI-NASHTA et al., 2007; FERNANDES et al., 2014). A originalidade do presente trabalho embora não tenha sido o foco principal da tese foi

investigar pela primeira vez a influência da linhaça (rica em ácidos graxos poli-insaturados) (BERNACCHIA; PRETI; VINCI, 2014) sobre o desenvolvimento *in vitro* de folículos antrais iniciais, a subsequente fertilização oocitária e o desenvolvimento embrionário inicial *in vitro*.

4 HIPÓTESES CIENTÍFICAS

Diante do exposto foram formuladas as seguintes hipóteses científicas:

a) GH e VEGF tanto associados como adicionados sequencialmente em meio suplementado com insulina em uma concentração fisiológica e sem adição de FSH influenciam no desenvolvimento *in vitro* de folículos isolados caprinos, bem como na secreção de hormônios esteroides e níveis de RNAm para *CYP17*, *CYP19A1*, *3βHSD*, *HAS2*, *PTGS2*, *Amh* e *LHR*;

b) Folículos pré-antrais e antrais iniciais apresentam comportamento diferente quando são submetidos as mesmas condições experimentais;

c) O sistema de MIV individual não afeta negativamente a maturação de oócitos caprinos;

d) A duração do cultivo de folículos antrais iniciais caprinos influencia a taxa de maturação oocitária *in vitro*.

e) O cultivo de folículos antrais iniciais oriundos de animais alimentados com uma dieta rica em ácidos graxos poli-insaturados afeta a fecundação e o desenvolvimento embrionário *in vitro*.

5 OBJETIVOS

5.1 OBJETIVOS GERAIS

a) Avaliar o efeito da adição de GH e VEGF isoladamente ou em associação, bem como na forma fixa ou sequencial sobre o cultivo *in vitro* de folículos pré-antrais e antrais iniciais isolados caprinos;

b) Verificar se folículos pré-antrais avançados e antrais iniciais apresentam comportamento semelhante quando são cultivados nas mesmas condições;

c) Analisar o efeito do sistema de MIV (individual vs. em grupo) sobre a produção de oócitos maturos;

d) Investigar o efeito do tempo de cultivo sobre o desenvolvimento de folículos antrais iniciais isolados caprinos.

e) Verificar se o uso de animais alimentados com uma dieta rica em ácidos graxos poli-insaturados afeta a eficiência da MOIFOPA.

5.2 OBJETIVOS ESPECÍFICOS

a) Investigar os efeitos de GH (50 ng/mL) e VEGF (100 ng/mL) adicionados sozinhos, em associação ou sequencialmente, na presença de baixas concentrações de insulina (10 ng/mL) e ausência de FSH sobre folículos pré-antrais e antrais iniciais isolados caprinos durante 18 dias de cultivo, analisando a morfologia e crescimento folicular, viabilidade e maturação oocitária, produção de estradiol, progesterona e testosterona e ainda os níveis de RNAm para *CYP17*, *CYP19A1*, *3βHSD*, *HAS2*, *PTGS2*, *Amh*, e *LHR*;

b) Analisar se o sistema de MIV individual (1 CCO/10 µL de meio) afeta negativamente a maturação meiótica em comparação com o sistema de MIV em grupo (10 CCOs/100 µL de meio);

c) Avaliar a influência do período de cultivo de folículos antrais iniciais caprinos isolados (12 ou 18 dias) sobre a morfologia e o crescimento folicular, bem como sobre a viabilidade e configuração da cromatina de oócitos antes e depois do cultivo folicular (antes MIV) e após MIV;

d) Correlacionar a dinâmica de crescimento folicular com a viabilidade e maturação oocitária *in vitro*.

e) Investigar o efeito da dieta sobre o desenvolvimento de folículos antrais inicias isolados de caprino, bem como sobre a subsequente fecundação oocitária e desenvolvimento embrionário *in vitro*, analisando a morfologia e o crescimento folicular, a produção de estradiol, o diâmetro oocitário, as taxas de fecundação e as de desenvolvimento embrionário.

6 CAPÍTULO 1

Os requisitos do folículo ovariano caprino diferem entre os estágios pré-antral e antral inicial após cultivo *in vitro* em meio suplementado com GH e VEGF sozinhos ou em combinação.

"Caprine ovarian follicle requirements differ between preantral and early antral stages after *in vitro* culture in medium supplemented with GH and VEGF alone or in combination."

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RESUMO

O objetivo do presente estudo foi avaliar o efeito do hormônio do crescimento (GH) e do fator de crescimento endotelial vascular (VEGF) adicionado de forma isolada, sequencialmente ou em combinação, na presença de insulina na concentração fisiológica (10 ng/mL) no cultivo *in vitro* de duas categorias foliculares diferentes: pré-antral (Experimento 1; Exp.1) e antral inicial (Experimento 2; Exp.2). Os folículos isolados foram cultivados individualmente por 24 (Exp.1) e 18 dias (Exp.2) nos seguintes tratamentos: αMEM + (Controle), ou meio Controle suplementado com 50 ng / mL de GH (GH), 100 ng / mL de VEGF (VEGF), a combinação de ambos (GH + VEGF), GH nos primeiros doze dias e VEGF a partir do dia 12 até o final do cultivo (GH / VEGF) e vice-versa (VEGF / GH). No final do cultivo, os complexos *cumulus*-oócito (CCOs) de folículos cultivados *in vitro* foram recuperados e submetidos a maturação *in vitro* (MIV). Foram avaliados os seguintes parâmetros: morfologia folicular, taxas de crescimento e formação de antro, produção de estradiol, progesterona e testosterona, viabilidade e estágio meiótico dos oócitos, bem como a expressão relativa *de LHR, Amh*, HAS2, *PTGS2*, *CYP17, CYP19A1* e *3βHSD*. Uma quantidade considerável de oócitos viáveis totalmente crescidos foram recuperados após o cultivo *in vitro* de folículos antrais iniciais em todos os tratamentos. No entanto, o tratamento com GH apresentou a maior porcentagem de oócitos totalmente crescidos (60%), diâmetro médio do oócito (117,74 ± 2,61 μm) e retomada da meiose (50%). Além disso, o tratamento com GH produziu maiores ($P < 0,05$) taxas de oócitos em MII do que todos os outros tratamentos, e níveis similares de *LHR*, *Amh* e *PTGS2* ao controle *in vivo*. Ao contrário dos folículos antrais iniciais, os folículos pré-antrais não foram afetados pela suplementação no meio de cultivo. Em conclusão, a adição de GH a um meio de cultivo contendo concentrações fisiológicas de insulina, melhora o crescimento e a maturação dos oócitos após o cultivo i*n vitro* de folículos antrais inicias de cabra.

Palavras-chave: GH; insulina; maturação de oócitos; folículo ovariano; VEGF.

Caprine ovarian follicle requirements differ between preantral and early antral stages after *in vitro* culture in medium supplemented with GH and VEGF alone or in combination.

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Abstract

The aim of the present study was to evaluate the effect of growth hormone (GH) and vascular endothelial growth factor (VEGF) added alone, sequentially or in combination, in the presence of insulin at physiological concentration (10 ng/mL) on the *in vitro* culture of two different follicular categories: preantral (Experiment 1; Exp.1) and early antral (Experiment 2; Exp.2). Isolated follicles were individually cultured for 24 (Exp.1) and 18 days (Exp.2) in the following treatments: αMEM^{+} (Control), or Control medium supplemented with 50 ng/mL GH (GH), 100 ng/mL VEGF (VEGF), the combination of both (GH+VEGF), GH during the first twelve days and VEGF from day 12 until the end of the culture (GH/VEGF) and vice versa (VEGF/GH). At the end of the culture, cumulus-oocyte complexes (COCs) from *in vitro* grown follicles were recovered and subjected to *in vitro* maturation (IVM). The following end points were evaluated: Follicle morphology, growth rates and antrum formation, production of estradiol, progesterone and testosterone, oocyte viability and meiotic stage, as well as relative expression of *LHR*, *Amh*, *HAS2*, *PTGS2*, *CYP17*, *CYP19A1* and *3βHSD*. A considerable amount of viable fully grown oocytes were recovered after the *in vitro* culture of early antral follicles in all treatments. Nevertheless, the GH treatment presented the highest percentage of fully grown oocytes (60%), mean oocyte diameter (117.74 ± 2.61 µm) and meiotic resumption (50%). Furthermore, GH treatment produced higher ($P < 0.05$) rates of MII oocytes than all the other treatments, and similar *LHR*, *Amh* and *PTGS2* transcript levels to *in vivo*. Contrary to early antral follicles, preantral follicles were not affected by medium supplementation. In conclusion, the addition of GH to a culture medium containing physiological concentrations of insulin, improves oocyte growth and maturation after the *in vitro* culture of goat early antral follicles.

Keywords: *GH*; *insulin; oocyte maturation*; *ovarian follicle; VEGF*.

1. Introduction

It is known that mammalian ovaries contain a large population of oocytes, being the vast majority enclosed in preantral follicles (PFs; i.e. primordial, primary and secondary follicles). However, 99.9% of them never reach ovulation, but rather, become atretic during their growth or maturation. A developing biotechnology called *in vitro* follicle culture (IVFC) might increase the number of potentially fertilizable oocytes by recovering, preserving and *in vitro* culturing those follicles, for the purpose of prevention of atresia [1].

The IVFC biotechnology has succeeded in mice, where birth of live offspring was obtained after fertilization of oocytes from *in vitro* cultured PFs [2]. On the other hand, in large animals such an achievement has not been reported. Instead, the results have been limited to the production of a low and variable number of metaphase II (MII) oocytes and embryos [3–6].

In caprine, the highest MII oocyte rate obtained from PFs grown *in vitro* so far is 29.4% [7], and the latest stage of embryo development attained is morulae, which was also the only embryo produced in that study [5]. Those results were obtained using culture medium supplemented with vascular endothelial growth factor (VEGF), and growth hormone (GH), respectively. Both culture media contained high insulin concentration (10 µg/mL) and follicle stimulating hormone (FSH) in increasing concentrations (from 100 to 1000 ng/mL). Nonetheless, the proper balance between FSH and insulin concentration *in vitro* is still under discussion. Chaves et al. [8] reported that a lower concentration of insulin (10 ng/mL) was more efficient in promoting meiotic resumption. This finding is consistent with the idea that the combination of both, FSH and insulin in high concentration, impacts oocyte competence and induces abnormal patterns of expression of genes related to oocyte maturation and cumulus cells differentiation, as already suggested by other authors [9,10].

Despite the improvements made on IVFC in caprine in the last years, the current *in vitro* systems are still unable to produce an amount of MII oocytes similar to those from follicles grown *in vivo*. One possible reason could be that follicle requirements are continuously changing throughout the culture period. Magalhães-Padilha et al. [11] pointed out this by microarray analysis; they observed the temporal changes in transcriptional profiles of secondary and early antral follicles in caprine ovaries. In this study, gene expression profiles showed that three major metabolic pathways (lipid metabolism, cell death, and hematological system) were significantly differentiated between the two follicle stages.

Consequently, we infer that secondary and early antral follicles behave differently under the same culture conditions; hence, they have different supplementation needs *in vitro*. This fact could be a key factor in order to develop a future culture system that reproduces complete folliculogenesis *in vitro*.

Based on the information above, the aim of this study was to test for the first time the effects of GH and VEGF alone, in association or sequentially, in the presence of low concentration of insulin (10 ng/mL) and absence of FSH on two different follicular categories: caprine preantral and early antral follicles. The following end points will be evaluated: (i) follicular growth and morphology, (ii) oocyte maturation and viability, (iii) estradiol, progesterone and testosterone production, and (iv) gene expression levels of: *CYP17*, *CYP19A1* and *3βHSD* in follicle walls, and *HAS2*, *PTGS2*, *Amh* and *LHR* in cumulus cells.

2. Materials and methods

All experiments were performed according to the recommendations of the Committee of Animal Handling and Ethical Regulation from the State University of Ceara, Fortaleza, Ceara, Brazil.

2.1 Chemicals and media

Unless mentioned otherwise, the reagents and chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

2.2 Source of ovaries

Ovaries from 48 adult mixed-breed goats (1 to 3 years old) were collected at a local abattoir, and used to perform experiments 1 (24 pairs) and 2 (24 pairs). Immediately after the slaughter, the ovaries were immersed in 70% alcohol, followed by two washes in minimum essential medium (MEM) plus HEPES (MEM HEPES), supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin, and then transported to the laboratory at 4 °C [12] within 3 h since they were collected.

2.3 Isolation and selection of caprine preantral and early antral follicles

In the laboratory, the surrounding fat and connective tissue were removed from the ovaries. Ovarian cortical slices (1 mm thick) were cut using a surgical blade, and placed in holding medium (MEM-HEPES) and then, preantral and early antral follicles were isolated. Two experiments were performed: Experiment 1 (Exp.1) with preantral follicles and

Experiment 2 (Exp.2) with early antral follicles. For Exp.1, preantral follicles (100 to 200 μm) were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan), manually dissected from the slices of ovarian cortex using 26-gauge (26-G) needles, and transferred to the culture medium for further evaluation of follicular quality. Follicles with a visible central oocyte, surrounded by two or more granulosa cell layers and with an intact basement membrane and no antral cavity, were selected for *in vitro* culture. In Exp.2, the procedure was the same as in the previous experiment, with the difference that only early antral follicles (300 to 400 μm) were selected for *in vitro* culture.

2.4 In vitro culture of caprine preantral and early antral follicles

Immediately after follicle isolation, the isolated follicles from all animals were pooled and randomly assigned to the six treatments, so every treatment is supposed to have follicles from all animals (Fig.1). A total amount of 240 preantral (Exp.1) and 253 early antral follicles (Exp.2) were used in experiment 1 and 2, respectively. Regardless of the experiment, follicles were individually cultured in 100 μL drops of culture medium on Petri dishes under mineral oil (60×15 mm; Corning, USA) for 24 days (Exp.1) or 18 days (Exp.2). Since in our culture system antrum formation begins by day 6 of culture, for Exp.2, whose follicles already had antrum at day 0, we decided to diminish the culture time in 6 days. The base medium used was α-MEM (pH 7.2 to 7.4), supplemented with 3 mg/mL bovine serum albumin (BSA), 10 ng/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine and 50 μg/mL ascorbic acid, referred as α-MEM^{+} (Control treatment). In both experiments (preantral and early antral follicles), medium was supplemented with 50 ng/mL bovine GH from bovine pituitary gland (GH) (MP Biomedicals, Solon, OH, USA; Purity ≥ 97%) and 100 ng/mL human recombinant VEGF-A_{165} expressed in *Escherichia coli* (VEGF) alone, in association or sequentially (Fig.1). The culture was carried out at 39°C in 5% CO_2. Fresh media were prepared and pre-equilibrated for 1 h prior to use. Medium was replaced partially (60 μL) in all treatments every other day, and totally (100 μL) on day 12, only in the sequential treatments. The concentrations of GH and VEGF were chosen based on previous studies performed in our laboratory [5,7]. The experiments were replicated four times and at least 40 follicles were used per treatment (approximately, 10 follicles/replicate/treatment).

2.5 Morphological evaluation of follicle development

Follicles were deemed degenerated if they presented darkened oocyte and/or misshapen surrounding granulosa cells. Follicle diameter and antrum formation were

evaluated only in morphologically intact follicles every 6 days of culture. The follicular diameter was calculated as the mean of two perpendicular measures of each follicle, using an ocular micrometer attached to a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; X 100 magnification). Follicle growth rates were expressed as follows: i) the diameter of viable follicles on Day 12 of culture minus the diameter of viable follicles on Day 0, divided by the total number of viable follicles on Day 12 (D0 to D12) (after which sequential treatments had GH replaced with VEGF and vice versa), ii) the diameter of viable follicles at the end of the culture minus the diameter of viable follicles on Day 12, divided by the total number of viable follicles at the end of the culture (D12 to D24 (Exp.1); D12 to D18 (Exp.2)), iii) the diameter of viable follicles at the end of the culture minus the diameter of viable follicles on Day 0, divided by the total number of viable follicles at the end of the culture (Overall).

Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers.

2.6 Hormonal Assay

To evaluate the relationship between follicle development and hormone production, the conditioned culture media were collected on days 6 and 18 of *in vitro* culture and stored at -80°C for measurement of estradiol (E2), progesterone (P4) and testosterone (T) levels. All steroidal determinations were performed at the Laboratory for Hormonology and Tumormarkers, UZ Brussel, Brussels, Belgium. All conditioned media used had prior been checked for linearity upon dilution and matrix effects. For all hormone assays, Electrochemiluminescence Immunoassay (COBAS 6000; Roche Diagnostics, Belgium) was used. The sensitivity and total precision were: 10 ng/L and < 6% for E2, 0.1 μg/L < 4% for P4, and 0.1 μg/L and < 5% for T, respectively.

2.7 In vitro maturation of caprine oocytes from preantral and early antral follicles cultured in vitro

After the culture, all morphologically intact follicles were mechanically opened with 26-G needles under a stereomicroscope. Only oocytes (≥110 μm) with homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for *in vitro* maturation (IVM) [13]. The percentage of fully grown oocytes was calculated by dividing the number of oocytes ≥110 μm (zona pellucida not included) by the number of viable follicles at the end of the culture and multiplying this value by 100. The selected COCs were pooled by treatment and washed three times in IVM medium, which consists of tissue

culture medium 199 (TCM 199) supplemented with 1 μg/mL 17β-estradiol, 5 μg/mL luteinizing hormone (LH), 0.5 μg/mL rFSH (bovine), 10 ng/mL epidermal growth factor (EGF), 1 mg/mL BSA, 1 mM pyruvate, 50 ng/mL insulin-like growth factor 1 (IGF-I), and 100 μM cysteamine. After being washed, COCs were transferred to fresh 100-μl drops of IVM medium (approximately 10 COCs per drop) on culture dishes (30 x 15 mm) under mineral oil and then incubated for 32 h at 39°C with 5% CO_2.

2.8 Assessment of oocyte viability and chromatin configuration

Oocyte viability and chromatin configuration were assessed by fluorescence microscopy (Eclipse 80i, Nikon, Tokyo, Japan). After IVM, oocytes were mechanically denuded and incubated for 30 min in 100 μL of PBS supplemented with 4 mM calcein-AM and 2 mM ethidium homodimer-1, (Molecular Probes - LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells – L3224, Invitrogen, Karlsruhe, Germany), 10 mM Hoescht 33342 and 0.5% glutaraldehyde. Oocytes were classified as viable when cytoplasm stained with calcein-AM (green; emission at 488 nm) and chromatin did not label with ethidium homodimer-1 (red; emission at 568 nm). Oocyte chromatin was stained by Hoescht 33342 (emission at 483 nm) and classified as germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase I (MI) and metaphase II (MII) (meiotic resumption: GVBD, MI and MII).

2.9 Cumulus cells recovery

After IVM, COCs originating from *in vitro* grown follicles as well as *in vivo* grown follicles (positive control) were mechanically denuded by gentle pipetting. Cumulus cells were recovered and centrifuged at 12000 G for 5 min at 4 °C. Supernatant was carefully discarded, and 100 μL Trizol were added to pellet and frozen at - 80 ºC for further quantitative real-time PCR analysis (qPCR).

2.10 Quantitative real-time PCR analysis for LHR, Amh, HAS2 and PTGS2 in cumulus cells, and CYP19A1, CYP17, and 3βHSD in follicular walls

For RNA isolation: three pools of 10 viable follicular walls (granulosa and theca cells) were collected from antral follicles of each experimental group after 24 or 18 days of culture (Exp.1 and 2, respectively); also, after IVM, cumulus cells were pooled per treatment and replication (4 pools / treatment). Follicles grown *in vivo*, with the same final diameter of experiments 1 and 2 were isolated, oocytectomized, and served as *in vivo* controls.

Samples were stored in microcentrifuge tubes (1.5 mL) with 100 µL Trizol at -80 ºC. Total RNA from follicular walls and cumulus cells was isolated and purified with Trizol® Plus Purification kit (Invitrogen, São Paulo, Brazil). The RNA preparations were treated with DNase I and Pure Link RNA Mini Kit (Invitrogen, São Paulo, Brazil). Complementary DNA (cDNA) was synthesized from the isolated RNA using Superscript II RNase H-Reverse Transcriptase (Invitrogen, São Paulo, Brazil). The qPCR reaction was performed in a final volume of 20 µL, containing 1 µL of each cDNA, 1 x Power SYBR Green PCR Master Mix (10 µL) (PE Applied Biosystems, Foster City, CA, USA), 5.5 µL of ultrapure water, and 0.5 µM of both sense and anti-sense primers. The gene-specific primers used for the amplification of different transcripts are shown in Table 1. Transcript levels in follicles and cumulus cells were normalized to the content of peptidylprolyl isomerase I (*PPIA)* and glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH).* Primer specificity and amplification efficiency were verified for each gene. The RT-PCR cycling conditions consisted of an initial denaturation and polymerase activation step at 94 ºC for 15 min, followed by 40 cycles of 15 s at 94 ºC, 30 s at 60 ºC, and 45 s at 72 ºC, and then a final extension for 10 min at 72º C. After amplification, melting curve analysis was performed between 60 ºC and 95 ºC for all genes. All amplifications were carried out in a Bio-Rad iQ5 (Hercules, CA, USA). The delta-delta-CT method was used to transform threshold cycle values into normalized relative expression levels [14].

2.11 Statistical analysis

All statistical analyses were performed using Sigma Plot 11 (Systat Software Inc., USA). Data for end points that were not normally distributed (Shapiro-Wilk test) were submitted to logarithmic transformation. Comparison of means (follicle and oocyte diameter, follicular growth rate, and hormones levels) among treatments were compared by Kruskal-Wallis test, while the Wilcoxon signed test was used to analyze the effect of treatment within days of culture. The proportion of follicular variables (morphologically intact, antrum formation, viability, and meiotic resumption) among treatments and days of culture were analyzed by Fisher's exact test. Differences among treatments from the data obtained by qPCR were determined using the Mann-Whitney test. Data are presented as mean (±SEM) and percentage and the results were considered different when $P < 0.05$. Probability values > 0.05 and ≤ 0.1 indicated that a difference approached significance.

3. Results

3.1 Morphology and growth of caprine preantral (Exp.1) and early antral (Exp.2) follicles

The percentage of morphologically intact follicles during the culture period decreased ($P < 0.05$) in all treatments at the end of the culture when compared to Day 0, however the VEGF/GH treatment was significantly lower ($P < 0.05$) than controls on Day 12 (Fig.2).

Follicle diameters increased over time in all treatments and experiments (Fig.3). In Exp.1, preantral follicles in VEGF treatment showed lower ($P < 0.05$) diameters than control treatment on Day 12. At the end of the culture, the diameter of preantral follicles in GH+VEGF treatment was significantly greater than in VEGF treatment and tended to be higher ($P = 0.07$) than control treatment (Fig.3A). In Exp.2, no differences were observed among treatments at any evaluated time point (Fig.3B).

Growth rate of preantral follicles in the first interval was significantly lower in VEGF treatment than the other treatments except for VEGF/GH. The overall growth rate was lower ($P < 0.05$) in VEGF treatment than in control treatment. Moreover, when comparing between intervals, VEGF and VEGF/GH treatments grew more ($P < 0.05$) in the second interval (Fig.4A). In Exp. 2 (early antral follicles), the growth rate was lower ($P < 0.05$) in GH+VEGF treatment than in control and GH treatments only in the first interval (Fig.4B).

All treatments presented lower ($P < 0.05$) percentage of antrum formation than control treatment, except GH treatment on Days 12 and 24. Furthermore, from Day 12 to Day 24, antrum formation increased only in VEGF and VEGF/GH treatments (Table 2).

3.2 Levels of estradiol, progesterone and testosterone during the in vitro culture of isolated caprine preantral and early antral follicles

All three steroid hormones were produced by preantral and early antral follicles on Days 6 and 18 of culture, independent of treatment (Tables 3 and 4). Differences among treatments were found only in preantral follicles, where VEGF and VEGF/GH treatments on Day 6 produced significantly lower E2 than the rest of the treatments. On Day 6, the levels of P4 were higher ($P < 0.05$) in GH and GH/VEGF treatments than in control and GH+VEGF treatments. Moreover, those treatments with VEGF either alone or in combination (VEGF, VEGF/GH and GH+VEGF treatments) on Day 6 produced higher ($P < 0.05$) levels of T than treatments with GH alone (GH and GH/VEGF treatments). On Day 18, GH/VEGF, VEGF/GH and GH+VEGF treatments produced higher ($P < 0.05$) T levels than control treatment (Table 3).

When the culture periods were compared within the same treatment using preantral follicles, GH treatment and treatments with VEGF from the beginning (VEGF, GH+VEGF and VEGF/GH) had elevated ($P < 0.05$) E2 from Day 6 to Day 18 (Table 3). In early antral follicles, GH/VEGF and GH+VEGF treatments had greater ($P < 0.05$) levels of T from Day 6 to Day 18 of culture (Table 4).

3.3 Recovery rate and chromatin configuration of oocytes from both caprine preantral and early antral follicles grown in vitro

The percentage of fully grown oocytes (≥ 110 μm) and meiotic stages after IVM from Exp.1 (preantral) and Exp.2 (early antral) are displayed in Tables 5 and 6, respectively. After 24 days of *in vitro* culture of preantral follicles, only a few oocytes (n = 8) from control, GH and GH+VEGF treatments grew to a minimum of 110 μm in diameter. Of these 8 oocytes, 4 remained viable from GH and GH+VEGF, and were able to resume meiosis, and two reached MII (Table 5).

After *in vitro* culture of early antral follicles, a considerable amount of fully grown oocytes were recovered from all treatments. This parameter tended to be higher ($P = 0.06$) in GH treatment than in control. Furthermore, mean oocyte diameter was higher ($P < 0.05$) in GH and GH/VEGF treatments than in control and VEGF treatments. The vast majority of viable oocytes greater than 110 μm resumed meiosis after IVM, with the highest rate in the GH treatment (50%), greater ($P < 0.05$) than control (29.6%) and GH+VEGF (22.5%) treatments. In addition, GH treatment presented higher ($P < 0.05$) percentage of MII oocytes than all treatments (42.5 %) (Table 6). In the *in vivo* control, the rate of MII oocytes was 73% (data not shown). Considering that all *in vivo* grown oocytes were viable fully grown oocytes, assuming that only the viable oocytes ≥ 110 μm are used to calculate the MII rate for GH treatment, the percentage of oocyte maturation in this treatment is 81%, which is very similar to the *in vivo* grown oocytes.

3.4 Gene expression in follicular walls

The relative expression of three steroidogenic enzymes *3βHSD, CYP17, CYP19A1* was measured in follicular walls (theca and mural granulosa cells) at the conclusion of the *in vitro* culture of preantral (Exp.1) and early antral follicles (Exp.2) (Fig.5). After preantral follicle culture, *3βHSD* was significantly more expressed in VEGF and GH/VEGF treatments than in control treatment (Fig.5A) while for early antral follicles, there were no differences among

treatments (Fig.5D). The mRNA expression of *CYP17* did not differ among treatments regardless the experiment, although it was lower ($P < 0.05$) in GH and control treatments for Exp.1 when compared with *in vivo* expression (Fig.5B), and similar to GH+VEGF for Exp.2 (Fig.5E). Finally, independent of the experiment, the mRNA levels for *CYP19A1* did not differ among treatments, and all treatments were similar to *in vivo*, except control and GH+VEGF treatments for Exp.1 (Fig.5C). In Exp.2, all cultured treatments differed ($P < 0.05$) from *in vivo* except for GH/VEGF treatment (Fig.5F).

3.5 Gene expression in cumulus cells

The mRNA levels of *LHR*, *Amh*, *HAS2* and *PTGS2* were quantified in both experiments in cumulus cells recovered from COCs after their IVM (Fig.6). *LHR* levels were equivalent to *in vivo* in control and GH treatments after preantral follicle culture (Fig.6A), and only in GH treatment after early antral follicle culture (Fig.6E). Expression of *Amh* was similar to *in vivo* in the control and GH+VEGF treatments for Exp.1 (Fig.6B), and in the control, GH and VEGF treatments for Exp.2 (Fig.6F). For *HAS2*, the mRNA levels were similar between the control and VEGF treatments and *in vivo* in both experiments (Figures 6C and 6D). Lastly, *PTGS2* expression was only detected *in vivo* in Exp.1 (Fig.6D), while in Exp.2 GH, VEGF and GH+VEGF treatments expressed similar levels to *in vivo* (Fig.6H).

4. Discussion

The present study developed an efficacious culture medium for the *in vitro* culture of isolated caprine early antral follicles, and demonstrated for the first time that preantral and early antral follicles behave differently under the same culture conditions. This approach may be very useful to develop a sequential medium for late secondary follicles and early antral follicles.

In our study, the percentage of morphologically intact follicles decreased at the end of the culture compared to Day 0 regardless of the treatment in both experiments, as observed in previous published papers with the same culture period [15].

All treatments increased follicle diameter in both experiments although statistical differences among them were found only in Exp.1 (Preantral follicles); here, VEGF treatment showed lower diameter than Control, GH and GH+VEGF treatments on Day 12. At the end of the culture, the association GH+VEGF presented higher diameter than VEGF alone, and tended to differ from Control and sequential treatments. This fact might be explained by the

cell-proliferation-related pathways that both substances share, such as MAPK/Erk, PLC-ɣ, and PI3-K [16–19].

Growth rate was calculated by dividing the culture period into two intervals which coincided with the sequential treatment media changes. There were no differences among medium supplements to improve either growth or antrum formation in preantral follicles. This result might be due to the composition of the Control medium, which contains among others, physiological concentrations of insulin, and showed better results in a previous study performed by our team [8]. Preantral follicles in the VEGF treatment showed lower growth rate than all other treatments in the first interval excluding VEGF/GH, but recovered in the second one, being higher than GH/VEGF. Interestingly, it draws our attention that treatments containing VEGF from the beginning (VEGF and VEGF/GH treatments) grew more in the second interval, while in the rest of treatments the growth was homogeneous, increasing at the same average pace throughout the *in vitro* culture. Similar results were observed on antrum formation and E2 production. In addition, the low E2 levels produced by these treatments on Day 6 were accompanied by high T levels, which might indicate that T was not converted into E2. It seems that in goats, VEGF is more important for antral than preantral follicle development, which is consistent with the observations of Exp.2 (early antral follicles), where no differences between the two culture intervals were observed. To confirm this statement in rats, it has been shown that the protein expression of its receptor (VEGFR2) is very low in preantral follicles and strong in antral follicles, i.e., VEGFR-2 increases during follicular development [20].

Results with GH and VEGF alone in the present study differed from previous papers that reported the beneficial effects of supplementing culture media with those substances on preantral follicle development [5–7]. However, unlike in our system (no FSH and low insulin; 10 ng/mL), those authors used medium containing FSH in increasing concentrations and higher insulin concentration (10 μg/mL). This fact could be important since previous studies in mice described that VEGF and its receptor (VEGFR-2) are essential for gonadotropin-dependent follicle development [21].

Only a few oocytes originating from preantral follicles cultured *in vitro* in the presence of GH either alone or combined with VEGF, were capable to grow to a minimum of 110 μm (fully grown) and remain viable. Additionally, a majority of those were able to resume meiosis and even reach metaphase II. Conversely, a considerable number of viable, fully

grown oocytes were recovered after the *in vitro* culture of early antral follicles no matter the treatment. Notwithstanding, it can be highlighted the GH treatment, because this treatments resulted in the highest percentage of fully grown oocytes (60%), mean oocyte diameter (117 ± 2.61 μm), meiotic resumption (50%) and rate of MII oocytes (42.5%). It is important to clarify that these values were calculated out of the total amount of follicles cultured *in vitro.* Therefore, if one considers only viable oocytes greater than 110 μm, the MII rate in this treatment amount 81%, which is similar to that obtained after the IVM of *in vivo* grown oocytes in our study (73%). In other words, the ability of fully grown oocytes, from *in vivo* and *in vitro* grown follicles, to maturity *in vitro* was similar, at least with respect to oocyte nuclear maturation. GH is an important factor for the survival and growth of ovarian follicles in several species [5,22,23]. It has been described that its direct action could be mediated by its receptor (GH-R), which is present in caprine antral follicles but not preantral, although it is present in the somatic ovarian cells [24]. In our culture system, preantral follicles are isolated together with a small amount of ovarian tissue in order to maintain basement membrane integrity. Hence, GH could act indirectly on preantral follicles via somatic cells. As a matter of fact, the few oocytes from *in vitro* culture of preantral follicles (Exp.1) that resumed meiosis were cultured in the presence of GH from the beginning (GH and GH+VEGF treatments), although this hormone did not improve significantly any evaluated end point. Recent data suggest that isolated caprine preantral follicles cultured *in vitro* in the presence of 50 ng/mL GH, should be supplemented with 100 μg/mL FSH and an elevated insulin concentration (10 μg/mL) [25]. Nonetheless, based on our findings, unlike preantral follicles, early antral follicles might not require FSH supplementation in the presence of GH *in vitro*.

Several genes have been identified in cumulus cells as biomarkers to evaluate the efficiency of a culture system. Among them, *Amh* and *LHR* are associated with cumulus cell differentiation [26,27]; and *HAS2* and *PTGS2* are related to oocyte maturation [28,29]. We observed that after the IVM of COCs from early antral follicles cultured *in vitro* in the GH treatment, cumulus cells expressed similar *LHR*, *Amh* and *PTGS2* transcript levels to those from COCs originating from antral follicles grown *in vivo*. Therefore, the gene expression similarity for key factors related to cumulus cells differentiation and oocyte maturation between the *in vitro* and *in vivo* COCs may support the use of GH for the *in vitro* culture of isolated early antral follicles.

4.1 Conclusions

The present paper clearly proves that caprine preantral and early antral follicles behave differently under the same culture conditions. Furthermore, a base medium with insulin at physiological concentration (10 ng/mL), associated with 50 ng/mL GH is able to maintain growth and maturation of oocytes from isolated early antral follicles cultured *in vitro* to levels similar to their *in vivo* counterparts.

Acknowledgments

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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.

Treatment identification	α-MEM+ supplementation		
	Day 0	Day 12	Day 24 (Exp.1) / Day 18 (Exp.2)
Control	None	None	
GH	GH	GH	
VEGF	VEGF	VEGF	
GH+ VEGF	GH+VEGF	GH+VEGF	
GH/VEGF	GH	VEGF	
VEGF/GH	VEGF	GH	

Fig.1. Treatments were tested according to medium supplementation for IVC of isolated caprine preantral follicles and early antral follicles. GH, growth hormone; MEM, minimum essential medium; VEGF, vascular endothelial growth factor.

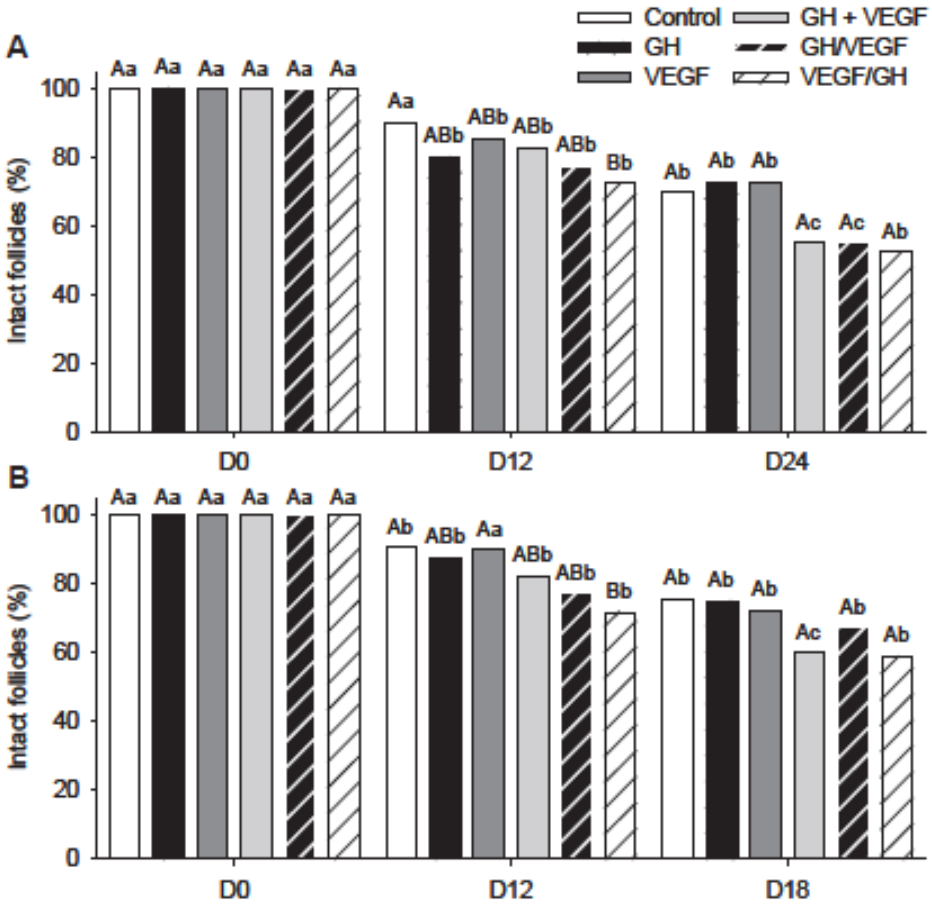

Fig.2. Percentage of caprine morphologically intact preantral follicles (A) and early antral follicles (B) throughout IVC in αMEM+ (Control), supplemented with GH and VEGF alone, in combination, or sequentially for 24 days and 18 days, respectively. A,B Differs among treatments within the same day of culture ($P < 0.05$). a,b,c Differs among days of culture within the same treatment ($P < 0.05$). GH, growth hormone; MEM, minimum essential medium; VEGF, vascular endothelial growth factor.

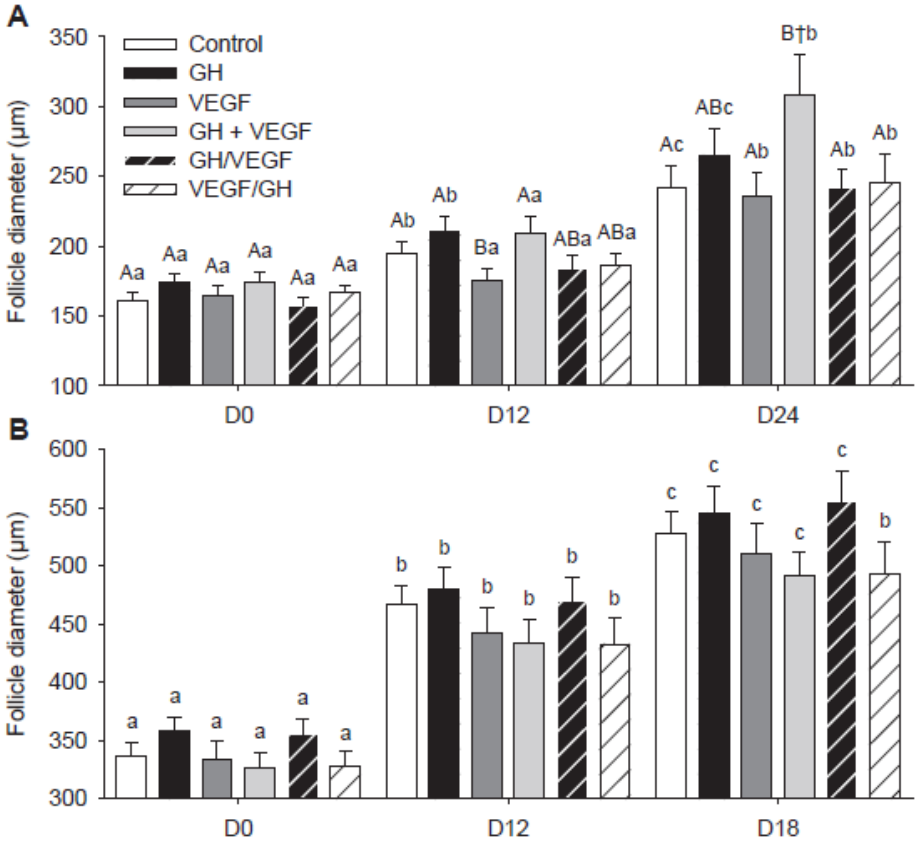

Fig.3. Mean (± standard error of the mean) diameter (µm) of caprine preantral follicles (A) and early antral follicles (B) cultured in αMEM^{+} (Control), supplemented with GH and VEGF alone, in combination, or sequentially for 24 days and 18 days, respectively. ^{A,B} Differs among treatments within the same day of culture ($P < 0.05$). ^{a,b,c} Differs among days of culture within the same treatment ($P < 0.05$). † Tended to differ from Control ($P = 0.07$), GH/VEGF ($P = 0.08$), and VEGF/GH ($P = 0.09$). GH, growth hormone; MEM, minimum essential medium; VEGF, vascular endothelial growth factor.

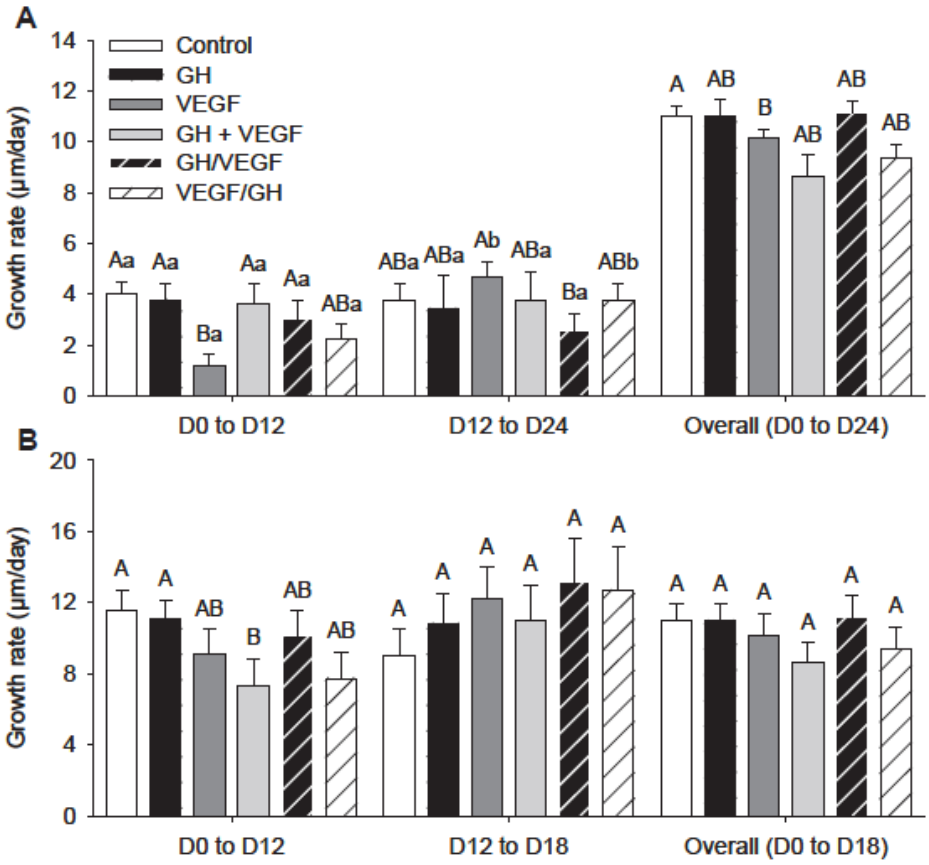

Fig.4. Mean (± standard error of the mean) follicular growth rate (µm/day) of caprine preantral follicles (A) and early antral follicles (B) cultured in αMEM^{+} (Control), supplemented with GH and VEGF alone, in combination, or sequentially for 24 days and 18 days, respectively, in different culture intervals. ^{A,B} Differs among treatments within the same culture interval ($P < 0.05$). ^{a,b} Significant differences among culture intervals within the same treatment ($P < 0.05$). GH, growth hormone; MEM, minimum essential medium; VEGF, vascular endothelial growth factor.

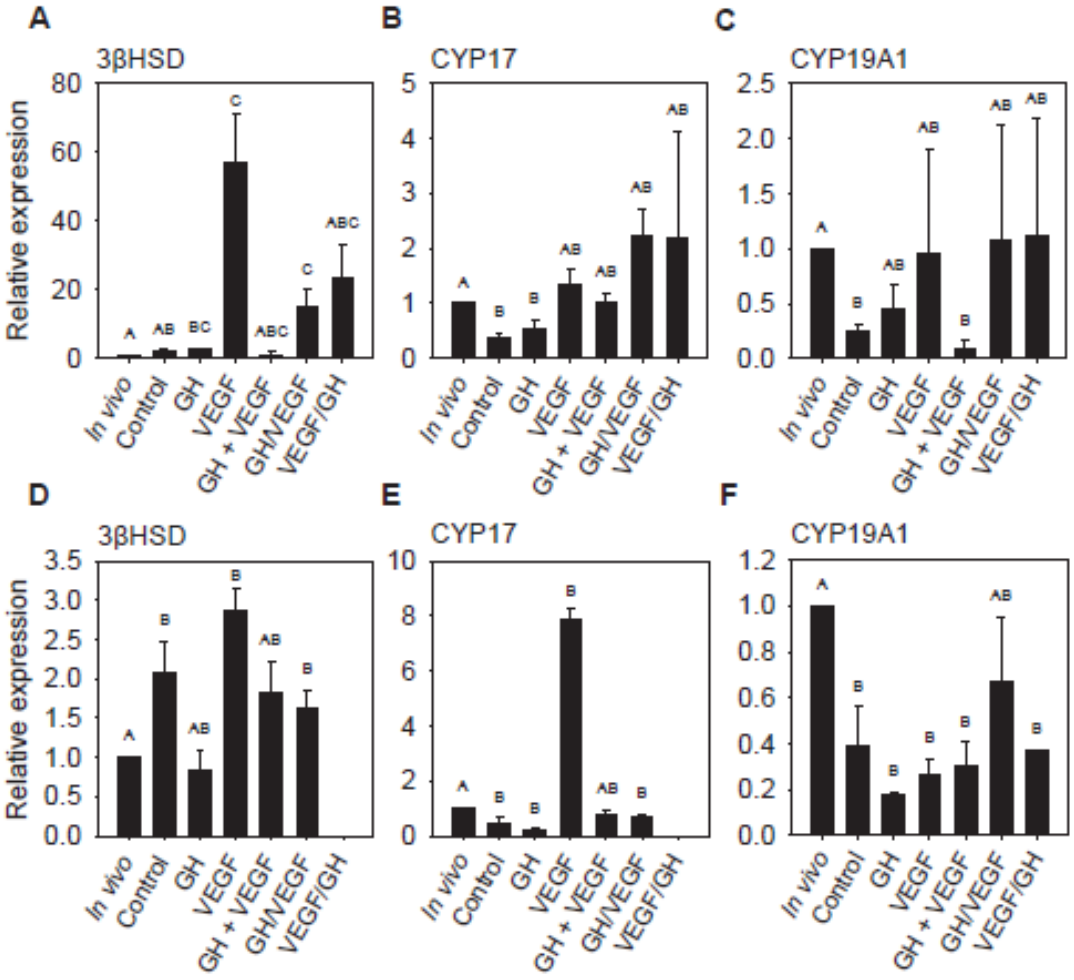

Fig. 5. Relative mean (± standard error of the mean) expression of mRNA of *3βHSD* (A and D), *CYP17* (B and E), and *CYP19A1* (C and F) in caprine preantral (A–C) and early antral (D–F) follicles cultured in αMEM$^+$ (Control) supplemented with GH and VEGF alone, in combination, or sequentially (n = 3). Different letters denote significant differences ($P < 0.05$). GH, growth hormone; MEM, minimum essential medium; VEGF, vascular endothelial growth factor.

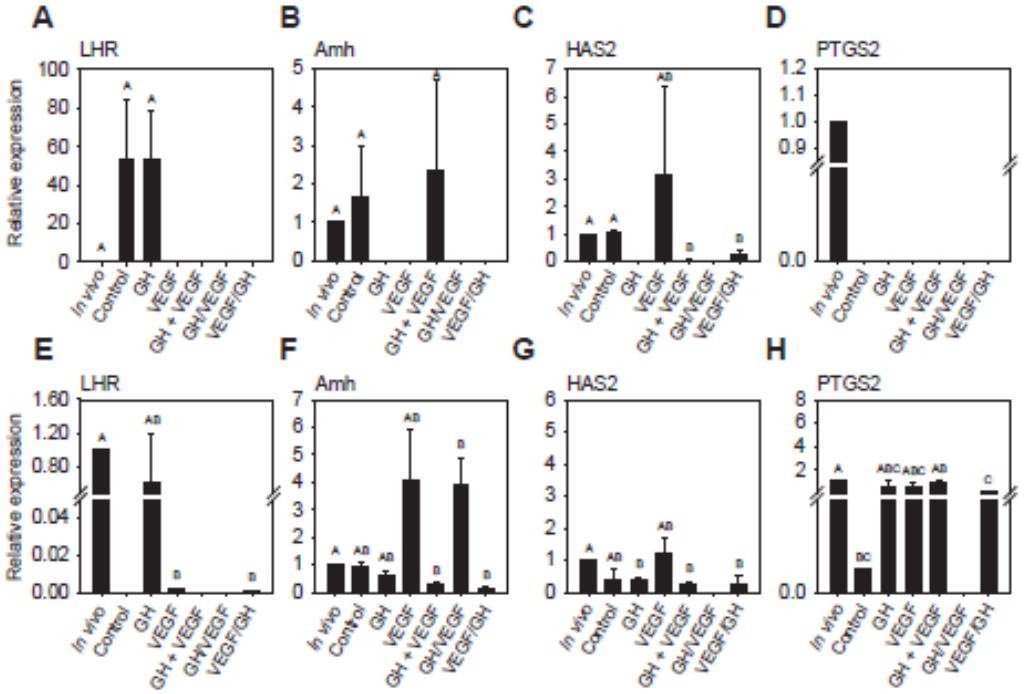

Fig. 6. Relative mean (± standard error of the mean) expression of mRNA of of *LHR* (A and E), *Amh* (B and F), *HAS2* (C and G), and *PTGS2* (D and H) from cumulus cells recovered from COCs from preantral (A–D) and early antral follicles (E–H) cultured in αMEM^{+} (Control) supplemented with GH and VEGF alone, in combination, or sequentially (n = 3) after IVM. Different letters denote significant differences ($P < 0.05$). GH, growth hormone; MEM, minimum essential medium; VEGF, vascular endothelial growth factor.

Table 1
Oligonucleotide primers used for PCR analysis

Target gene	Primer sequence (5'→3')	Orientation	Genbank Accession no.
LHR (Luteinizing hormone/choriogonadotropin receptor)	CTGGATGCCACACTGACTTACC TTTGGTCTCCTTGCTGTGCTT	Sense Anti-sense	GI:936975959 *(Capra hircus)*
Amh (Anti-Müllerian hormone)	AGCGGGACACTCATCTTTCAG GGTCAAGTCACTCAGGCCC	Sense Anti-sense	GI:926696403 (*Capra hircus*)
HAS2 (hyaluronan syshase 2)	CTCACAGCATGTCACCCAGT GGGTCAAGCATGGTGTCTGA	Sense Anti-sense	GI:926708392 *(Capra hircus)*
PTGS2 (Prostaglandin-endoperoxide synthase 2)	TGGGTTTAAGGCTGCATGGT GTTCTCTGTGGGCTGGTGAA	Sense Anti-sense	GI:926711906 *(Capra hircus)*
CYP17 (Cytochrome P450 17alpha-hydroxilase / 17, 20 lyase	ACTGAATGCCTTTGCCCTGT CTGATTATGTTGGTGATCC	Sense Anti-sense	GI:7649267 *(Capra hircus)*
CYP19A1 (Cytochrome P450, family 19, subfamily A, polypeptide 1)	CGGCATGCATGAGAAAGGCATCAT ACACGTCCACATAGCCCAAGTCAT	Sense Anti-sense	GI: 23506123 *(Capra hircus)*
3βHSD (3-beta-hydroxysteroid dehydrogenase)	CGGCATCCTGACCAATTAC GGCCACATTGCCAACATAGA	Sense Anti-sense	GI:550822252 *(Capra hircus)*
PPIA (Peptidylprolyl isomerase A)	TCATTTGCACTGCCAAGACTG TCATGCCCTCTTTCACTTTGC	Sense Anti-sense	GI:548463626 *(Capra hircus)*
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	ATGCCTCCTGCACCACCA AGTCCCTCCACGATGCCAA	Sense Anti-sense	GI:298676424 *(Ovis aries)*

Table 2
Percentage of antrum formation of caprine preantral follicles cultured for 24 days (Exp.1) in α-MEM^{+} (Control) supplemented with GH and VEGF alone, in combination, or sequentially on days 0, 12 and 24

Treatment (n)*	D0	D12	D24
Control (40)	0.00 (0/40)^{Aa}	75.0 (30/40)^{Ab}	80.0 (32/40)^{Ab}
GH (40)	0.00 (0/40)^{Aa}	57.5 (23/40)^{ACb}	65.0 (26/40)^{ABb}
VEGF (40)	0.00 (0/40)^{Aa}	35.0 (14/40)^{Bb}	60.0 (24/40)^{Bc}
GH+VEGF (40)	0.00 (0/40)^{Aa}	47.5 (19/40)^{BCb}	57.5 (23/40)^{Bb}
GH/VEGF (40)	0.00 (0/40)^{Aa}	40.0 (16/40)^{BCb}	47.5 (19/40)^{Bb}
VEGF/GH (40)	0.00 (0/40)^{Aa}	32.5 (13/40)^{Bb}	52.5 (21/40)^{Bc}

^{A,B,C} Differs among treatments within the same day of culture ($P < 0.05$). ^{a,b,c} Significant differences among days of culture within the same treatment ($P < 0.05$).* Total number of follicles.

Table 3
Levels of E2, P4 and T on days 6 and 18 of in vitro culture of preantral follicles (Exp.1)

Treatment	E2 (µg/L)		P4 (µg/L)		T (µg/L)	
	D6	D18	D6	D18	D6	D18
Control	0.041 ± 0.011 ^{Aa}	0.026 ± 0.011 ^{ABa}	0.079 ± 0.001 ^{Aa}	0.088 ± 0.004 ^{Aa}	0.082 ± 0.004 ^{ABa}	0.074 ± 0.002 ^{Aa}
GH	0.021 ± 0.007 ^{Aa}	0.072 ± 0.008 ^{Ab}	0.092 ± 0.003 ^{Ba}	0.100 ± 0.015 ^{Aa}	0.079 ± 0.003 ^{Aa}	0.098 ± 0.015 ^{ABa}
GH/VEGF		0.039 ± 0.006 ^{Ba}		0.087 ± 0.005 ^{Aa}		0.090 ± 0.001 ^{Ba}
VEGF	0.009 ± 0.001 ^{Ba}	0.041 ± 0.009 ^{ABb}	0.094 ± 0.008 ^{ABa}	0.085 ± 0.003 ^{Aa}	0.095 ± 0.004 ^{Ba}	0.087 ± 0.005 ^{ABa}
VEGF/GH		0.040 ± 0.009 ^{ABb}		0.092 ± 0.012 ^{Aa}		0.091 ± 0.001 ^{Ba}
GH+VEGF	0.014 ± 0.001 ^{Aa}	0.065 ± 0.015 ^{ABb}	0.079 ± 0.001 ^{Aa}	0.086 ± 0.008 ^{Aa}	0.096 ± 0.002 ^{Ba}	0.095 ± 0.005 ^{Ba}

All data are expressed as mean ± SEM. ^{a,b} Within a row and the same hormone ($P < 0.05$). ^{A,B} Within a column ($P < 0.05$).

Table 4
Levels of E2, P4 and T on days 6 and 18 of *in vitro* culture of early antral follicles (Exp.2)

Treatment	E2 (µg/L)		P4 (µg/L)		T (µg/L)	
	D6	D18	D6	D18	D6	D18
Control	1.052 ± 0.778 ^{a}	7.290 ± 6.690 ^{a}	0.110 ± 0.010 ^{a}	0.130 ± 0.040 ^{a}	0.180 ± 0.030 ^{a}	0.490 ± 0.270 ^{a}
GH	1.784 ± 0.960 ^{a}	1.071 ± 0.169 ^{a}	0.116 ± 0.009 ^{a}	0.100 ± 0.030 ^{a}	0.200 ± 0.002 ^{a}	0.250 ± 0.010 ^{a}
GH/VEGF		4.476 ± 2.907 ^{a}		0.130 ± 0.002 ^{a}		0.400 ± 0.100 ^{b}
VEGF	1.251 ± 0.614 ^{a}	3.391 ± 2.389 ^{a}	0.117 ± 0.009 ^{a}	0.130 ± 0.003 ^{a}	0.190 ± 0.002 ^{a}	0.320 ± 0.070 ^{a}
VEGF/GH		4.041 ± 3.164 ^{a}		0.130 ± 0.010 ^{a}		0.320 ± 0.009 ^{a}
GH+VEGF	0.722 ± 0.537 ^{a}	2.463 ± 1.439 ^{a}	0.100 ± 0.010 ^{a}	0.130 ± 0.005 ^{a}	0.150 ± 0.010 ^{a}	0.290 ± 0.040 ^{b}

All data are expressed as mean ± SEM. ^{a,b} Within a row and the same hormone ($P < 0.05$). Within a column ($P > 0.05$).

Table 5
Percentage of fully grown oocytes, oocyte diameter, viability rate, percentage of meiotic resumption, and meiotic stages of oocytes from caprine preantral follicles (Exp.1) cultured in αMEM^{+} (Control), supplemented with GH and VEGF alone, in combination, or sequentially for 24 days.

Treatment (n)*	(%) Fully grown oocytes (≥110 μm/n)	Oocyte diameter (μm) (mean ± SEM)	(%) Viability rate (viable/≥110 μm)	(%) Meiotic resumption (n/total)	(%) GV (n/total)	(%) GVBD (n/total)	(%) MI (n/total)	(%) MII (n/total)
Control (40)	2.5 (1/40)^{AB}	85.40 ± 2.87^{AB}	0.0 (0/1)^{A}	-	-	-	-	-
GH (40)	5.0 (2/40)^{AB}	83.77 ± 3.82^{A}	100.0 (2/2)^{A}	5.0 (2/40)^{A}	-	2.5 (1/40)	-	2.5 (1/40)^{A}
VEGF (40)	0.0 (0/40)^{A}	79.88 ± 2.90^{A}	-	-	-	-	-	-
GH+VEGF (40)	12.5 (5/40)^{B}	91.95 ± 3.75^{B†}	60.0 (3/5)^{A}	5.0 (2/40)^{A}	2.5 (1/40)	-	2.5 (1/40)	2.5 (1/40)^{A}
GH/VEGF (40)	0.0 (0/40)^{A}	89.46 ± 2.10$^{B\#}$	-	-	-	-	-	-
VEGF/GH (40)	0.0 (0/40)^{A}	79.42 ± 3.09^{A}	-	-	-	-	-	-

^{A,B} Differs significantly among treatments ($P < 0.05$).† Tended to differ from GH ($P = 0.07$). $^{\#}$ Tended to differ from GH ($P = 0.06$).* Total number of follicles.

Table 6
Percentage of fully grown oocytes, oocyte diameter, viability rate, percentage of meiotic resumption, and meiotic stages of oocytes from caprine early antral follicles (Exp.2) cultured in αMEM^{+} (Control), supplemented with GH and VEGF alone, in combination, or sequentially for 18 days.

Treatment (n)*	(%) Fully grown oocytes (≥110 μm/n)	Oocyte diameter (μm) (mean ± SEM)	(%) Viability rate (viable/≥110μm)	(%) Meiotic resumption (n/total)	(%) GV (n/total)	(%) GVBD (n/total)	(%) MI (n/total)	(%) MII (n/total)
Control (54)	40.7 (22/54)^{A}	107.56 ± 2.60^{A}	84.4 (17/22)^{A}	29.6 (16/54)^{A}	1.9 (1/54)^{A}	-	5.6 (3/54)^{A}	24.1 (13/54)^{A}
GH (40)	60.0 (24/40)^{B†}	117.74 ± 2.61^{B}	87.5 (21/24)^{A}	50.0 (20/40)$^{B\#}$	2.5 (1/40)^{A}	-	7.5 (3/21)^{A}	42.5 (17/40)^{B}
VEGF (40)	42.5 (17/40)^{AB}	107.53 ± 3.24^{A}	70.6 (12/17)^{A}	30.0 (12/40)^{A}	-	2.5 (1/40)^{A}	5.0 (2/40)^{A}	22.5 (9/40)^{A}
GH+VEGF (40)	35.0 (14/40)^{A}	111.65 ± 3.72^{AB}	64.3 (9/14)^{A}	22.5 (9/40)^{A}	-	2.5 (1/40)^{A}	5.0 (2/40)^{A}	15.0 (6/40)^{A}
GH/VEGF (40)	52.5 (21/40)^{AB}	116.01 ± 2.43^{B}	71.4 (15/21)^{A}	37.5 (15/40)^{AB}	-	5.0 (2/40)^{A}	10.0 (4/40)^{A}	22.5 (9/40)^{A}
VEGF/GH (39)	33.3 (13/39)^{A}	110.21 ± 3.00^{AB}	84.6 (11/13)^{A}	25.6 (10/39)^{AB}	2.6 (1/39)^{A}	2.6 (1/39)^{A}	5.1 (2/39)^{A}	17.9 (7/39)^{A}

^{A,B} Differs among treatments ($P < 0.05$). † Tended to differ from Control ($P = 0.06$). $^{\#}$ Tended to differ from VEGF ($P = 0.06$) * Total number of follicles.

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7 CAPÍTULO 2

Relação entre as dinâmicas foliculares e a maturação oocitária durante o cultivo *in vitro* como um marcador não-invasivo de competência meiótica de oócitos caprinos.

"Relationship between follicular dynamics and oocyte maturation during *in vitro* culture as a non-invasive sign of caprine oocyte meiotic competence"

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RESUMO

A busca de marcadores não-invasivos de competência meiótica de ovócitos é muito importante para o desenvolvimento de sistemas de cultivo folicular *in vitro* (CFIV). Os objetivos do presente estudo foram: (1) investigar o efeito da maturação *in vitro* (MIV), em grupo ou individualmente, de complexos *cumulus*-oócito (CCOs) de cabra crescidos *in vivo*, na configuração de cromatina do oócito (Experimento 1) e (2) a influência do período de cultivo (12 vs. 18 dias) sobre a capacidade do oócito para retomar a meiose imediatamente após CFIV (antes da MIV) ou após MIV (Experimento 2). No experimento 1, os CCOs crescidos *in vivo* foram submetidos à IVM em grupos (10 CCOs / gota de 100 μL) ou individualmente (1 CCO / gota de 10 μL), e a configuração da cromatina foi avaliada. No experimento 2, os folículos isolados foram cultivados individualmente por 12 ou 18 dias, e submetidos posteriormente a MIV individual. Foram avaliados os seguintes parâmetros: crescimento e morfologia folicular, diâmetro, viabilidade e configuração da cromatina dos oócitos, bem como produção de estradiol folicular individual. Foram obtidas taxas de maturação semelhantes entre os CCOs crescidos *in vivo* maturados individualmente e em grupo (66,7% vs. 63,6%, respectivamente) (Experimento 1). Somente após 18 dias de IVFC, os oócitos foram capazes de crescer durante a MIV, atingindo um diâmetro oocitário médio de 119 μm. Além disso, esse tratamento produziu a maior taxa de oócitos em metafase II (46,2% em relação ao número total de folículos cultivados). Finalmente, observou-se que os folículos com uma taxa de crescimento diária > 7,1 μm (crescimento rápido) e que atingiram pelo menos 600 μm de diâmetro, foram mais propensos ($P < 0,05$) a produzir oócitos capazes de atingir a MII. Em conclusão, os oócitos caprinos podem ser maturados individualmente *in vitro*, tão eficientemente quanto em grupos. Este resultado foi essencial para estudar a relação do desenvolvimento folicular *in vitro* e a maturação oocitária *in vitro* com folículos individuais específicos. Usando essa abordagem, foi possível estabelecer marcadores não invasivos de eficiência do CIVF com base na taxa de crescimento diário e diâmetro folicular, bem como no diâmetro do oócito: crescimento diário do folículo > 7 μm, diâmetro do folículo de pelo menos 600 μm e diâmetro do oócito ≥ 120 μm. Além disso, 18 dias parece ser o período de cultivo mais adequado para os folículos antrais iniciais caprinos.

Palavras-chave: Caprino; período de cultivo; folículo antral inicial; MIV individual; maturação oocitária.

Relationship between follicular dynamics and oocyte maturation during *in vitro* culture as a non-invasive sign of caprine oocyte meiotic competence.

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Abstract

The search for non-invasive signs of oocyte meiotic competence is very important for the development of *in vitro* follicle culture (IVFC) systems. The aims of the present study were: (1) to investigate the effect of *in vitro* maturation (IVM) of *in vivo* grown goat COCs, in group or individually, on oocyte chromatin configuration (Experiment 1), and (2) the influence of IVFC period (12 vs. 18 days) on the ability of the oocyte to resume meiosis immediately after IVFC (before *in vitro* maturation; IVM), or after IVM (Experiment 2). In experiment 1, *in vivo* grown cumulus-oocyte complexes (COCs) were submitted to IVM in groups (10 COCs/100 μL-drop) or individually (1 COC/10 μL-drop), and chromatin configuration was assessed. In experiment 2, isolated follicles were individually cultured for 12 or 18 days, and submitted to individual IVM afterwards. The following end points were evaluated: follicular growth and morphology, oocyte diameter, viability and chromatin configuration, as well as individual follicular estradiol production. Similar maturation rates were obtained between *in vivo* grown COCs matured individually and in groups (66.7% vs. 63.6%, respectively) (Experiment 1). Only after 18 days of IVFC, oocytes were able to grow during IVM, reaching a mean oocyte diameter of 119 μm. Also, this treatment produced the highest rate of metaphase II oocytes (46.2% out of the total number of cultured follicles). Finally, it was observed that follicles with a daily growth rate > 7.1 μm/day (fast-growing) and that reached at least 600 μm in diameter, were more likely ($P < 0.05$) to produce oocytes capable of attaining MII. In conclusion, caprine oocytes can be individually matured *in vitro*, as efficiently as in groups. This result was essential to pair *in vitro* follicle development and in vitro oocyte maturation with specific individual follicles. Using this approach, it was possible to establish non-invasive signs for the efficiency of IVFC based on follicle daily growth rate and diameter, and oocyte diameter: follicle daily growth > 7 μm, follicle diameter of at least 600 μm, and oocyte diameter ≥ 120 μm. In addition, 18 days seems to be the most suitable culture time for caprine early antral follicles.

Key words: Caprine; culture period; early antral follicle; individual IVM; oocyte maturation.

1. Introduction

The possibility of recovering and *in vitro* culturing of immature oocytes could dramatically increase the availability of potentially fertilizable oocytes destined for assisted reproductive techniques. Consequently, among other applications it would help to conserve endangered species, and to preserve the fertility of women with cancer [1]. However, the future success of this technology depends on the advances on animal models. To this end, small ruminants have served as an excellent animal model for large mammals [2,3]. In particular, the goat has been described as a suitable model for the human ovary, due to similarities in the time needed for ovarian follicle development (~10 weeks) [4], as well as in follicular and oocyte diameters [5].

In farm animals, immature oocytes are usually cultured *in vitro* enclosed in preantral (primordial, primary and secondary) and antral (tertiary or early antral) follicles. This biotechnology is known as *in vitro* follicle culture (IVFC), and is mainly carried out in two forms: *in situ*, which means that ovarian follicles are cultured within the ovarian tissue [6–8], or as isolated follicles [9–11]. The performance of IVFC can be influenced by great number of variables, such as the animal model [3,12,13], the base media composition [14–16] and supplementation [11], the culture system [16–18], the oxygen tension [19,20], the follicular category [21], and the culture time interval [9,17]. The appropriate length of culture is crucial because the growing oocytes accumulates compounds essential for acquiring developmental competence [22]. In this sense, either an insufficient culture time period or an excessive culture period can impair oocyte maturation and development [23]. In addition, Hirao and Miyano [24] reported that a smaller oocyte at the onset of culture requires a longer culture time to acquire developmental competence

Even though live offspring have been produced in mice after IVFC of preantral follicles (PFs) [25], in large animals the best results have been obtained from early antral follicles (EAFs) [26–28]. In goats, oocytes originating from intact EAFs (300-400 μm in diameter) have shown a greater maturation rate (42.5%) compared to oocytes from PFs (2.5%) [21]. Nevertheless, contrary to PFs [17], the suitable culture time for intact EAFs has not been investigated in any species so far. Furthermore, we were concerned that the influence of culture time on oocyte ability to resume meiosis immediately after IVFC (before *in vitro* maturation; IVM), or after IVM has not been investigated.

In several different species (non-human primates [20], bovine [10], and caprine [29,30]), studies have shown large individual variations among isolated follicles cultured in the same experimental conditions. According to their dynamics *in vitro*, follicles can be classified as: non-growing, slow-growing and fast-growing [20,31,32]. In this regard, Apolloni et al. [30] reported a negative correlation between follicular growth rate and follicular extrusion or degeneration during IVFC. Notwithstanding, there are no studies correlating caprine follicular dynamics *in vitro* and oocyte maturation because after IVFC recovered cumulus-oocyte complexes (COCs) are matured in groups, i.e., it is impossible to match individual follicle growth patterns and its derived oocytes during IVM. Therefore, the development of an individual IVM system would provide invaluable data on every single oocyte in order to generate a better understanding of folliculogenesis *in vitro*. Also, it may describe a reliable follicular growth pattern that predicts oocyte maturation *in vitro*, which would help to improve the current IVFC systems. In mice, individual oocyte IVM showed lower maturation rates than oocytes in groups [33]. On the other hand, bovine oocytes have presented similar maturation rates when matured individually or in groups [34]. To the best of our knowledge, no information is available on the effect of IVM system on individual culture of goat oocytes.

Therefore, the present study aims to investigate the effect of IVM in groups or individually on oocyte chromatin configuration, and the influence of culture time on oocyte ability to resume meiosis immediately after IVFC (before IVM), or after IVM. Two different culture times were assessed, 12 and 18 days, and the following end points were evaluated: (1) follicular growth and morphology, (2) oocyte diameter, viability and chromatin configuration before IVFC, and before and after IVM, (3) and individual estradiol production.

2. Materials and methods

All experiments were performed according to the recommendations of the Committee of Animal Handling and Ethical Regulation from the State University of Ceara, Fortaleza, Ceara, Brazil.

2.1 Chemicals and media

Unless otherwise mentioned, the reagents and chemicals used in the present study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., USA).

2.2 Source of ovaries

Ovaries from 32 adult mixed-breed goats (1 to 3 years of age) were collected at a local abattoir. Immediately after the slaughter, the ovaries were immersed in 70% alcohol, followed by two washes in minimum essential medium (MEM) plus HEPES (MEM-HEPES), supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin, and then transported to the laboratory within 3 h of when they were collected. It is important to emphasize that the ovaries destined for IVM (Experiment 1) and those destined for IVFC (Experiment 2) were transported respectively at 33 °C [35,36] and 4 °C [37] as described previously.

2.3 Experiment 1: Caprine oocyte in vitro maturation in groups or individually

At the laboratory, ovaries from 12 animals were washed twice in TCM199-HEPES supplemented with 40 μg/mL gentamicin, 1 mg/mL bovine serum albumin (BSA), and 10 IU/mL heparin (holding medium). After washing, cumulus-oocyte complexes (COCs) were mechanically released from the ovaries into holding medium by slicing with a surgical blade. Only oocytes (≥110 μm) with homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for *in vitro* maturation (IVM) [38]. The selected COCs were pooled and washed three times in IVM medium, which consists of tissue culture medium 199 (TCM 199) supplemented with 1 μg/mL 17β-estradiol, 5 μg/mL luteinizing hormone (LH), 0.5 μg/mL rFSH (bovine), 10 ng/mL epidermal growth factor (EGF), 1 mg/mL BSA, 1 mM pyruvate, 50 ng/mL insulin-like growth factor 1 (IGF-I), and 100 μM cysteamine [21]. After being washed, COCs were transferred to fresh IVM medium on culture dishes (30 x 15 mm) under mineral oil and incubated for 27 h at 38.5°C with 5% CO_2 in two ways: 10 COCs per 100-μL droplet (IVM in group), or 1 COC per 10-μL droplet (Individual IVM). The experiment was replicated three times, and a total of 24 ovaries were used (8 ovaries/replicate).

2.3.1 Assessment of oocyte viability and chromatin configuration

Oocyte viability and chromatin configuration were assessed by fluorescence microscopy (Eclipse 80i, Nikon, Tokyo, Japan). After IVM, oocytes were mechanically denuded and incubated either individually or in group for 30 min in 10 or 100 μL drops respectively of PBS supplemented with 4 μM calcein-AM and 2 μM ethidium homodimer-1, (Molecular Probes - LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells – L3224,

Invitrogen, Karlsruhe, Germany), 20 μM Hoechst 33342 (emission at 483 nm) and 0.5% glutaraldehyde. Oocyte chromatin was classified as degenerated (DEG), germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII). Oocytes were classified as viable when cytoplasm stained with calcein-AM (green; emission at 488 nm), chromatin did not show abnormal configuration and/or did not label with ethidium homodimer-1 (red; emission at 568 nm).

2.4 Experiment 2: In vitro culture of isolated caprine early antral follicles: Influence of follicle culture time on oocyte maturation

2.4.1 Isolation and selection of caprine early antral follicles

Ovaries from 20 animals were collected and transported from the abattoir as described above. In the laboratory, the surrounding fat and connective tissue were removed from the ovaries. Ovarian cortical slices (1 mm thick) were cut using a surgical blade, and placed in holding medium (MEM-HEPES) and then, early antral follicles were isolated. Early antral follicles (300 to 400 μm) were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan), manually dissected from the slices of ovarian cortex using 26-gauge (26-G) needles, and transferred to the culture medium for further evaluation of follicular quality. Follicles with a visible oocyte, surrounded by several granulosa cell layers and with an intact basement membrane and antral cavity, were selected for *in vitro* culture (Fig.1a).

2.4.2 In vitro culture of caprine early antral follicles

Immediately after follicle isolation, the isolated follicles from all animals were pooled. A fraction of them were immediately opened, and their oocytes were denuded and fixed to evaluate oocyte diameter and chromatin configuration before IVFC (non-cultured control). The remaining follicles were randomly assigned to the two treatments: *in vitro* follicle culture for 12 (IVFC-12) or 18 days (IVFC-18). Culture periods were selected based on a preliminary experiment performed in our laboratory where no differences were found between 18 and 24 days (unpublished data). It is important to emphasize that every treatment had follicles from all animals. The experimental setup is summarized in Figure 2. A total amount of 199 early antral follicles were used, 157 for *in vitro* follicle culture, and 42 for non-cultured control. Follicles were individually cultured in 100 μL drops of culture medium on Petri dishes under mineral oil (60×15 mm; Corning, USA). At the end of both culture times, approximately half of recovered oocytes were immediately denuded and fixed, and the other half were submitted

to individual IVM in order to check oocyte diameter, viability and chromatin configuration before and after IVM. The medium used was previously described by our group and consisted in α-MEM (pH 7.2 to 7.4), supplemented with 3 mg/mL BSA, 10 ng/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 50 μg/mL ascorbic acid, and 50 ng/mL GH [21]. The culture was carried out at 38.5°C in 5% CO_2 in air. Fresh medium was prepared and pre-equilibrated overnight prior to use. Medium was replaced partially (60 μL) every other day. The experiment was replicated four times.

2.4.3 Morphological evaluation of follicle development

Follicles were classified as intact, degenerated or extruded. Degenerated follicles presented darkened oocyte and/or misshapen cytoplasm of the oocyte and surrounding granulosa cells, while extruded follicles were those that experienced rupture of the basement membrane. The percentages of morphologically intact follicles and follicle diameter were calculated by excluding extruded and degenerated follicles. The follicle diameter was calculated as the mean of two perpendicular measures of each follicle, using an ocular micrometer attached to a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; X 100 magnification). Follicle daily growth rates during culture were expressed by dividing the culture time into three thirds: 1) the diameter of intact follicles on Day 6 of culture minus the diameter of intact follicles on Day 0, divided by six (D0-D6), 2) the diameter of intact follicles on Day 12 of culture minus the diameter of intact follicles on Day 6, divided by six (D6-D12), and 3) the diameter of intact follicles on Day 18 of culture minus the diameter of intact follicles on Day 12, divided by six (D12-D18). Noting that on Day 12 of culture, approximately half of follicles were removed from the IVFC and the other half remained until Day 18. Furthermore, an overall follicle daily growth rate was calculated as the diameter of intact follicles at the end of the culture (12 or 18 days) minus their respective diameter on Day 0, divided by the days they were cultured (12 or 18). Based on their daily growth rates, follicles were classified as: non-growing, follicles that did not grow during the culture; slow-growing, follicles with a daily growth between 0.1 and 7.0 μm/day; and fast-growing follicles with a daily growth between > 7.1 μm/day [30].

2.4.4 Hormonal Assay

To evaluate the relationship between follicle/oocyte development and estradiol (E2) production, the conditioned culture media were individually collected on days 12 and 18 of IVFC after *in vitro* maturation, and stored at -80°C for further analysis. The concentration of

E2 was analyzed by radioimmunoassay kits (MP Biomedicals, LLC-Orangeburg, NY, USA). The assay sensitivity and intra-assay coefficient were 5 pg/mL and 7%, respectively.

2.4.5 In vitro maturation of caprine oocytes from early antral follicles cultured in vitro

After the culture, all recovered COCs were selected for individual IVM, as described above (Experiment 1). COCs were individually transferred to fresh 10-µl drops of IVM medium on culture dishes (30 x 15 mm) under mineral oil and then incubated for 32 h at 39°C with 5% CO_2 in air. The percentages of oocytes ≥ 110 and ≥ 120 µm in diameter (zona pellucida not included) before and after IVM were calculated out of the total number of cultured follicles removed from the *in vitro* culture at each evaluated time point, and multiplying this value by 100. Furthermore, oocytes were classified according to size as: small (<110 µm), medium (between 110.1 and 120 µm), and large (>120.1 µm) to perform association analysis between oocyte size and MII rate. Oocyte viability and chromatin configuration were assessed before IVFC (non-cultured control), and after both culture periods (12 and 18 days) before and after IVM following the methodology described in the Experiment 1.

2.7 Statistical analysis

All statistical analyses were performed using Sigma Plot 11 (Systat Software Inc., USA). Comparison of means among treatments was analyzed by Kruskal–Wallis test, whereas the Wilcoxon signed test was used to analyze the effect of treatment between days of culture. The percentage of intact, degenerated and extruded follicles and chromatin configuration among days of culture and treatments were analyzed by chi-square or Fisher's exact tests. A linear regression analysis was performed to evaluate the association of chromatin configuration with oocyte diameter and follicular growth category. In addition, the association between oocyte diameter and follicular growth category was evaluated by Spearman rank test. A logistic regression was performed to analyze the relationship between oocyte viability and follicular growth (µm/day). Odds ratio and confidence interval (CI) were calculated to determine: (i) the effect of *in vitro* culture time and follicular growth category on oocyte viability and (ii) the oocyte size after *in vitro* maturation on MII rates. Data are presented as mean (± SEM) and percentage, and the statistical significance was defined as $P < 0.05$ (two-sided).

3. Results

3.1 Experiment 1: Caprine oocyte in vitro maturation in groups or individually

No differences were found between IVM in groups or individually regarding chromatin status: Degeneration rate from IVM in groups and individually was 14.3% vs. 17.5%, respectively, and the MII rates were 63.6% vs. 66.7%, respectively (Table 1).

3.2 Experiment 2: In vitro culture of isolated caprine early antral follicles: Influence of follicle culture time on oocyte maturation

3.2.1 Follicle morphology and growth

The percentage of morphologically intact follicles decreased ($P < 0.05$) throughout the culture period. Follicle degeneration and extrusion augmented ($P < 0.05$) from day 0 to day 12 of culture, and remained unchanged until day 18. Also, there was a progressive increase ($P < 0.05$) in the mean follicle diameter during culture (Fig.1). In contrast, mean daily growth increased ($P < 0.05$) only from the first (D0-D6) to the second (D6-D12) third of culture (Table 2).

3.2.2 Oocyte parameters

The data regarding oocyte growth, viability and chromatin configuration are summarized (Table 3). Both culture periods (12 and 18 days) increased ($P < 0.05$) oocyte diameter when compared to non-cultured control. Moreover, the IVFC-18 treatment presented greater ($P < 0.05$) mean oocyte diameter after IVM than before IVM (119.0 ± 2.2 µm vs. 108.9 ± 2.3 µm, respectively) and the IVFC-12 treatment. Accordingly, the highest percentage of fully grown oocytes (≥ 110 µm in diameter, excluding zona pellucida) was obtained in the IVFC-18 treatment after IVM, being greater ($P < 0.05$) than in IVFC-12 treatment.

When the total number of oocytes were analyzed based on chromatin configuration at the end of the culture period regardless their size, degeneration rate only exceeded ($P < 0.05$) non-cultured control after 18 days of culture. Similar percentages of GVBD were observed in all treatments and non-cultured control, despite the IVFC or the IVM. Finally, comparing both culture periods 12 and 18 days after IVM, IVFC-18 treatment presented lower ($P < 0.05$) percentage of oocytes with GV configuration (7.7% vs. 53.8%) and higher ($P < 0.05$) percentage of oocytes at MII stage than IVFC-12 treatment (46.2% vs. 12.8%) (Fig.3). Maturation rate of *in vivo* grown oocytes was 66.7% (Table 1) and all oocytes were ≥ 110 µm.

3.2.3 Relationship among follicular final diameter, daily growth rate, oocyte viability, diameter, and chromatin configuration.

Based on the data from individual IVM, we observed by linear regression that the probability of having a viable oocyte was positively related to follicular growth rate ($P < 0.01$) (Fig.4). Furthermore, when dividing the culture time into thirds, the odds ratio (OR) analysis revealed that this positive association was significant only in the second (D6 to D12) (OR = 6.6) and the last third of culture (D12 to D18) (OR = 7.5) ($P < 0.0001$ and 0.0003, respectively), and not in the first third of culture (D0 to D6) (Table 4). Moreover, daily follicular growth rate was correlated positively ($P < 0.01$) with oocyte diameter (Fig.5), as well as with the ability of an oocyte to progress in meiosis from GV to MII stage after IVM (Fig.6). Also, by grouping intact follicles by their final diameters into four intervals (< 400, 400.1-500.0, 500.1-600, and > 600.1 µm) the percentage of follicles that harbored MII oocytes was significantly greater in those follicles that attained more than 600 µm in diameter (90.9%), regardless the culture time (Fig.7).

3.2.4 Relationship between oocyte diameter and chromatin configuration.

Oocyte diameter was positively related ($P < 0.001$) with meiotic progression, as shown by linear regression (Fig.8). Hence, the probability of reaching MII was higher ($P < 0.01$) in large oocytes (> 120.1 µm) compared to small (< 110 µm) (OR = 360.0) and medium (110.1 to 120 µm) oocytes (OR = 42.0). Nevertheless, no differences were found between small and medium oocytes (Table 5).

3.2.5 Relationship between estradiol production, chromatin configuration, and follicular dynamics.

Since the IVFC-12 treatment did not produce enough number of MII oocytes to study the possible correlation between estradiol (E2) production at the end of the culture period, chromatin configuration, specifically the GV and MII configurations, and follicular dynamics, levels of estradiol (E2) were individually measured only in conditioned medium after 18 days of IVFC. In this regard, E2 production was not related ($P > 0.05$) either to chromatin configuration or follicular dynamics. However, it tended to be correlated positively ($P = 0.1$) with follicular daily growth rate.

4. Discussion

The present study showed, for the first time, that individual caprine COCs can be matured *in vitro* as efficiently as in groups. Also, our individual IVM system allowed us to describe for the first time a positive correlation between *in vitro* follicle growth (daily growth rate and final diameter) and oocyte features (viability, diameter, and maturation). In addition, 18 days has been set as the appropriate length of *in vitro* culture of isolated caprine EAFs (300 to 400 μm in diameter) under our experimental conditions.

The appropriate number of COCs (individual vs. group) per drop during IVM is a controversial subject. In our study, individual IVM system did not adversely affect caprine oocyte maturation. Many papers have described that COCs cultured in group showed better results than COCs cultured individually [33,39,40], while others described no differences between both systems [34,41,42]. The differences among studies might be a consequence of the use of different culture systems, base media, supplements, and species. During IVM in group, COCs may help each other given that oocyte and cumulus cells secrete autocrine and paracrine factors that are essential for oocyte development [43]. However, high COC density may also increase the amount of reactive oxygen species and diminish nutrient availability, promoting oocyte degeneration. Furthermore, degenerating oocytes secrete factors that may harm other viable oocytes [39].

An important tool to evaluate the efficiency of any IVFC system is the rate of MII oocytes after IVM. In the present study, the fact that caprine COCs could mature individually *in vitro* as efficiently as in group (Experiment 1) enabled us to pair follicle development and oocyte *in vitro* maturation (Experiment 2). In experiment 2, two different culture periods were evaluated for the *in vitro* culture of isolated caprine EAFs: 12 and 18 days. The results showed that the percentage of morphologically intact follicles decreased significantly from Day 0 to Day 12, and subsequently to Day 18. Despite that, intact follicles continued to grow until Day 18, as shown by the increase in follicle diameter. The reduction in the percentage of morphologically normal follicles [11,13,18,21,23] and the maintenance of growth of the remaining intact follicles [12,17,21] is a common event observed after IVFC in many species.

The results of our study confirm the findings of previous studies reporting a positive correlation between oocyte size and meiotic competence [30,38,45–47], with better outcome obtained with the use of oocytes greater than 120 μm, as reported in bovine [28,48,49]. Taking into account that oocyte diameter is crucial for the success of IVM, the present paper stablished a completely new approach to study the influence of IVFC, i.e., checking the

oocytes at the onset of IVFC (non-cultured control), right after the IVFC (before IVM), and after IVM. The greatest mean final oocyte diameter was obtained in the IVFC-18 treatment after IVM, where oocyte diameter increased ($P < 0.05$) from 108.9 µm to 119.0 µm (excluding zona pellucida) during IVM. In addition, higher maturation rate was obtained after 18 days compared to 12 days of IVFC (46.2% vs. 12.8%). It is important to highlight that these values were calculated out of the total amount of follicles cultured *in vitro*. As mentioned before, the maturation rate of *in vivo* grown oocytes was 66.7% and all oocytes were ≥ 110 µm. Therefore, if one considers only the *in vitro* grown oocytes with similar size to the *in vivo* grown oocytes i.e., ≥ 110 µm, the maturation rate in the IVFC-18 treatment will amount to 75% (18 MII oocytes out of 24 oocytes ≥ 110 µm), which is comparable to the maturation rate of *in vivo* grown oocytes (66.7%). Considering the similarities of oocyte diameter from both IVFC times before IVM, we can infer that the differences between the treatments with regard to the ability of oocytes to grow and mature during IVM probably rely on oocyte and its companion follicular cell features. As a matter of fact, due to their larger diameter, follicles from day 18 of culture are supposed to have a greater number of granulosa and cumulus cells, which could help to improve oocyte meiotic competence. To support this statement, previous studies have shown that the number of follicular cells is intimately associated with energy sufficiency of the oocyte, and therefore with its growth and developmental ability [50,51]. The growth of oocytes during IVM was also reported in human, but only when they were retrieved from growing follicles [47].

In most studies, the authors presume that at the onset of IVFC all follicles with normal morphology harbor viable oocytes. However, for the first time we investigated the quality of oocytes before IVFC. Despite all selected follicles looked morphologically normal, 14.3% of them presented degenerate oocytes (fluorescence analysis) even before the IVFC (non-cultured control), which indicates that those oocytes were doomed from Day 0. This observation could partially explain the limited results obtained so far after the in IVFC from large mammals [2,52–55]. Also, we observed a similar percentage of GVBD oocytes, regardless the culture time of IVFC (D0, D12 and D18) and IVM (0h and 32h). We believe that the presence of GVBD oocytes at the onset of IVFC or IVM is due to the mechanical opening of the follicles [56], since an ongoing experiment in our laboratory showed that when the whole intact EAFs were fixed right after the isolation, no meiotic resumption was observed (unpublished data).

In spite of the culture period (12 and 18 days), we observed several correlations between follicular dynamics and oocyte parameters based on individual follow up data. The probability of having a viable oocyte was positively related to follicular daily growth rate from day 6 onwards. A previous study in isolated ovine preantral follicles cultured for 6 days recommended the elimination from the *in vitro* culture follicles that did not increase in size during the first two days [2]. According to our results, such approach cannot be applied for the *in vitro* culture of caprine EAFs, since the measurement of daily growth rate during the first six days of culture was not a good sign to predict oocyte viability after culture. Moreover, levels of E2 were individually measured in conditioned media collected on day 18 of IVFC in the interest of correlate its production at the end of the culture period with meiotic progression and/or follicular growth. Our results did not show any relationship between E2 production and any of those parameters, although it tended to be positively correlated ($P = 0.1$) with the overall follicular growth rate. Indeed, a positive relation between E2 and follicular growth has been described in other species as well (human [57], and non-human primates [20,58,59]).

Finally, we wondered if it would be possible to establish a relationship between follicle size and oocyte maturation. Interestingly, follicles with a daily growth > 7.1 µm/day (fast-grow) and that reached at least 600.1 µm in diameter, were more likely ($P < 0.05$) to have greater oocytes capable to attain MII. Likewise, fast-grow follicles have been associated with oocyte growth [57] and maturation [20] in human, and non-human primates, respectively. Conversely, Apolloni et al. [30] reported that high follicle growth rate (> 7.1 µm/day) was negatively related to follicular viability and oocyte meiotic resumption. Nonetheless, this conclusion was merely based on morphological analysis, since extruded follicles were considered degenerated, and follicles were not paired to their respective oocytes. Unlike them, we have seen that most of extruded follicles have viable oocytes (8 out of 10) after the IVFC, and half of them reached MII after IVM (5 out of 10). This result indicate that the presence of basement membrane is not essential for the oocyte to grow and mature *in vitro*. To support this statement, previous studies in mice and bovine have shown the production of live offspring after *in vitro* culture of oocyte-granulosa cells complexes [25–27].

4.1 Conclusions

Caprine oocytes can be individually matured *in vitro*, as efficiently as in groups. This result was essential to pair *in vitro* follicle development and oocyte maturation. Using this

approach, under the culture conditions described for caprine EAFs in the present paper, we can state that follicle daily growth rate and diameter and oocyte diameter can be used as non-invasive signs of caprine oocyte meiotic competence. Hence, the cut-point figures that allow the production of *in vitro* grown meiotically competent oocytes are: follicle daily growth of 7 µm, follicle diameter of 600 µm and oocyte diameter of 120 µm. In addition, 18 days seems to be the most suitable culture time for caprine EAFs.

Acknowledgments

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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.

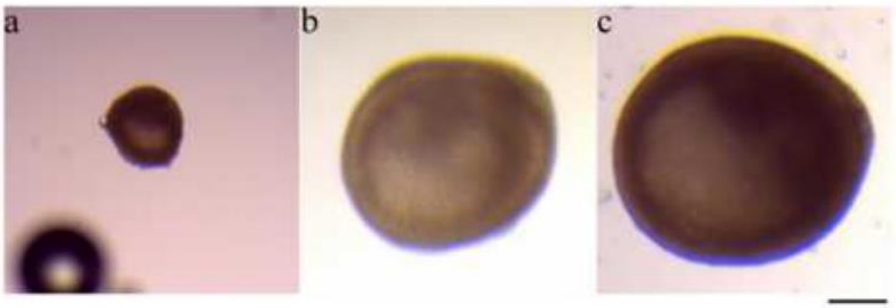

Fig.1. Follicular development during *in vitro* culture. (a-c) Images of the same follicle on days 0, 12 and 18 of *in vitro* culture, respectively. Scale bar = 200 µm

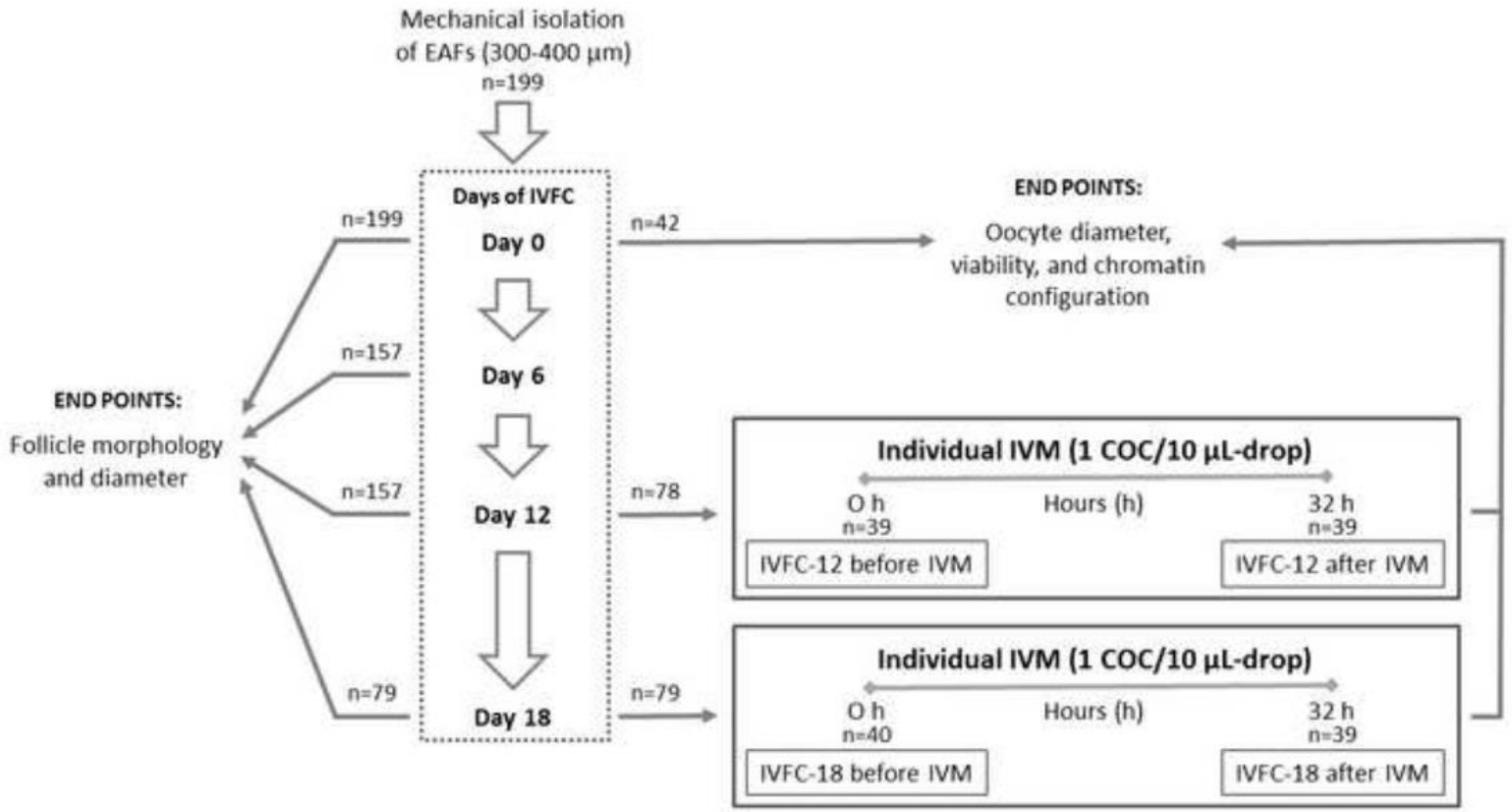

Fig.2. Experimental design (Experiment 2). Two different culture times were evaluated: 12 and 18 days. On day 0, 199 early antral follicles (EAFs) were mechanically isolated and selected for *in vitro* follicle culture (IVFC). Forty-two of them were destined for evaluation of their oocytes before IVFC by light microscopy (oocyte diameter), and fluorescent microscopy (Ethidium homodimer-1 and Calcein-AM staining for oocyte viability, and Hoechst 33342 staining for chromatin configuration). On day 12, 78 follicles were opened and their oocytes were destined for *in vitro* maturation (IVM). Half of them (39), were evaluated (oocyte diameter, viability and chromatin configuration) before IVM (IVFC-12 before IVM) and the other half after IVM (IVFC-12 after IVM). The same procedure was applied on day 18 (IVFC-18 before IVM, and IVFC-18 after IVM). On days 0,6,12, and 18, the morphology of all follicles was assessed and their diameter determined. The sample number (n) represents the number of follicles.

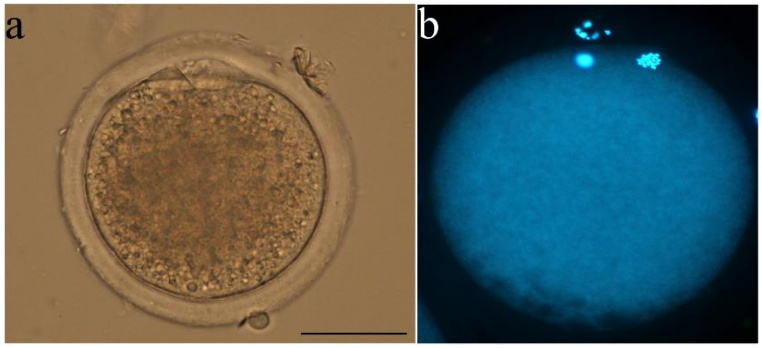

Fig.3. Metaphase II oocytes obtained after the *in vitro* maturation of oocytes retrieved from early antral follicles cultured *in vitro*. (a) Direct observation by light microscopy; and (b) chromatin stained with Hoechst 33342 assessed by fluorescence microscopy. Scale bar = 50 µm.

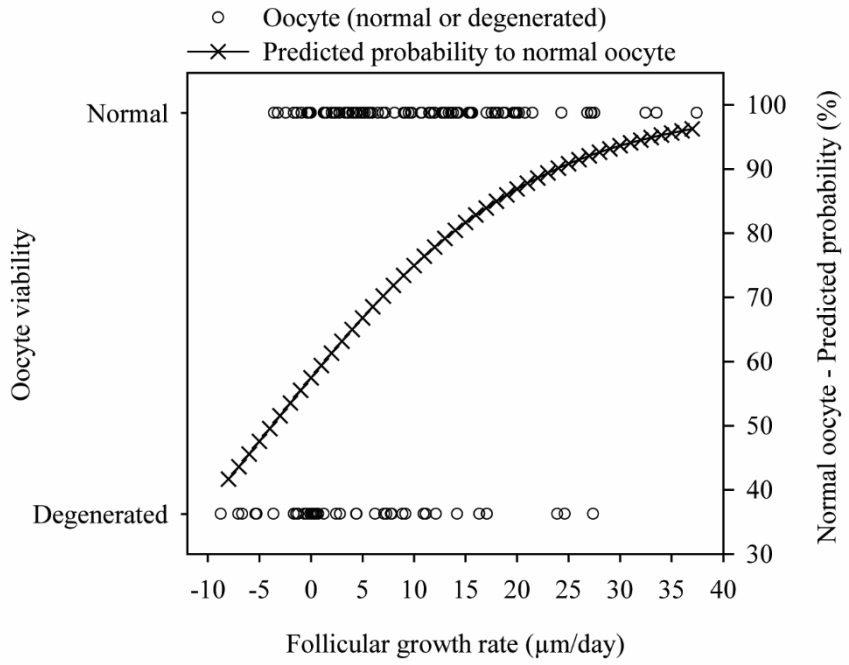

Fig.4. Relationship between oocyte viability and follicular growth rate. Each circle of the graph is an oocyte evaluated (n = 147). The oocytes analyzed were defined by binary values (0 = degenerated; 1 = normal). A logistic regression is represented by "×" mark and the equation [Logit P = 0.301 + (0.0796 × follicular growth rate); $P < 0.01$].

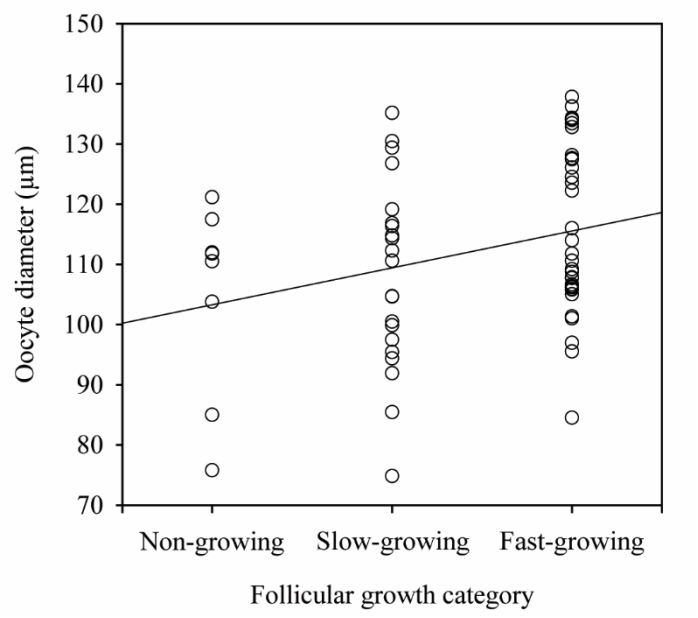

Fig.5. Correlation analysis between oocyte diameter and follicular growth category. Each circle on the chart represents an oocyte evaluated after *in vitro* culture (n = 132). The follicles were classified as: non-growing, follicles that did not grow during the culture; slow-growing, follicles with a daily growth between 0.1 and 7.0 µm/day; and fast-growing, follicles with a daily growth between 7.1 and 37.4 µm/day. Pearson correlation coefficient ($r = 0.24$; $P < 0.01$).

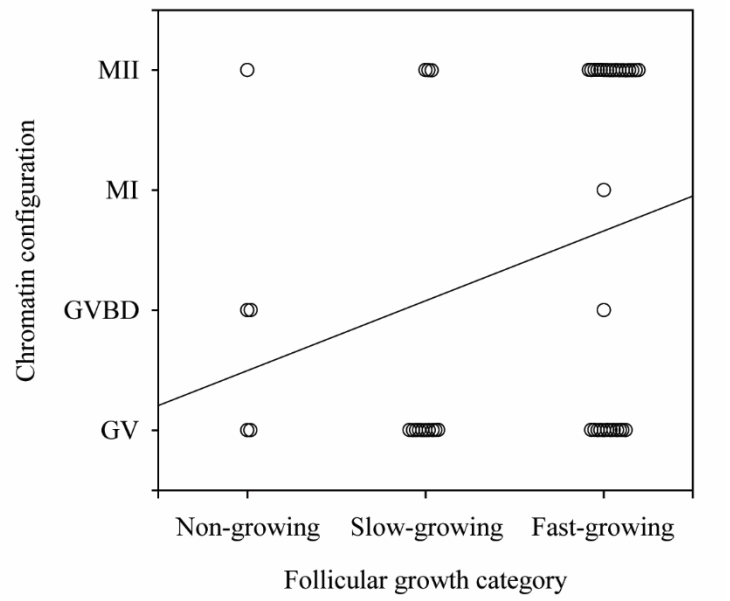

Fig.6. Relationship between oocyte chromatin configuration after *in vitro* maturation and follicular growth category. Each circle on the chart represents an oocyte evaluated after *in vitro* maturation (n = 49). The oocytes evaluated were classified by values (1 = germinal vesicle - GV; 2 = germinal vesicle breakdown - GVBD; 3 = metaphase I - MI; and 4 = metaphase II - MII) to chromatin configuration (dependent variable). The follicles *in vitro* cultured were classified according to growth category in: 1 = non-growing, follicles that did not grow during the culture; 2 = slow-growing, follicles with a daily growth between 0.1 and 7.0 µm/day; and 3 = fast-growing, follicles with a daily growth > 7.1 µm/day. A linear regression is represented by the line (black) and equation [chromatin configuration = 0.915 + (0.582 × follicular growth category); $r = 0.27$; $R^2 = 0.07$; $P < 0.05$].

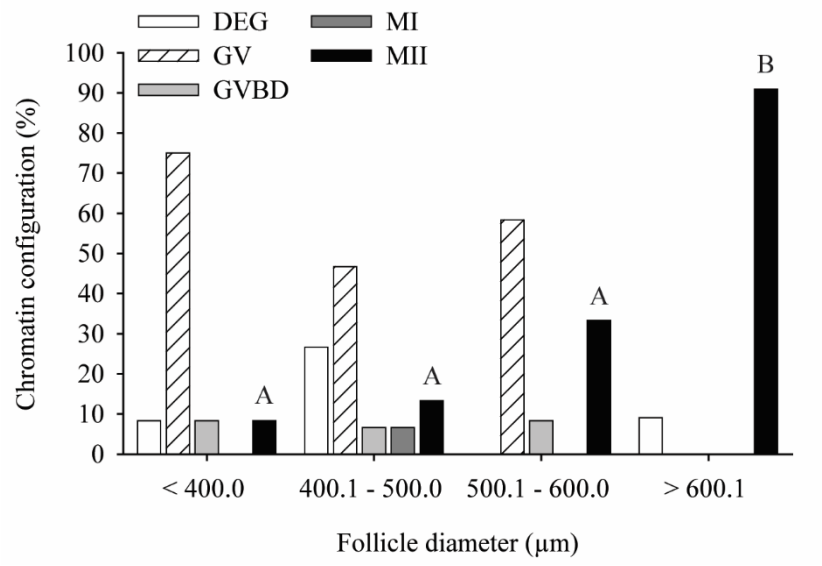

Fig.7. Percentage of intact follicles (n = 50) within a specific range of diameter harboring oocytes with different chromatin configuration (11 to 15 intact follicles per group) (germinal vesicle - GV; germinal vesicle breakdown - GVBD; metaphase I - MI; metaphase II - MII). ^{A,B} Differs among ranges of follicle diameter within the same chromatin configuration ($P < 0.05$).

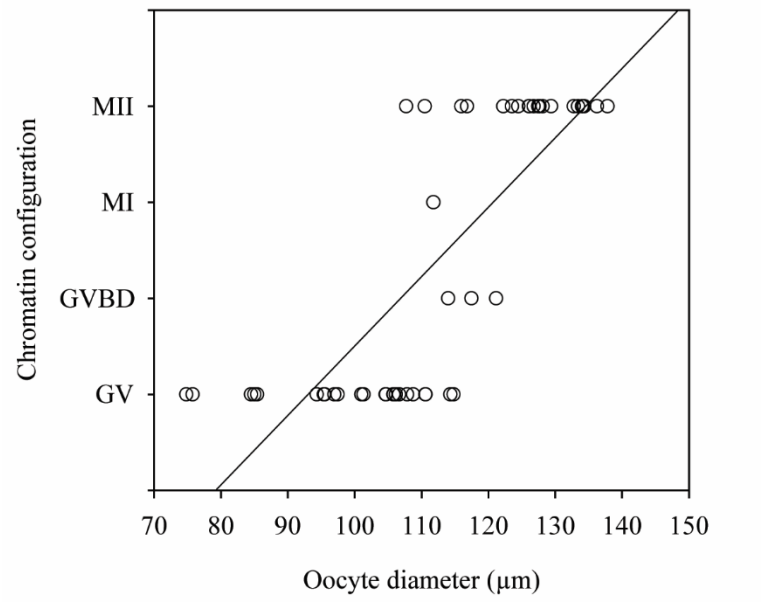

Fig.8. Relationship between chromatin configuration and oocyte diameter. Each circle on the chart represents an oocyte evaluated after *in vitro* maturation (n = 50). The oocytes evaluated were classified by values (1 = germinal vesicle - GV; 2 = germinal vesicle breakdown - GVBD; 3 = metaphase I - MI; and 4 = metaphase II - MII) according to chromatin configuration (dependent variable). A linear regression is represented by the line (black) and equation [chromatin configuration = -5.7326 + (0.0723 × oocyte diameter); r = 0.79; R^2 = 0.63; $P < 0.001$].

Table 1

Chromatin stage after caprine *in vitro* maturation in groups or individually

IVM (n)	DEG (%)	GV (%)	GVBD (%)	MI (%)	MII (%)
In groups (77) (10 COCs/100µL-drop)	14.3 (11/77)	7.8 (6/77)	5.2 (4/77)	9.1 (7/77)	63.6 (49/77)
Individually (57) (1 COC/10µL-drop)	17.5 (10/57)	3.5 (2/57)	0.0 (0/57)	12.3 (7/57)	66.7 (38/57)

Within a column ($P > 0.05$). n represents the number of cumulus-oocyte complexes (COCs).

Abbreviations: IVM, *in vitro* maturation; DEG, degenerated; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

Table 2

Morphologically intact follicles (%), mean diameter (µm), degeneration rate (%), and extruded follicles (%) on days 6, 12 and 18, as well as growth rate (µm/day) in the first (D0-D6), second (D6-D12) and last third (D12-D18) of culture of caprine early antral follicles.

End points	D0 (n=157)	D6 (n=157)	D12 (n=157)	D18 (n=79)
Morphologically intact follicles (%)	100.0a (157/157)	93.6b (147/157)	72.6c (114/157)	58.2d (46/79)
Degeneration rate (%)	0.0a (0/157)	4.5b (7/157)	19.1c (30/157)	27.8c (22/79)
Extruded follicles (%)	0.0a (0/157)	1.9a (3/157)	8.3b (13/157)	13.9b (11/79)
Diameter (µm) (mean ± SEM)	341.2 ± 4.6a	364.8 ± 6.2b	449.3 ± 11.7c	574.7 ± 22.4d
		D0 to D6	D6 to D12	D12 to D18
Growth rate (µm/day) (mean ± SEM)	-	3.6 ± 0.7a	14.5 ± 1.2b	17.8 ± 1.8b

Different lowercase letter (a,b,c,d) differs within a row and the same end point ($P < 0.05$). n represents the number of follicles.

Table 3

Mean oocyte diameter (µm), oocytes ≥ 110 µm (%) and ≥ 120 µm (%), and chromatin configuration after 12 or 18 days of *in vitro* follicle culture, before and after *in vitro* maturation.

Treatments	n	IVM	Mean oocyte diameter (µm) (mean ± SEM)	Oocytes ≥ 110 µm (%)	Oocytes ≥ 120 µm (%)	Chromatin configuration				
						DEG (%)	GV (%)	GVBD (%)	MI (%)	MII (%)
Non-cultured control	42		87.0 ± 1.9 A	0.0 A (0/42)	0.0 A (0/42)	14.3 A (6/42)	76.2 A (32/42)	9.5 A (4/42)	-	-
IVFC-12	39	Before	104.8 ± 2.3 B	20.5 B (8/39)	10.3 B (4/39)	20.5 AB (8/39)	69.2 AB (27/39)	10.3 A (4/39)	-	-
	39	After	104.7 ± 2.2 B	33.3 BD (13/39)	12.8 B (5/39)	28.2 AB (11/39)	53.8 BC (21/39)	2.6 A (1/39)	2.6 A (1/39)	12.8 A (5/39)
IVFC-18	40	Before	108.9 ± 2.3 B	42.5 CD (17/40)	15.0 B (6/40)	40.0 B (16/40)	40.0 C (16/40)	15.0 A (6/40)	5.0 A (2/40)	-
	39	After	119.0 ± 2.2 C	61.5 C (24/39)	43.6 C (17/39)	41.0 B (16/39)	7.7 D (3/39)	5.1 A (2/39)	-	46.2 B (18/39)

Different uppercase letters (A, B, C, D) differs within a column and the same end point ($P < 0.05$). n represents the number of follicles.

Abbreviations: IVM, *in vitro* maturation; DEG, degenerated; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

Table 4

Association analyses between follicular growth and oocyte viability in different intervals of *in vitro* follicle culture.

Comparisons	Oocyte viability (%)	Odds ratio (95% C.I)	P - value
In vitro culture - D0 to D6			
non-growing follicles	60.0 (35/53)	1.4 (0.6 - 2.9)	0.4509
†growing follicles	73.4 (69/94)		
In vitro culture - D6 to D12			
non-growing follicles	43.7 (21/48)	6.6 (3.0 - 14.5)	0.0001
†growing follicles	83.8 (83/99)		
In vitro culture - D12 to D18			
non-growing follicles	36.6 (11/30)	7.5 (2.5 - 21.9)	0.0003
†growing follicles	81.3 (35/43)		

C.I: 95% confidence interval.

† Growing follicles: the data of slow- and fast-growing follicles were combined.

Table 5

Association analyses between †oocyte size after *in vitro* maturation and metaphase II rates.

Comparisons among oocyte size	Metaphase II (%)	Odds ratio (95% C.I)	P - value
Small oocyte	4.7 (1/21)	8.5 (0.7 - 96.5)	0.165
Medium oocyte	30.0 (3/10)		
Small oocyte	4.7 (1/21)	360.0 (20.9 - 6186.4)	0.001
Large oocyte	94.7 (18/19)		
Medium oocyte	30.0 (3/10)	42.0 (3.7 - 475.0)	0.001
Large oocyte	94.7 (18/19)		

C.I: 95% confidence interval.

† Oocytes were classified according to size as: small, < 110 µm of diameter; medium, between 110.1 and 120.0 µm; and large, > 120.1 µm.

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8 CAPÍTULO 3

In vitro embryo production from early antral follicles of goats fed with a whole flaxseed based diet

"Produção de embriões *in vitro* a partir de folículos antrais iniciais de cabras alimentadas com uma dieta suplementada com sementes de linhaça"

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RESUMO

O presente estudo teve como objetivo a utilização da biotécnica do cultivo *in vitro* de folículos ovarianos (CIVF) como ferramenta para avaliar a influência da suplementação com semente de linhaça na dieta no desenvolvimento *in vitro* de folículos antrais iniciais (FAs) caprinos e a subsequente produção in vitro de embriões. Um total de 18 cabras adultas foram distribuídas homogeneamente em duas dietas: Controle e Linhaça. FAs de ambos os grupos experimentais (300-400 μm) foram isolados e cultivados *in vitro* durante 18 dias. Depois disso, os complexos *cumulus*-oócitos recuperados foram submetidos à MIV, e subsequentemente à FIV e ao cultivo embrionário *in vitro*. Os seguintes parâmetros foram avaliados: crescimento folicular e morfologia, produção de estradiol, taxa de recuperação e diâmetro oocitário, penetração espermática, formação de pronúcleos e desenvolvimento embrionário. A adição de semente de linhaça na dieta não afetou o crescimento folicular e nem o diâmetro. Uma maior ($P < 0,05$) porcentagem de oócitos ≥ 110 μm foi recuperada do tratamento com linhaça. No entanto, a taxa de fecundação foi maior ($P < 0,05$) no controle (40%), mas não foram encontradas diferenças nas taxas de fecundação normal ou de clivagem. Em conclusão, a linhaça na dieta aumentou a taxa de recuperação de oócitos ≥ 110 μm, mas afetou negativamente a taxa de fertilização total, embora não tenha afetado a taxa de clivagem.

Palavras-chave: cultivo *in vitro,* embriões, FIV, folículo ovariano, linhaça.

In vitro embryo production from early antral follicles of goats fed with a whole flaxseed based diet

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Abstract

The present study aimed to use the *in vitro* follicle culture (IVFC) biotechnique as a tool to evaluate the influence of whole flaxseed as feed supplementation in the diet on the *in vitro* development of caprine early antral follicles (EAFs) and further embryo production. A total of 18 adult goats were homogeneously allocated into two diet groups: Control and Flaxseed. EAFs from both experimental groups (300-400 µm) were isolated and cultured *in vitro* for 18 days. After that, recovered cumulus-oocyte complexes (COCs) were submitted to IVM, and subsequently to IVF and *in vitro* embryo culture. The following end points were evaluated: Follicular growth and morphology, oocyte recovery rate and diameter, sperm penetration, pronuclei formation, embryo development, and estradiol production. The addition of the whole flaxseed in the diet did not affect follicular growth and diameter. A greater ($P < 0.05$) percentage of oocytes ≥ 110 µm were recovered from the flaxseed treatment. However, fertilization rate was higher ($P < 0.05$) in the Control treatment (40%), but no differences were found regarding normal fertilization nor cleaved embryos. In conclusion, dietary flaxseed increased the recovery rate of fully grown oocytes, but it did negatively affect the sperm penetration rate, even though with no further effect on the cleavage rate.

Keywords: embryos, FIV, flaxseed, *in vitro* culture, ovarian follicle.

Introduction

The developing *in vitro* follicle culture (IVFC) biotechnique aims to mimic ovarian folliculogenesis *in vitro*. This biotechnique could be used to maximize the number of potentially fertilizable oocytes for assisted reproductive technologies in human, and for *in vitro* embryo production in livestock (Figueiredo et al., 2011). Also, IVFC may serve as an *in vitro* model for toxicological research (Stefansdottir et al., 2014), limiting the number of experimental animals needed.

It has been shown that the IVFC efficacy may be influenced by many variables, such as the culture system (Araújo et al., 2015, 2014; Pessoa et al., 2014), the base media composition (Araújo et al., 2015; Castro et al., 2014; Rossetto et al., 2013) and supplementation (Ferreira et al., 2016), the culture time length (Pessoa et al., 2014; Sun and Li, 2013), the animal model (Magalhães et al., 2011b; Rocha et al., 2014; R. Rossetto et al., 2013), the oxygen tension (Gigli et al., 2006; Xu et al., 2011), and the follicular category (Cadenas et al., 2017). Nonetheless, the vast majority of the studies have been performed using ovaries from a slaughterhouse. As a result, the ovaries used in the IVFC are from animals of different ages , breeds , and physiological status (Silva et al., 2014a, 2014b; Souza-Fabjan et al., 2014). Under these conditions, it is impossible to determine, for instance, the impact of nutrition, another essential variable, on *in vitro* follicle development.

In vivo, the association between nutrition and reproduction has been widely described, and it is accepted that a diet with a deficiency, excess or imbalance in energy, proteins, vitamins and minerals may compromise reproductive efficiency (Bindari et al., 2013). In this sense, dietary fatty-acids may positively affect reproduction in ruminants (Mattos et al., 2000). However, the relationship between the lipid content in diet, specifically polyunsaturated fatty acids (PUFAs) and oocyte quality is still controversial (Adamiak et al., 2005; Fernandes et al., 2014; Fouladi-Nashta et al., 2007), although its presence in follicular fluid has been positively correlated with oocyte competence (Matoba et al., 2014). Nevertheless, despite the importance of PUFAs for reproduction, to the best of our knowledge, there is no information about the effect of a diet rich in PUFAs on the *in vitro* follicle development. Among several possible diet supplements, flaxseed has been highlighted as an important source of PUFAs (Bernacchia et al., 2014).

Therefore, the present study used for the first time the IVFC biotechnology as a tool to

study the influence of the whole flaxseed supplementation in the diet on the *in vitro* development of caprine early antral follicles. The following end points were evaluated: follicular daily growth and diameter, oocyte recovery rate and diameter, sperm penetration, pronuclei formation, embryo development, and estradiol production.

Materials and methods

Unless mentioned otherwise, all chemicals and reagents used in the present study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., USA).

A total of 18 Anglo-Nubian cross-bred, non-lactating adult does, between second and third parity, and selected for cyclicity were allocated to two diet groups, according to homogeneity ($P > 0.05$) in body weight, (33.4 ± 3.6 kg; overall mean ± SD), body condition scores (2.7 ± 0.3, from 1 to 5), and ages (45.5 ± 7,25 months): Control and Flaxseed. In the control treatment (n=9), the diet consisted of Elephant Grass hay (*Pennisetum purpureum*) and commercial concentrate with corn and soybean meal as feed basis (52.8% corn, 22.7% soybean meal, 18.4% wheat bran, 6.0% minerals). In the flaxseed treatment (n=9), the diet consisted of Elephant Grass hay (*Pennisetum purpureum*) and concentrate containing 30.9% corn, 29.8% flaxseed, 20.2 wheat bran, 13.3% soybean meal, 5.9% minerals. All concentrate rations were isonitrogenous (18% Crude protein on Dry Matter (DM) basis). The average composition of the experimental concentrate-based diets, and the chemical composition of dietary ingredients are presented in Table 1 and Table 2, respectively.

Animals from each group, homogeneously segregated for use in three replicates were kept in collective stalls (Fernandes et al., 2014), receiving mineral salt and water *ad libitum*. A total of 7, 6 and 5 animals were used in the first, second and third replicates, respectively. In all experimental groups, diets were provided twice a day (07:00 and 15:00 h) for 30 days, up to follicle recovery. All animals received the diets to satisfy their energy requirements for maintenance and breeding according to the National Research Council (NRC, 2007) for adult non-dairy does.

The experimental design is summarized (Figure 1). Ovaries from both groups were collected and transported to the laboratory as previously described (Chaves et al., 2008). For the IVFC and oocyte IVM we used the methodology previously described by our group (Cadenas et al., 2017). Briefly, in the laboratory, early antral follicles (300 to 400 µm) from each dietary treatment, i.e., control and flaxseed were isolated and individually cultured *in vitro* in 100µL drops of α-MEM (pH 7.2 to 7.4), supplemented with 3 mg/mL bovine serum

albumin (BSA), 10 ng/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 50 µg/mL ascorbic acid, and 50 ng/mL growth hormone (GH) for 18 days at 38.5°C and 5% CO_2 in air under mineral oil. Fresh medium was prepared and pre-equilibrated overnight prior to use. Medium was replaced partially (60 µL) every other day. At the end of the culture, levels of estradiol were measured in spent media using a competitive immunoassay commercial kit (enzyme linked fluorescence assay VIDAS, Biomerieux, Marcy l'Etoile, France). The analytical sensitivity of the E2 was 9 pg/mL (range, 9 – 3000 pg/mL) and the intra-assay coefficient of variation was 5%. All follicles were mechanically opened and only those oocytes ≥ 110 µm in diameter (zona not included) were submitted to IVM in groups (10 COCs/100 µL-drop) for 30 h at 38.5°C and 5% CO_2 in air under mineral. The IVM medium consisted of tissue culture medium 199 supplemented with 1 µg/mL 17b-estradiol, 5 µg/mL luteinizing hormone, 0.5 µg/mL rFSH (bovine), 10 ng/mL epidermal growth factor, 1 mg/mL BSA, 1 mM pyruvate, 50 ng/mL, insulin-like growth factor 1, and 100 mM cysteamine.

After the IVM, all COCs were washed and transferred in groups of 10 into 100 µL drops fertilization medium under mineral oil. Fertilization medium consisted on IVF-TALP (Parrish et al., 1986) supplemented with 30 µg/mL heparin (Calbiochem 375095), 15 µM hypotaurine and 5 µg/mL gentamicin. The IVF-TALP medium was pre-equilibrated for at least 2 h prior to use. Refrigerated semen diluted in extender from two fertile bucks were pooled and motile sperm were selected by swim-up procedure (Fukui et al., 2000) in Sperm-TALP medium (Papa et al., 2015). Viable sperm were diluted in the appropriate volume of fertilization medium to achieve a final concentration of 2×10^6 sperm/mL. Spermatozoa and COCs were co-incubated for 18 h at 38.5 °C in a humidified atmosphere of 5% CO_2. After the IVF, all presumptive zygotes were washed and transferred to microdrops of embryo culture medium (G1™, Vitrolife, Gothemburg, Sweden) (10 zygotes: 10 µL medium) under mineral oil, and incubated for 2 days at 38.5 °C in a humidified atmosphere of 5% CO_2 and 5% O_2. On day 3 post-IVF, all cleaved embryos were surgically transferred into the oviduct of 3 synchronized recipient goats. Pregnancy diagnosis was performed on day 36 after transfer by transrectal ultrasonography. Statistical analyses were carried out using Sigma Plot 11 (Systat Software Inc., USA). Comparison of means (follicle and oocyte diameters, estradiol and daily growth rate) between treatments were analyzed by t-test. One-way repeated measures analysis of variance (Holm-Sidak pos-hoc test) was performed to compare the effect of treatment among days of culture. When appropriate, chi-square or Fisher's exact tests were used to

evaluate the percentage variables (in vitro fertilization parameters, intact, degenerated, and extruded follicles) between treatments. Data are presented as mean (± SEM) and percentage, and the statistical significance was defined as $P < 0.05$ (two-sided).

Results and Discussion

Main results are summarized (Table 3). As mentioned before, flaxseed is rich in PUFAs (Bernacchia et al., 2014). Our results showed that there was no influence of the administration of the whole flaxseed in the diet on follicle morphology, growth rate and estradiol production. Similar results were shown *in vivo* where dietary PUFAs did not affect either follicular dynamics or steroid production in bovine (Bilby et al., 2006; Childs et al., 2008). Also, PUFAs had no effect on granulosa cell proliferation and steroidogenesis during *in vitro* culture in ovine (Wonnacott et al., 2010) and bovine (Lammoglia et al., 1997). Conversely, several authors have shown increasing dietary PUFAs increased the size of preovulatory follicles (Beam and Butler, 1997; Lucy et al., 1993) and the total number of follicles (Beam and Butler, 1997; Lammoglia et al., 1997; Lucy et al., 1993; Thomas and Williams, 1996).

Both treatments, control and flaxseed increased ($P < 0.05$) follicle diameter from day 0 (368.12 ±7.8 and 376.28 ± 8.1 µm, respectively) to the end of the culture period (779.70 ± 25.6 and 783.71 ± 25.7 µm, respectively). Also, follicle daily growth rate was lower ($P < 0.05$) in the first six days of culture than from day 6 onwards (10.36 ± 1.1 and 10.90 ± 1.1 vs. 34.76 ± 2.2 and 34.35 ± 2.0 µm/day, respectively). These growth patterns (first third of culture vs. second and last third) are in agreement with previous published papers under the same culture conditions (Cadenas et al., 2018). However, compared to a previous study performed by our group, the end points: percentage of morphologically normal follicles (~80 vs. 65%), follicle diameter (~780 vs. ~550 µm) and growth rates (~23 vs. ~11 µm/day) showed higher figures when using ovaries from controlled well-nourished animals (present study) compared to ovaries from slaughterhouse (Cadenas et al., 2017). It is well known that animal nutritional status (Abecia et al., 2006) and age (Ottolenghi et al., 2004) can profoundly affect *in vivo* follicle development. Therefore, the use of a homogenous group of animals (same body weight, body condition score, and age) could contribute to provide a high quality starting material for the IVFC.

After the IVFC, the recovery rate of oocytes ≥ 110 µm was higher ($P < 0.05$) in the flaxseed treatment. The beneficial effect of the whole flaxseed in the diet on oocyte growth

may be due to the fact that among other important functions, fatty acids are stored within the oocyte and cumulus cells, providing a potent source of energy via β-oxidation (Dunning et al., 2014). However, oocytes from goats fed with flaxseed based diet showed lower ($P < 0.05$) overall sperm penetration. This result is consistent with other studies that associated elevated fatty acid concentration in diet with low oocyte competence (Nolan et al., 1998; O'Callaghan et al., 2000; Wakefield et al., 2008). The relationship between fatty acids and oocyte quality is still controversial since some studies showed positive effects (Fouladi-Nashta et al., 2007; Matoba et al., 2014), while in others no effect was observed (Bilby et al., 2006; Fernandes et al., 2014).

Percentages of normal fertilization (2PN) and cleaved embryos on day 3 post-IVF were not affected by the presence of the whole flaxseed in the diet. Similar results were described in caprine when another important source of PUFAs, cashew walnuts, was added to the diet (Fernandes et al., 2014).

Regardless of the diet, most of the fertilized oocytes showed only 1 pronucleus (MPN) and only five embryos were produced on day 3 post-IVF, three at the 4- to 6-cell stage, and two at the 2- to 3-cell stage. All five embryos were surgically transferred into the oviduct of 3 recipient goats. Thirty-six days after the embryo transfer, none of the recipient showed signs of estrous behavior, however, no pregnancy was detected after ultrasonography examination. The low embryo production when using *in vitro* grown caprine oocytes has been described before (Magalhães et al., 2011a; Saraiva et al., 2010; Silva et al., 2014a). However, those previous studies showed low oocyte nuclear maturation as well, unlike our system, which was able to produce a considerable amount of metaphase II oocytes (Cadenas et al., 2017). Here, 40% of the *in vitro* grown oocytes were fertilized. Even though most of the fertilized oocytes formed only MPN, to our knowledge, this is the highest fertilization rate described in this specie so far for *in vitro* grown oocytes. These findings indicate that the oocytes were penetrated by the spermatozoa and were able to contribute toward MPN formation. However, the second meiotic resumption did not occur appropriately, preventing normal female pronuclei formation and subsequent cleavage. Taken together, these facts suggest that oocyte developmental competence (oocyte cytoplasmic maturation) (Mao et al., 2014) should be improved.

In conclusion, except for the increasing in the percentage of fully grown oocytes, in general, dietary flaxseed did not affect goat *in vitro* folliculogenesis. However, it did negatively affect the sperm penetration rate, even though with no further effect on the

cleavage rate. Also, our current IVFC system was able to produce a considerable amount of meiotically matured oocytes that were able to be fertilized. Nevertheless, the low percentage of oocytes showing normal fertilization and embryo production highlight the need for further research in order to improve oocyte cytoplasmic maturation.

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Statement of Interest

None

Ethical Standards

All experiments were performed according to the recommendations of the Committee of Animal Handling and Ethical Regulation from the State University of Ceara, Fortaleza, Ceara, Brazil.

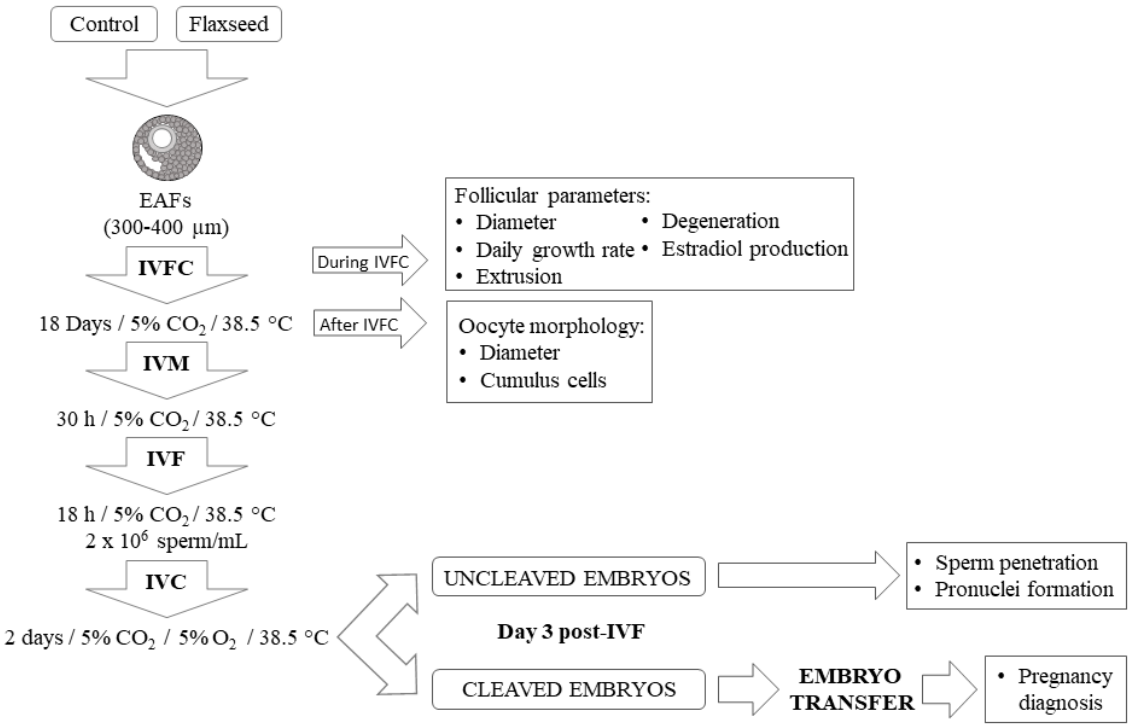

Figure 1. Experimental design. Abbreviations: EAFs, early antral follicles; IVFC, *in vitro* follicle culture; IVM, oocyte *in vitro* maturation; *IVF*, *in vitro* fertilization; IVC, *in vitro* embryo culture**.**

Table 1
Ingredient composition of the concentrate-based diets

Ingredient (% DM)	Diet	
	Control	Linseed
Soybean meal	22.70	13.03
Ground corn	52.83	30.97
Flaxseed	-	29.83
Wheat bran	18.43	20.20
Vitamin mineralized premix	6.04	5.93

Abbreviations: DM, dry matter.

Table 2
Chemical composition of the dietary ingredients

Ingredients	Composition (% DM)					
	DM	EE	CP	NDF	ADF	Ash
Elephant Grass hay (*Pennisetum purpureum*)	86.26	1.60	6.89	58.30	35.20	8.04
Flaxseed	88.58	28.39	20.30	33.11	23.10	3.42
Concentrate–based suplements						
Control diet	89.56	2.51	18.30	16.82	6.11	7.87
Flaxseed diet	89.37	10.26	18.17	23.47	11.74	7.89

Abbreviations: DM, dry matter; EE, ether extract; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber.

Table 3

Percentages of morphologically normal, extruded and degenerated follicles, daily growth, estradiol production, oocyte diameter, percentage of oocytes ≥ 110 µm, sperm penetration, male pronucleus formation, two pronuclei formation and cleaved embryos after the IVFC of caprine EAFs.

Treatments	End points										
	MIF (%)	Ext (%)	Deg (%)	Daily growth (µm) (mean ± SEM)	E2 (ng/mL) (mean ± SEM)	Oocyte diameter (mean ± SEM)	Oocytes ≥ 110 µm (%)	† Oocyte fertilization (%)			Cleaved* (%)
								Sperm penetration	MPN*	2PN*	
Control (n=100)	78.0 (78/100)	8.0 (8/100)	14.0 (14/100)	22.57 ± 1.6	36.37 ± 13.1	120.1 ± 1.0	62.0 (62/100) A	40.3 (25/62) B	76.0 (19/25)	24.0 (6/25)	12.0 (3/25)
(Flaxseed) (n=76)	85.5 (65/76)	6.6 (5/76)	7.9 (6/76)	23.77 ± 1.4	53.69 ± 37.7	119.0 ± 0.9	76.3 (58/76) B	20.7 (12/58) A	75.0 (9/12)	25.0 (3/12)	16.7 (2/12)

† Only oocytes ≥ 110 µm were submitted to IVM, IVF and IVC.

* Values calculated out of the overall fertilized oocytes.

A,B Within a column ($P < 0.05$)

Abbreviations: IVFC, *in vitro* follicle culture; EAFs, early antral follicles; MIF, morphologically intact follicles; Ext, extruded follicles; Deg, degenerated follicles; E2, estradiol; MPN, male pronucleus; 2PN, two pronuclei.

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9 CONCLUSÕES

Nas condições de cultivo descritas no presente trabalho, podemos afirmar que:

a) O meio de cultivo para folículos antrais iniciais isolados caprinos com insulina na concentração fisiológica (10 ng/mL), associado a 50 ng/mL de GH, é capaz de manter níveis de crescimento e maturação oocitária semelhantes aos seus homólogos crescidos *in vivo*.

b) Folículos pré-antrais e antrais iniciais caprinos se comportam de forma diferente quando submetidos as mesmas condições de experimentais.

c) Os oócitos caprinos podem ser maturados individualmente *in vitro*, tão eficientemente quanto em grupos.

d) A taxa de crescimento diário e o diâmetro folicular, bem como no diâmetro oocitário podem ser usados como sinais não invasivos de competência meiótica de oócitos caprinos. Assim, os pontos de corte que permitem a produção de oócitos meioticamente competentes são: crescimento folicular diário de 7 μm, diâmetro folícular de 600 μm e diâmetro oocitário de 120 μm. Além disso, 18 dias parece ser o tempo de cultivo mais adequado para folículos antrais iniciais caprinos.

e) Os oócitos crescidos *in vitro* após o cultivo de folículos antrais iniciais caprinos tem a capacidade ser fecundados e produzir embriões *in vitro*.

f) A dieta rica em ácidos graxos poli-insaturados não afeita o desenvolvimento folicular nem a competência oocitária *in vitro*.

10 PERSPECTIVAS

Sabendo que a foliculogênese é um processo dinâmico, a presente tese abordou o cultivo folicular *in vitro* a partir de uma perspectiva completamente nova, que consiste em estudar as diferenças entre folículos pertencentes à diferentes categorias foliculares, bem como rastrear cada oócito após a MIV com o seu folículo correspondente. Assim, demonstrou-se que o requerimento dos folículos cultivado *in vitro* varia de acordo com o estágio do desenvolvimento folicular, sendo que o GH tem um papel fundamental durante a fase antral da foliculogênese *in vitro*, e que as dinâmicas de crescimento folicular estão correlacionadas com a maturação nuclear oocitária *in vitro* na espécie caprina.

Em termos gerais, o meio de cultivo de base desenvolvido no presente trabalho bem como os marcadores foliculares não invasivos de maturação oocitária poderão ser utilizados para o desenvolvimento de novos meios e sistemas de cultivo visando melhorar as atuais baixas taxas de produção embrionária *in vitro* e posterior geração de crias vivas.

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