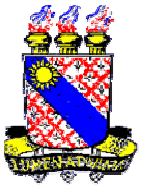

UECE

UNIVERSIDADE ESTADUAL DO CEARÁ

PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS (PPGCV)

MARIA HELENA TAVARES DE MATOS

CULTIVO IN VITRO DE FOLÍCULOS PRÉ-ANTRAIS CAPRINOS UTILIZANDO ÁCIDO 3-INDOL-ACÉTICO (IAA), FATOR DE CRESCIMENTO FIBROBLÁSTICO-2 (FGF-2) E HORMÔNIO FOLÍCULO ESTIMULANTE (FSH)

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Orientador: Prof. Dr. José Ricardo de Figueiredo

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Aos meus pais,

Aos meus irmãos,

Ao meu esposo,

por tudo que representam em minha vida.

Com amor, dedico.

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RESUMO

Os objetivos deste estudo foram investigar o efeito do Ácido 3-Indol Acético (IAA), Fator de Crescimento Fibroblástico-2 (FGF-2) e Hormônio Folículo Estimulante (FSH) sobre a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos *in vitro*, utilizando técnicas de histologia clássica e microscopia eletrônica de transmissão. Na Fase I, verificou-se o efeito de diferentes concentrações de IAA (10, 20, 40 e 100 ng/ml), FGF-2 (10, 50 e 100 ng/ml) ou FSH (10, 50 e 100 ng/ml). As concentrações que apresentaram os melhores resultados foram: IAA (20 ng/ml), FGF-2 (50 ng/ml) e FSH (50 ng/ml). As maiores taxas de sobrevivência folicular foram observadas após 7 dias de cultivo com FSH (65,6%). Somente após cultivo com IAA (1 e 7 dias) e MEM (7 dias), folículos considerados como morfologicamente normais após análise histológica, revelaram-se degenerados quando examinados ultra-estruturalmente. Ao contrário, os folículos cultivados em FSH e FGF-2 apresentavam-se ultra-estruturalmente normais. Diante deste resultado, o IAA não foi utilizado na Fase II deste estudo, sendo testados somente o FSH e o FGF-2 sozinhos ou a interação entre FSH e FGF-2. Nesta fase, o cultivo com FSH sozinho apresentou as maiores percentagens de folículos normais e a interação FSH + FGF-2 foi mais eficiente para promover a ativação folicular e o crescimento oocitário após 7 dias. Em função deste resultado, o FSH sozinho e a associação FSH + FGF-2 foram utilizados para suplementação do meio no cultivo de longa duração (Fase III). Além disso, no tratamento com FSH, testou-se dois intervalos de troca do meio de cultivo (a cada 2 ou 7 dias). Os resultados mostraram que após 28 dias de cultivo, o FSH (com troca de meio a cada 2 dias) aumentou a sobrevivência, a formação de folículos primários, bem como os diâmetros oocitário e folicular. Em conclusão, este estudo demonstrou que o FSH estimula a ativação de folículos primordiais caprinos e o crescimento de folículos primários após cultivo *in vitro* de 28 dias. Além disso, a adição de FSH ao meio de cultivo e a troca do meio a cada dois dias mantêm a morfologia de folículos pré-antrais caprinos cultivados por um longo período.

ABSTRACT

The aims of this study were to investigate the effect of Indol-3-Acetic Acid (IAA), Fibroblast Growth Factor-2 (FGF-2) and Follicle Stimulating Hormone (FSH) on the survival, activation and growth of caprine preantral follicles *in vitro*, using classical histology and trasmission electron microscopy. In Phase I, the effects of different concentrations of IAA (10, 20, 40 and 100 ng/ml), FGF-2 (10, 50 and 100 ng/ml) or FSH (10, 50 and 100 ng/ml) were analysed. The best results were found in the following concentrations: IAA (20 ng/ml), FGF-2 (50 ng/ml) e FSH (50 ng/ml). The highest percentages of follicular survival were observed after 7 days of culture with FSH (65,6%). Only after culture with IAA (1 and 7 days) and MEM (7 days), follicles judged as morphologically normal after histological analysis were ultrastructurally degenerated. On the other hand, follicles cultured in FSH and FGF-2 were ultrastructurally normal. Then, IAA was not used in Phase II of this study and only FSH and FGF-2 alone or the interaction between FSH and FGF-2 was tested. In this phase, culture with FSH alone showed the highest percentages of normal follicles and the interaction FSH + FGF-2 was more efficient to promote follicular activation and oocyte growth after 7 days. Based on these results, FSH alone and the association FSH + FGF-2 were used to supplement the medium in the long-term culture (Phase III). In addition, in the treatment with FSH, two intervals of medium change were tested (at each 2 or 7 days). The results showed that after 28 days of culture, FSH (with the medium changed at each 2 days) increased the survival, formation of primary follicles, as well as oocyte and follicular diameters. In conclusion, this study demonstrated that FSH stimulates the activation of primordial follicles and the growth of primary follicles after *in vitro* culture for 28 days. Furthermore, addition of FSH to the culture medium and the replacement of this medium every two days maintain caprine preantral follicles morphology after long-term culture.

SUMÁRIO

LISTA DE ABREVIATURAS E SÍMBOLOS

A -	:Antro
ATP -	:Adenosina Trifosfato
b -	:basement membrane (membrana basal)
BMP-15 -	:Bone Morphogenetic Protein-15 (Proteína morfogenética do osso-15)
BrdU-	:Bromo-deoxiuridina
CG -	:Células da granulosa
CGP -	:Células germinativas primordiais
CNPq -	:Conselho Nacional de Desenvolvimento Científico e Tecnológico
CT -	:Células da teca
DNA -	:Deoxyribonucleic Acid (Ácido Desoxirribonucléico)
EGF -	:Epidermal Gorwth Factor (Fator de crescimento epidermal)
er -	:endoplasmic reticulum (retículo endoplasmático)
FGF-2 -	:Fibroblast Growth Factor-2 (Fator de Crescimento Fibroblástico-2)
FINEP -	:Financiadora de Estudos e Projetos
FSH -	:Follicle Stimulating Hormone (Hormônio Folículo Estimulante)
FUNCAP -	:Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnlógico
GDF-9 -	:Growth Differentiation Factor-9 (Fator de crescimento de diferenciação-9)
GH -	:Growth Hormone (Hormônio do crescimento)
GC -	:Granulosa Cells (Células da granulosa)
HC -	:Histologia Clássica
HE -	:Hematoxilina-Eosina
IAA -	:Indol 3-Acetic Acid (Ácido 3-indol Acético)
IGF-2 -	:Insulin Like Growth Factor-2 (Fator de Crescimento Semelhante à Insulina-2)
IGFPB -	: Insulin Like Growth Factor Binding Protein (Proteína de ligação do IGF)
KGF -	:Keratinocyte Growth Factor (Fator de crescimento keratinócito)
KL -	:Kit Ligand
l -	:Lipid droplets (gotas lipídicas)
L -	:Litro
LAMOFOPA -	:Laboratório de Manipulação de Oócitos e Folículos Ovarianos Pré-Antrais
LFCR -	:Laboratório de Fisiologia e Controle da Reprodução

LH - :Luteinizing Hormone (Hormônio Luteinizante)

LIF - :Leukemia Inhibitory Factor (Fator inibidor de leucemia)

LM - :Light microscopic (Microscopia de luz)

m - :Mitocôndrias

M - :Molar

mL - :Mililitro

mm - :Milímetros

mM - :Milimolar

MEM - :Meio Essencial Mínimo

MET - :Microscopia Eletrônica de Transmissão

MOIFOPA - :Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-Antrais

ng - :Nanograma

nm - :Nanômetros

Nu - :Núcleo

no - :nucleolus (nucléolo)

O - :Oocyte (Oócito)

ORS - :Oxygen Reactive Species (Espécies Reativas de Oxigênio)

PAS - :Periodic Acid Schiff (Ácido Periódico de Schiff)

PCNA - :Proliferation Cell Nuclear Antigen (Antígeno Nuclear de Proliferação Celular)

REL - :Retículo Endoplasmático Liso

RER - :Retículo Endoplasmático Rugoso

RNAm :Ácido Ribonucléico mensageiro

s - :Stroma

ser - :Smooth endoplasmic reticulum (Retículo endoplasmático liso)

TEM - :Transmission Electron Microscopy (Microscopia Eletrônica de Transmissão)

TGF-α - :Transforming Growth Factor- α (Fator de crescimento de transformação-α)

TGF-β - : Transforming Growth Factor-β (Fator de crescimento de transformação-β)

TUNEL - :Terminal deoxynucleotidil tranferase-mediated deoxyuridine triphosphate biotin nick end-labeling

UECE - :Universidade Estadual do Ceará

UFC - :Universidade Federal do Ceará

UnB - :Universidade deBrasília

Unesp - :Universidade Estadual Paulista

v - :vesicles

VEGF - :Vascular Endotelial Growth Factor (Fator de crescimento do endotélio vascular)

VG - :Vesícula Germinativa

ZP - :Zona pelúcida

µg - :Micrograma

µm - :Micrômetro

µM - :Micromolar

^{o}C :Graus Celsius

1. INTRODUÇÃO

Diversas tecnologias da reprodução assistida têm sido desenvolvidas com o intuito de aumentar a utilização do potencial reprodutivo em fêmeas mamíferas, tais como a fecundação *in vitro*, a transferência de embriões, a transgenia e a clonagem. Entretanto, a utilização em larga escala destes procedimentos depende da disponibilidade de oócitos maturos, que constituem uma pequena porção dos oócitos presentes no ovário (MURUVI *et al.*, 2005). Por outro lado, o ovário mamífero contém milhares de oócitos inclusos em folículos pré-antrais em diversos estádios de desenvolvimento (primordiais, transição, primários e secundários) (SILVA *et al.*, 2004a). Em qualquer fase deste desenvolvimento, o folículo pode continuar a desenvolver normalmente ou, mais freqüentemente, sofrer atresia, que ocorre com aproximadamente 99,9% dos folículos (KATSKA-KSIAZKIEWICK, 2006).

Neste sentido, a biotécnica de Manipulação de Oócitos Inclusos em Folículos Pré-Antrais (MOIFOPA) vem sendo desenvolvida com o objetivo de recuperar um grande número de oócitos inclusos nestes folículos e, posteriormente, cultivá-los *in vitro* até sua completa maturação, prevenindo-os assim da atresia. Em associação com outras tecnologias reprodutivas, a MOIFOPA poderá fornecer milhares de oócitos maturos, que podem ser utilizados, por exemplo, para a multiplicação de animais de alto valor genético, programas de conservação de espécies raras ou ameaçadas de extinção e, ainda, para a preservação da fertilidade e reprodução assistida em humanos (MURUVI *et al.*, 2005). Além disso, a MOIFOPA poderá contribuir para uma melhor compreensão acerca dos fatores e mecanismos implicados na foliculogênese inicial e no processo de atresia folicular (FIGUEIREDO *et al.*, 2003).

Vários sistemas de cultivo *in vitro* têm sido desenvolvidos a fim de produzir oócitos maturos a partir de oócitos inclusos em folículos nos estádios iniciais de desenvolvimento. Em camundongos, já foi relatada a obtenção de crias saudáveis a partir do cultivo *in vitro* de folículos pré-antrais (O'BRIEN et al, 2003). Entretanto, em espécies domésticas, o sucesso tem sido mais lento e limitado à investigação da viabilidade e do crescimento folicular pós-cultivo.

No decorrer desta tese, serão abordados aspectos como oogênese, foliculogênese, atresia folicular, importância e aplicações da MOIFOPA, utilização do Ácido 3-indol Acético (IAA), Fator de Crescimento Fibroblástico-2 (FGF-2) e do Hormônio Folículo Estimulante (FSH) no cultivo *in vitro* de folículos pré-antrais.

2. REVISÃO DE LITERATURA

2.1. O ovário mamífero

O ovário mamífero é o órgão principal do sistema reprodutivo das fêmeas e exerce duas funções fisiológicas importantes, sendo responsável pela: 1) diferenciação e liberação do oócito maturo para posterior fecundação (função exócrina); 2) síntese de hormônios e diversos peptídeos (função endócrina) que são essenciais para o desenvolvimento folicular, ciclicidade e manutenção da gestação (BARNETT *et al.*, 2006). Ele é composto por vários tipos celulares diferenciados, é circundado por uma superfície epitelial, comumente conhecida como epitélio germinal e possui duas regiões: cortical e medular.. A medula ovariana é localizada na porção mais interna do ovário, com exceção dos eqüídeos, e consiste de um arranjo irregular de tecido conjuntivo fibroelástico e um extenso sistema nervoso e vascular. A região cortical contém folículos ovarianos em vários estádios de desenvolvimento ou regressão, bem como corpos lúteos (LIU *et al.*, 2006). O tecido conjuntivo do córtex consiste de fibroblastos, colágeno e fibras reticulares (SILVA, 2005) (Figura 1).

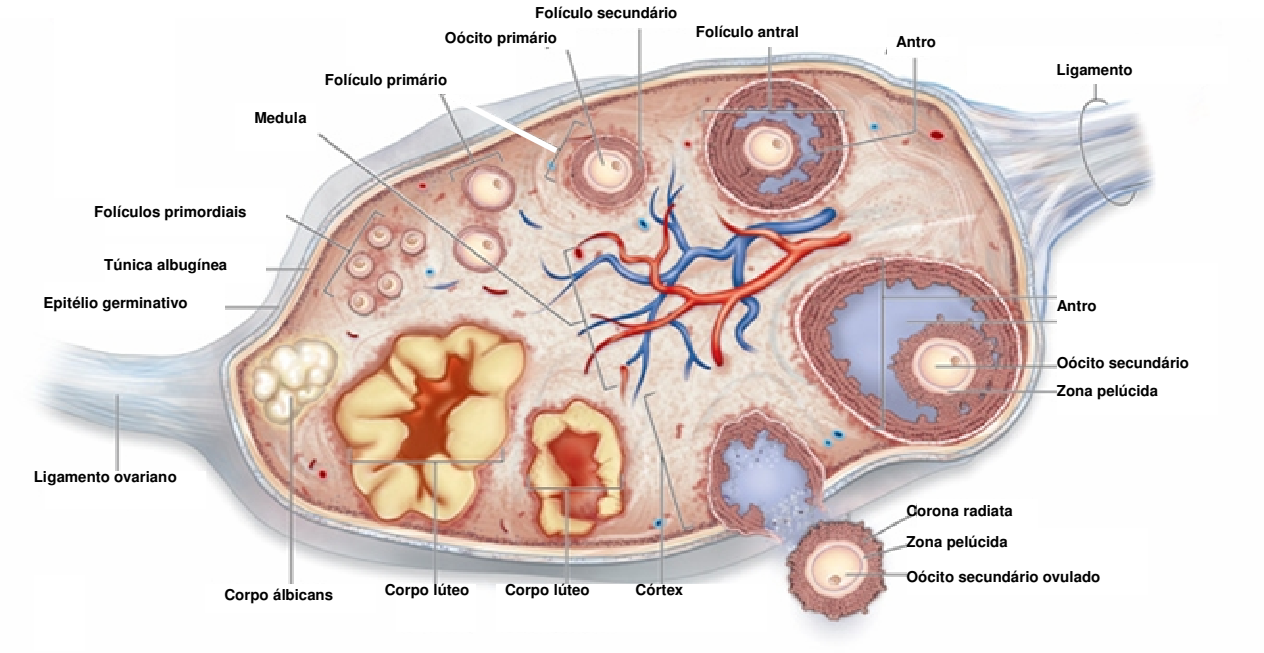

Figura 1. Representação esquemática do ovário mamífero.
Adaptado de http://academic.kellogg.cc.mi.us/herbrandsonc/bio201_McKinley/Reproductive.

2.2. Oogênese em ruminantes

A oogênese em ruminantes consiste na formação e diferenciação das células germinativas primordiais (CGP) até a formação do oócito haplóide fecundado. A oogênese inicia-se antes do nascimento, mas somente alguns oócitos conseguem completar este processo meses ou anos mais tarde no animal adulto, após a fecundação (FIGUEIREDO *et al.*, 2003). O processo de formação do oócito envolve sete etapas: (1) produção das células germinativas primordiais (CGP); (2) migração das CGP para as gônadas; (3) colonização das gônadas pelas CGP; (4) diferenciação das CGPs em oogônias; (5) proliferação das oogônias; (6) início da meiose das oogônias; (7) parada da meiose no estádio de diplóteno da prófase I (VAN DEN HURK & ZHAO, 2005).

Durante o início do desenvolvimento fetal, ocorre a migração das CGP do saco vitelínico para a região das gônadas primitivas (VAN DEN HURK & ZHAO, 2005). Em seguida, as CGP multiplicam-se e transformam-se em oogônias, as quais possuem uma alta atividade mitótica e transcricional (EPPIG *et al.*, 2004). Então, ocorre a replicação do DNA das oogônias e estas entram em meiose, tornando-se oócitos. Estes começam a primeira divisão meiótica, passando pelos estádios da prófase I (leptóteno, zigóteno e paquíteno), permanecendo no estádio de diplóteno ou vesícula germinativa até à puberdade. Neste período, o pico do Hormônio Luteinizante (LH) induz o oócito a retomar a meiose e então, ocorre o rompimento da vesícula germinativa, progressão para metáfase I, anáfase I e telófase I, expulsão do primeiro corpúsculo polar e formação do oócito secundário. Inicia-se a seguir a segunda divisão meiótica, em que o núcleo do oócito evolui até o estádio de metáfase II, quando ocorre a segunda interrupção da meiose (VAN DEN HURK & ZHAO, 2005). O oócito permanece neste estádio até ser fecundado pelo espermatozóide, quando então, completa a meiose e expulsa o segundo corpúsculo polar, formando o oócito haplóide fecundado (MOORE & PERSAUD, 1994).

2.3. Foliculogênese e características dos folículos ovarianos

A foliculogênese pode ser definida como o processo de formação, crescimento e maturação folicular, iniciando-se com a formação do folículo primordial e culminando com o estádio de folículo pré-ovulatório (VAN DEN HURK E ZHAO, 2005). Na maioria das espécies, a foliculogênese se inicia na vida fetal. Entretanto, em roedores, este processo têm

início durante os primeiros dias após o nascimento (FORTUNE, 2003). A foliculogênese pode ser dividida nas seguintes fases de desenvolvimento: (1) início do desenvolvimento de folículos primordiais (também conhecido como ativação folicular) e formação dos folículos primários, (2) transição de folículos primários para secundários, (3) crescimento de folículos secundários e formação de folículos antrais, e (4) crescimento e diferenciação de folículos antrais e formação de folículos pré-ovulatórios (SILVA, 2005).

O folículo é considerado a unidade morfológica e funcional do ovário mamífero, cuja função é proporcionar um ambiente ideal para o crescimento e maturação do oócito (CORTVRINDT e SMITZ, 2001), bem como produzir hormônios e peptídeos (BARNETT *et al.*, 2006). O folículo ovariano é composto por um oócito circundado por células somáticas (granulosa e tecais) (MAGOFFIN, 2005). De acordo com o grau de evolução, os folículos podem ser classificados em pré-antrais ou não cavitários (primordiais, transição, primários e secundários) e antrais ou cavitários (terciários e pré-ovulatórios) (FIGUEIREDO *et al.*, 2003) (Figura 2).

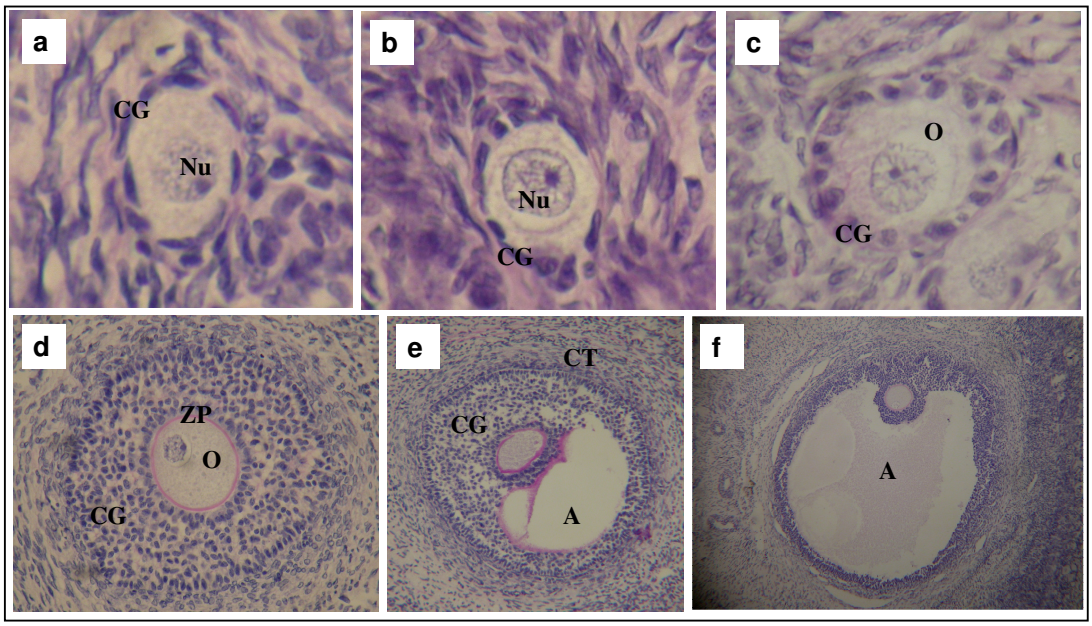

Figura 2. Folículos ovarianos caprinos após coloração com Ácido Periódico de Schiff (PAS)-Hematoxilina (400 x). Folículos pré-antrais: (a) primordial; (b) transição; (c) primário e (d) secundário. Folículo antrais: (e) terciário e (f) pré-ovulatório. Nu: núcleo do oócito; O: oócito; CG: células da granulosa; ZP: zona pelúcida; A: antro; CT: células da teca.

Após a formação dos oócitos, estes se associam em cachos ou ninhos de células germinativas. Ainda na vida pré-natal, na maioria das espécies, ocorre a apoptose de alguns oócitos dentro dos ninhos e o rompimento destes. Em seguida, os oócitos sobreviventes são circundados por células somáticas (células da pré-granulosa), formando os folículos primordiais (PEPLING & SPRADLING, 1998) (Figura 3). Sabe-se que um gene expresso no oócito chamado de Fig α é essencial para a formação dos folículos primordiais (BARNETT *et al.*, 2006).

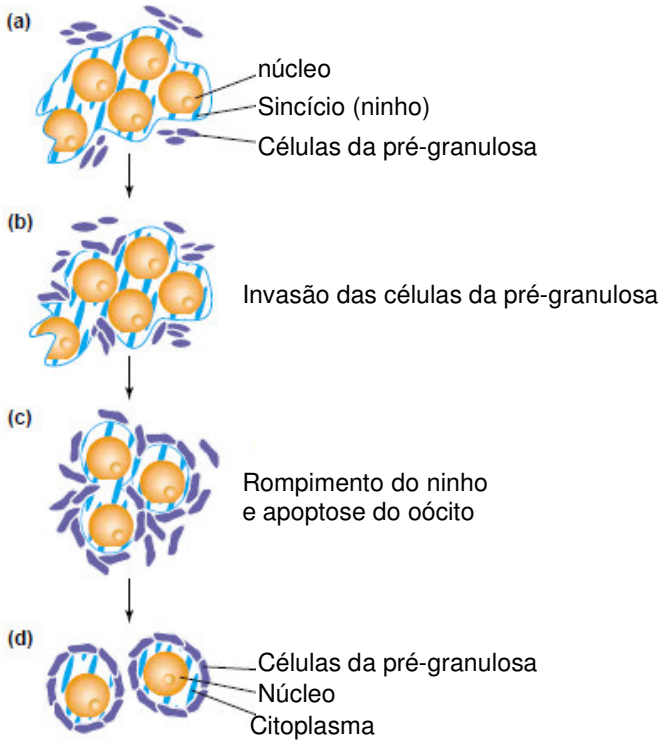

Figura 3. Formação de folículos primordiais. (a) Ninhos de células germinativas; (b) Células somáticas (pré-granulosa) surgem para invadir o sincício; (c) rompimento do sincício (ninho) acompanhado de intensa apoptose oocitária; (d) as células germinativas sobreviventes, no estádio de prófase I, são circundadas por uma camada de células da pré-granulosa e por uma membrana basal, formando os folículos primordiais. Adaptado de EPIFANO & DEAN (2002).

Os folículos primordiais são constituídos por um oócito (no estádio de diplóteno da prófase I) circundado por uma camada de células da pré-granulosa pavimentosas (SILVA, 2005). A zona pelúcida nesse estádio ainda não é observada, verificando-se apenas uma justaposição do oócito e células da granulosa, sem nenhuma junção específica (LUCCI *et al.*, 2001). Em ovários de cabras, os folículos primordiais têm um diâmetro médio de 33 μm e são observados a partir do 62º dia de desenvolvimento fetal (BEZERRA *et al.*, 1998). Uma vez formados, os folículos primordiais representam o pool de células germinativas disponíveis durante toda a vida reprodutiva (EPIFANO & DEAN, 2002), embora estudos recentes tenham sugerido que a oogênese pós-natal também pode ocorrer em fêmeas mamíferas (JOHNSON *et al.*, 2004). É importante ressaltar que os folículos primordiais correspondem a um total de 90% de todos os folículos presentes no ovário (SMITZ & CORTVRINDT, 2002).

Os folículos primordiais permanecem quiescentes até a ativação para o grupo de folículos em crescimento (VAN DEN HURK & ZHAO, 2005). Os mecanismos seletivos pelos quais alguns folículos crescem e outros permanecem quiescentes ainda não são completamente compreendidos (EPIFANO & DEAN, 2002). Sabe-se que o desenvolvimento folicular inicial, incluindo a transição de folículo primordial para folículo primário (ativação folicular) é independente de gonadotrofinas e que é regulado, principalmente, por fatores intra-ovarianos (VAN DEN HURK & ZHAO, 2005). Provavelmente, a ativação de folículos primordiais é regulada pelo balanço entre fatores inibitórios e estimulatórios presentes no próprio ovário. Dentre os fatores estimulatórios para a ativação folicular, destacam-se: kit ligand (KL – PARROTT & SKINNER, 1999), fator de crescimento de diferenciação-9 (GDF-9 – VITT *et al.*, 2000), fator de crescimento fibroblástico-2 (FGF-2 – NILSSON *et al.*, 2001), fator inibidor de leucemia (LIF – NILSSON *et al.*, 2002) e proteína morfogenética do osso-4 (BMP-4) (Nilsson & Skinner, 2003). Por outro lado, alguns estudos têm demonstrado que o hormônio Anti-Mülleriano inibe o recrutamento de folículos primordiais para o pool de folículos em crescimento (DURLINGER *et al.*, 2002).

O início do crescimento folicular é caracterizado pelo início da proliferação das células da granulosa e mudança na sua morfologia, bem como pelo crescimento do oócito (BARNETT *et al.*, 2006). Após a ativação, os folículos primordiais gradualmente adquirem células da granulosa de morfologia cúbica, tornam-se folículos de transição (oócito circundado por uma camada de células da granulosa pavimentosas e cúbicas) e, em seguida, folículos primários, quando são circundados por uma única camada de células da granulosa cúbicas (BARNETT *et al.*, 2006). Os folículos primários possuem diâmetro médio de 50 μm

e são observados em ovários de cabras no 71º dia de desenvolvimento fetal (BEZERRA *et al.*, 1998). Durante o crescimento de folículos primários, as células da granulosa proliferam e ocorre um aumento no tamanho do oócito e no seu conteúdo protéico (VAN DEN HURK & ZHAO, 2005). Ultra-estruturalmente, devido ao maior metabolismo e, conseqüentemente, da maior necessidade de energia e nutrientes, observa-se um aumento no número de mitocôndrias alongadas, bem como de retículos endoplasmáticos lisos e rugosos no oócito (FAIR *et al.*, 1997).

Os folículos secundários (também chamados de multilaminares) são formados quando duas a três camadas de células da granulosa estão presentes, as células da teca podem ser visualizadas em torno da membrana basal e a zona pelúcida pode ser identificada (VAN DEN HURK e ZHAO, 2005). As células da teca são células endócrinas que desempenham uma função importante na fertilidade, pois sintetizam o hormônio esteróide (MAGOFFIN, 2005). Os folículos secundários podem ser observados em ovários de cabras no 80º dia de gestação (BEZERRA *et al.*, 1998). Alguns fatores de crescimento locais estão envolvidos na transição de folículo primário para secundário, tais como GDF-9 (camundongos; DONG *et al.*, 1996), Ativina-A (vaca: HULSHOF *et al.*, 1997), proteína morfogenética do osso-15 (BMP-15) (ovelha: GALLOWAY *et al.*, 2000), fator de crescimento epidermal (EGF) (vaca: WANDJI *et al.*, 1996), fator de crescimento de transformação-β (TGF-β) (camundongo: LIU *et al.*, 1999) e fator de crescimento do endotélio vascular (VEGF) (YANG & FORTUNE, 2007). Recentemente, YANG & FORTUNE (2006) mostraram que a testosterona também pode influenciar a transição de folículos primários para secundários. Os folículos secundários, a partir do estádio de duas camadas de células da granulosa até o estádio antral, possuem diâmetro de 83 a 130 μm, respectivamente (BEZERRA *et al.*, 1998).

O desenvolvimento de folículos secundários está associado com fatores locais, tais como: ativina-A (rata: ZHAO *et al.*, 2001, ovelha: THOMAS *et al.*, 2003), fator de crescimento keratinócito (KGF) (rata: McGEE *et al.*, 1999), hormônio do crescimento (GH) (rata: LIU *et al.*, 1998), TGF-β (camundongo: LIU *et al.*, 1999), EGF (vaca: GUTIERREZ *et al.*, 2000), GDF-9 (camundongo: HAYASHI *et al.*, 1999) e BMP-15 (ovelha: JUENGEL *et al.*, 2002). Gonadotrofinas como hormônio folículo estimulante (FSH) vaca: (GUTIERREZ *et al.*, 2000) e hormônio luteinizante (LH) (camundongo: WU *et al.* 2000), além de outros hormônios, como a melatonina (ADRIAENS *et al.*, 2006) também podem promover o crescimento de folículos secundários. Além da importância da secreção de fatores de crescimento e hormônios, é importante ressaltar que o crescimento folicular depende da

comunicação bidirecional entre o oócito e as células da granulosa (ALBERTINI *et al.*, 2001; MATZUK *et al.*, 2002; LIU *et al.*, 2006), que pode ser mediada via projeções transzonais (ALBERTINI *et al.*, 2001) ou por junções gap (KIDDER & MHAWI, 2002). Essa interação parácrina entre as células permite a passagem de pequenas moléculas, fatores de crescimento, nutrientes e íons, que auxiliam no desenvolvimento tanto do oócito como das células da granulosa (KIDDER & MHAWI, 2002).

Com a intensa proliferação das células da granulosa, ocorre a formação de uma cavidade repleta de líquido folicular, entre as camadas de células granulosa, denominada antro. A partir deste estádio, os folículos passam a ser denominados terciários ou antrais. O fluido antral pode servir como uma importante fonte de substâncias regulatórias derivadas do sangue ou de secreções das células foliculares, i.e., gonadotrofinas, esteróides, fatores de crescimento, enzimas e lipoproteínas. Durante o desenvolvimento folicular, a produção de fluido antral é intensificada pelo aumento da vascularização folicular e permeabilidade dos vasos sangüíneos, os quais estão fortemente relacionados com o aumento do folículo antral (VAN DEN HURK & ZHAO, 2005).

O desenvolvimento dos folículos antrais é caracterizado por uma fase de crescimento, recrutamento, seleção e dominância (VAN DEN HURK e ZHAO, 2005), sendo a formação de folículos pré-ovulatórios um pré-requisito para a ovulação e formação do corpo lúteo, bem como para a manutenção da fertilidade. Em cabras, a competência meiótica completa é adquirida em folículos de 3 mm, que corresponde a um oócito de aproximadamente 110 µm de diâmetro (HYTTEL *et al.*, 2002). Os folículos antrais iniciais possuem RNAm para receptores de FSH nas células da granulosa, mas são relativamente independentes de gonadotrofinas durante seu período de crescimento inicial, uma vez que eles aumentam em tamanho na ausência ou presença de baixas concentrações de FSH e LH (VAN DEN HURK & ZHAO, 2005). Estudos ultrassonográficos indicaram que o ciclo estral de cabras é caracterizado por um padrão de ondas de desenvolvimento folicular nos ovários (de BULNES *et al.*, 1999). Uma onda folicular envolve um crescimento de um grupo de folículos antrais dos quais geralmente um ou dois folículos são selecionados para crescer até mais de 5 mm. De acordo com vários autores, o número de ondas foliculares por ciclo varia entre duas a cinco. Entretanto, o padrão predominante em cabras com ciclo estral de comprimento normal (19-22 dias) é de quatro ondas (de CASTRO *et al.*, 1999).

O aumento das concentrações plasmáticas de FSH constitui o estímulo necessário para o recrutamento e a emergência da onda folicular (ADAMS *et al.*, 1992). Em espécies

monovulatórias, apenas um folículo é selecionado dentre os recrutados para continuar a crescer e diferenciar-se em folículo ovulatório, enquanto os demais têm como destino a atresia. O folículo selecionado é conhecido como folículo dominante e suprime ativamente o crescimento dos subordinados pela secreção de estradiol e inibina (FORTUNE *et al.*, 2001; GINTHER *et al.*, 1996). Os folículos são considerados dependentes de FSH até a ocorrência da dominância, após o que eles se tornam dependentes de LH (FORTUNE *et al.*, 2001). A expressão de receptores de LH (LHR) em células da granulosa está associada à dominância folicular (BAO & GARVERICK, 1998).

A Figura 4 ilustra resumida e esquematicamente os diferentes fatores envolvidos em todas as fases do desenvolvimento de folículos ovarianos, desde a fase pré-antral até a formação do folículo pré-ovulatório.

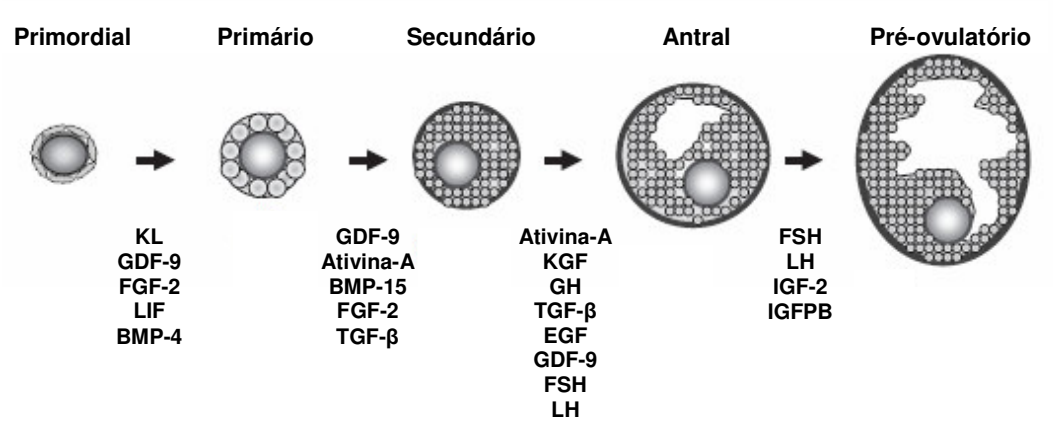

Figura 4. Desenho esquemático ilustrando os fatores envolvidos nas diferentes fases do desenvolvimento folicular. Adaptado de Barnett *et al.* (2006).

2.4. População e atresia folicular

Em caprinos, a população folicular foi estimada em 20.000 folículos por ovário (BEZERRA *et al.*, 1998). Vários fatores, além da variação individual, podem afetar o número de folículos presentes no ovário. Dentre eles, podem ser citados raça, idade, níveis

hormonais, genética, estado reprodutivo (fêmeas impúberes, púberes ou prenhes) e nutricional do animal (FIGUEIREDO *et al.*, 2003).

Os folículos pré-antrais representam 90% da população folicular e são responsáveis pela renovação contínua de folículos antrais no ovário. Entretanto, a grande maioria dos folículos pré-antrais não chega até a ovulação, mas sofre um processo conhecido por atresia folicular (KATSKA-KSIAZKIEWICK, 2006). A atresia é um fenômeno natural que é comum a todas as espécies domésticas, podendo ocorrer em qualquer estádio do desenvolvimento folicular, sendo mais comum nos estádios antrais mais avançados (GLAMOCLIJA *et al.*, 2005). Vários são os fatores que podem influenciar o processo de atresia, como idade, ciclo reprodutivo, gestação, hipofisectomia, ovariectomia unilateral, hormônios, nutrição e isquemia (INGRAM, 1962).

A atresia pode ocorrer por via degenerativa e/ou apoptótica (FIGUEIREDO *et al.*, 2003). A isquemia é uma das principais causas do desencadeamento da morte celular por degeneração (FARBER, 1982). A redução da oxigenação celular durante a isquemia resulta em diminuição da produção de ATP afetando o funcionamento da bomba de Na^+/K^+ presente na membrana celular. As mudanças na permeabilidade membranária provocam alterações nos níveis intracelulares de Na^+, K^+ e Ca^{++}. O aumento do influxo de Na^+ para o citoplasma, que ativa a Na^+/K^+ ATPase, associado com modificações na distribuição de Ca^{++} e com aumento de água intracelular podem levar ao aumento do volume celular, vacuolização citoplasmática e, conseqüentemente, degeneração (BARROS *et al.*, 2001). Com a evolução da degeneração, a morte celular é identificada histologicamente como necrose.

O processo de apoptose é altamente dependente da expressão de genes (BARNETT *et al.*, 2006). O balanço estabelecido entre os produtos dos genes pró-apoptóticos e anti-apoptóticos pode determinar a morte celular (HURWITZ & ADASHI, 1992). A progressão da apoptose pode ser dividida nas fases de iniciação, execução e terminação. A fase de iniciação pode ser promovida por fatores extrínsecos, tais como citocinas (ex. Fator de Necrose tumoral-α, Fas ligand) e proteínas virais ou pela remoção de fatores de crescimento. A morte celular também pode ser induzida por fatores intrínsecos, tais como o estresse oxidativo ou irradiação. Independentemente dos tipos de fatores envolvidos, ocorre o envolvimento de uma ou mais caspases de iniciação (caspase 8, caspase 9) (MORITA & TILLY, 1999; JOHNSON & BRIDGHAM, 2002). A fase de execução é caracterizada por mudanças na membrana celular, fragmentação nuclear, condensação da cromatina e degradação do DNA. Esta fase é considerada irreversível e ocorre devido à ativação das

caspases efetoras (caspase 3, caspase 6 e caspase 7). Finalmente, a fase de terminação consiste na fagocitose de corpos apoptóticos fragmentados através de um processo não inflamatório. No sentido de evitar a morte celular por apoptose, existem processos de sobrevivência celular que promovem a transcrição de várias proteínas anti-apoptóticas, tais como alguns membros da família Bcl-2, que bloqueiam a progressão da apoptose em diferentes etapas ao longo deste processo (JOHNSON, 2003).

Para avaliar as alterações morfológicas, bem como a eficiência do cultivo *in vitro* de folículos pré-antrais, podem-se utilizar técnicas que permitam verificar a qualidade, a ativação e o crescimento folicular pós-cultivo. No primeiro Capítulo desta tese, será mostrada uma revisão acerca das diferentes técnicas utilizadas para avaliar a qualidade folicular antes e após o cultivo *in vitro*, destacando a histologia clássica e a microscopia eletrônica de transmissão, que foram as técnicas utilizadas nos demais Capítulos desta tese. A importância da utilização concomitante de diferentes técnicas também é discutida na revisão apresentada no Capítulo I.

2.5. Biotécnica de MOIFOPA e suas aplicações

Conforme exposto anteriormente, uma quantidade mínima (cerca de 0,1%) dos oócitos inclusos em folículos pré-antrais irá ovular e, conseqüentemente, ter alguma possibilidade de ser fecundado. Em virtude desta grande perda folicular que ocorre naturalmente *in vivo*, e na tentativa de utilizar de maneira eficiente o potencial de gametas femininos no futuro, vem sendo desenvolvida a biotécnica de MOIFOPA. Esta biotécnica visa o resgate de folículos pré-antrais do ambiente ovariano, seguido das etapas de conservação (resfriamento e/ou criopreservação) e/ou cultivo *in vitro* até o estádio de maturação folicular (FIGUEIREDO *et al.*, 2003), prevenindo assim, a ocorrência da atresia.

A fim de resgatar os folículos pré-antrais dos ovários mamíferos antes que eles sejam atingidos pela atresia, procedimentos de isolamento mecânico e/ou enzimático podem ser utilizados. Os métodos de isolamento têm proporcionado a recuperação de milhares de folículos pré-antrais a partir de um único ovário (para revisão, ver FIGUEIREDO *et al.*, 2003).

2.6. Modelos *in vitro* para o estudo do desenvolvimento de folículos pré-antrais

Diferentes métodos têm sido desenvolvidos para manter a viabilidade e promover o crescimento de folículos pré-antrais *in vitro* (HARTSHORNE, 1997). Em roedores, devido ao pequeno tamanho do ovário, utiliza-se freqüentemente o cultivo *in vitro* do órgão inteiro, o que permite estudar os fatores que podem afetar a ativação folicular (FORTUNE, 2003). Por outro lado, o tamanho dos ovários de mamíferos domésticos não permite que estes sejam cultivados inteiros. Entretanto, vários métodos para cultivo de fragmentos de córtex ovariano, área em que está localizado o pool de folículos quiescentes, têm sido desenvolvidos. Estes métodos são importantes para o estudo da ativação de folículos primordiais e do crescimento de folículos primários em várias espécies (bovinos: BRAW-TAL & YOSSEFI, 1997; bubalinos: WANDJI *et al.* 1997; humanos: HOVATTA *et al.* 1997; caprinos: SILVA *et al.* 2004b). Outros trabalhos cultivam folículos pré-antrais isolados, permitindo o estudo dos efeitos *in vitro* de hormônios e fatores de crescimento sobre os folículos primordiais, primários e secundários (FORTUNE, 2003).

Vários autores têm utilizado estes modelos para estudar o desenvolvimento de folículos pré-antrais em roedores e ruminantes (FORTUNE, 2003; VAN DEN HURK & ZHAO, 2005). Em gatas (JEWGENOW & STOLTE, 1996), gambás (BUTCHER & ULLMAN, 1996) e macacas (FORTUNE *et al.*, 1998), já foi observado o crescimento de folículos pré-antrais após o cultivo *in vitro*, porém sem a formação de antro. Nas espécies bovina (GUTIERREZ *et al.*, 2000; McCAFFERY *et al.*, 2000), ovina (CECCONI *et al.*, 1999), caprina (HUAMIN & YONG, 2000) e humana (ROY & TREACY, 1993), folículos pré-antrais isolados foram cultivados *in vitro* e se desenvolveram até o estádio antral. Em suínos, folículos secundários crescidos *in vitro* chegaram até a ovulação, tiveram seus oócitos fecundados *in vitro*, com desenvolvimento até o estádio de blastocisto (WU *et al.*, 2001). Apenas em camundongos, o cultivo *in vitro* de folículos primordiais, seguido de maturação e fecundação *in vitro*, resultou no nascimento de 59 animais (O´BRIEN *et al.* 2003). O sucesso na obtenção de oócitos de boa qualidade após cultivo *in vitro* de folículos pré-antrais ainda tem sido limitado, especialmente em ruminantes e mais esforços são necessários para melhorar as condições de cultivo.

Nos Capítulos II a VI desta tese, foram realizados experimentos com cultivo de fragmentos de córtex ovariano caprino a fim de estudar o desenvolvimento *in vitro* de folículos pré-antrais desta espécie.

2.7. Hormônios e fatores de crescimento locais que podem influenciar a foliculogênese inicial em cabras

Durante a última década, o papel de hormônios e fatores de crescimento na foliculogênese ovariana tem sido bastante estudado, principalmente em roedores. Há uma grande evidência de que os membros da superfamília TGF-β, tais como TGF-β, TGF-α, ativinas, inibinas, BMPs, GDF-9, têm um papel importante no desenvolvimento e atresia folicular (FORTUNE, 2003; VAN DEN HURK & ZHAO, 2005). Além disso, outros fatores de crescimento como FGF, LIF e KL, bem como as gonadotrofinas FSH e LH também são importantes na foliculogênese (FORTUNE, 2003; VAN DEN HURK & ZHAO, 2005). Além disso, o efeito de algumas substâncias que originalmente não são produzidas no ovário também vem sendo testado na foliculogênese. Desta forma, nesta tese, estudou-se a possível importância de uma substância alternativa, o IAA, bem como do FGF-2 e do FSH na manutenção da viabilidade e no desenvolvimento de folículos pré-antrais caprinos *in vitro*.

2.7.1. Ácido 3-Indol-Acético

O Ácido 3-Indol-Acético (IAA) é um hormônio vegetal, pertencente ao grupo das auxinas, e é um importante componente da água de coco (TONIOLLI *et al.*, 1996). As auxinas controlam vários processos, tais como crescimento, tropismo, expansão e divisão celular (BECKER & HEDRICH, 2002). O IAA atua no crescimento de vegetais ligando-se às proteínas solúveis da seiva, sendo transportado a receptores transmembranários, provocando, direta ou indiretamente, respostas celulares variadas (TONIOLLI *et al.*, 1996), tais como o aumento da plasticidade da parede celular, aumento da entrada de água na célula, o que promove o crescimento da mesma (BARBIER-BRYGOO, 1995). O IAA pode ser obtido a partir de uma dieta rica em caules vegetais ou sintetizado pelo aminoácido triptofano (MILLS *et al.*, 1991). Este hormônio já foi demonstrado no fluido cerebrospinhal (HU & DRYHURST, 1997) e em vários órgãos, como pulmões, rins, fígado e cérebro (TUSELL *et al.*, 1984).

Em animais, foi demonstrado que o IAA presente na água de coco é eficiente para a preservação de espermatozóides caprinos (TONIOLLI *et al.*, 1996) e caninos (CARDOSO *et al.*, 2002). Além disso, o IAA foi utilizado com sucesso para a conservação de folículos pré-antrais caprinos durante o transporte de ovários até o laboratório a 4^{o} C por até 24 h,

mantendo as porcentagens de folículos normais similares àquelas observadas no controle (aproximadamente 95%) (FERREIRA *et al.*, 2001). Com relação ao cultivo *in vitro*, ainda há poucos relatos utilizando o IAA, principalmente, em animais domésticos. Estudos mostraram que baixas concentrações de IAA aumentaram o crescimento *in vitro* de talos de ervilha (MOORE *et al.*, 1983) e do microorganismo *Candida utilis* (AHMED *et al.*, 1994). Mais recentemente, mostrou-se que o IAA previne a degeneração de folículos pré-antrais ovinos cultivados *in vitro* quando associado ao FSH e ao EGF (ANDRADE *et al.*, 2005). Entretanto, os mecanismos moleculares de ação das auxinas nas células animais ainda não são claros.

No Capítulo II desta, avaliamos os efeitos de diferentes concentrações de IAA sobre a morfologia e ultra-estrutura, bem como sobre a ativação e o crescimento de folículos pré-antrais caprinos.

2.7.2. Fator de Crescimento Fibroblástico-2

O Fator de Crescimento Fibroblástico-2 (FGF-2), também conhecido como básico (FGFb) é importante na regulação de várias funções ovarianas, tais como: mitose (ROBERTS & ELLIS, 1999), esteroidogênese (VERNON & SPICER, 1994), diferenciação (ANDERSON & LEE, 1993) e apoptose (TILLY *et al.*, 1992) das células da granulosa. O FGF-2 já foi imunolocalizado em folículos ovarianos (ratas: NILSSON *et al.*, 2001; humanos: YAMAMOTO *et al.*, 1997, QUENNELL *et al.*, 2004, BEN-HAROUSH *et al.*, 2005) e corpos lúteos (ratas: ASAKAI *et al.*, 1993) de várias espécies. Há cinco tipos receptores de FGF (FGFR) conhecidos (Kim et al., 2001, Sleeman et al., 2001). Além disso, os receptores para o FGF-2 têm sido demonstrados em folículos em crescimento de vacas (WANDJI *et al.*, 1992), ratas (SHIKONE *et al.*, 1992; ASAKAI *et al.*, 1993, 1995) e mulheres (QUENNELL *et al.*, 2004, BEN-HAROUSH *et al.*, 2005), sugerindo uma possível relação entre o FGF-2 e o desenvolvimento folicular.

Alguns estudos *in vitro* mostraram que o FGF-2 promove a proliferação das células da granulosa e da teca de folículos pré-antrais de diferentes espécies (vacas: WANDJI *et al.*, 1996; gatas domésticas: JEWGENOW, 1996; galinhas: ROBERTS AND ELLIS, 1999). Além disso, o FGF-2 promoveu a ativação e o crescimento de folículos primordiais e primários de ratas (NILSSON *et al.*, 2001). Recentemente, um estudo mostrou que o FGF-2 estimulou a sobrevivência de oócitos caprinos *in vitro*, apesar de não ter tido efeito sobre o crescimento oocitário (ZHOU & ZHANG, 2005). Similarmente, DERRAR *et al.*, (2000)

mostraram que o FGF-2 não teve efeito na taxa de crescimento de folículos bovinos *in vitro*. Desta forma, sabe-se que o FGF-2 exerce um papel na regulação do crescimento e diferenciação de folículos pré-antrais. Entretanto, ainda são necessários mais estudos para compreender os resultados obtidos até o momento.

O experimento realizado no Capítulo III avalia os efeitos de diferentes concentrações de FGF-2 sobre a morfologia e ultra-estrutura, bem como sobre a ativação e o crescimento de folículos pré-antrais caprinos.

2.7.3. Hormônio Folículo Estimulante

O Hormônio Folículo Estimulante (FSH) é um crítico regulador da função ovariana. In vivo, o FSH é essencial para a esteroidogênese através da estimulação da atividade da enzima aromatase, bem como para a diferenciação das células da granulosa através da indução da expressão de receptores para LH (DEMEESTERE *et al.*, 2005). O FSH também regula a conexão transzonal entre o oócito e as células da granulosa (ALBERTINI *et al.*, 2001).

Estudos têm mostrado que os folículos ovarianos pré-antrais conseguem se desenvolver, independentemente da ação do FSH. Este fato foi observado em camundongos com deficiência nos genes FSHβ e FSHR (KUMAR *et al.*, 1997; DIERICH *et al.*, 1998) e também em pacientes com mutações que suprimiam a função do receptor para FSH (BEAU *et al.*, 1998; TOURAINE *et al.*, 1999). Os receptores de FSH se expressam nas células da granulosa (ULLOA-AGUIRRE *et al.*, 1995; O'SHAUGHNESSY *et al.*, 1996) e oócitos (MÉDURI *et al.*, 2002) a partir do estádio de folículos primários (OKTAY *et al.*, 1997). Apesar de os receptores de FSH não estarem presentes em folículos primordiais, o FSH parece exercer um efeito indireto sobre o desenvolvimento folicular inicial através da liberação de fatores parácrinos produzidos por folículos maiores ou pelas células do estroma ovariano (VAN DEN HURK AND ZHAO, 2005). Alguns trabalhos demonstraram que o FSH regula a expressão de vários fatores de crescimento, tais como KL, GDF-9 e BMP-15, que têm um papel importante na ativação e no posterior crescimento folicular (JOYCE *et al.*, 1999, THOMAS *et al.*, 2005).

Vários estudos *in vitro* têm mostrado que o FSH pode promover o desenvolvimento de folículos pré-antrais e a formação de antro (camundongos: SPEARS *et al.*, 1998; ratas: McGEE *et al.*, 1997; mulheres: WRIGHT *et al.*, 1999; vacas: GUTIERREZ *et al.*, 2000;

cabras: ZHOU & ZHANG, 2005; ovelhas: CECCONI *et al.*, 1999; porcas: MAO *et al.*, 2002). Além disso, o FSH inibe a apoptose em folículos pré-antrais cultivados *in vitro* (mulheres: ROY & TREACY, 1993; camundongos: BAKER & SPEARS, 1997; ratas: McGEE *et al.*, 1997). Recentemente, SILVA *et al.*, (2004a) relataram que o FSH, na concentração de 100 ng/mL, promoveu aumento dos diâmetros oocitário e folicular, sem contudo alterar a ativação e a viabilidade de folículos pré-antrais caprinos. Além disso, NUTTINCK et al. (1996) cultivaram pequenos folículos pré-antrais bovinos por 7 dias e observaram que o FSH aumentou a degeneração oocitária. Esses resultados contraditórios podem ser devido a diferenças entre espécies, metodologias de cultivo *in vitro*, substâncias adicionadas ao meio de cultivo e concentrações de FSH utilizadas nos experimentos. Além disso, é importante ressaltar que a maioria destes trabalhos utilizou o FSH numa única concentração.

No Capítulo IV desta tese, foi testado o efeito de diferentes concentrações de FSH sobre os mesmos parâmetros descritos anteriormente para o IAA e o FGF-2.

3. JUSTIFICATIVA

Diversas pesquisas sobre o desenvolvimento folicular têm focado principalmente nos estádios finais da foliculogênese, ou seja, em folículos antrais. Sabe-se que os folículos primordiais representam cerca de 90% de todos os folículos presentes no ovário. Entretanto, os mecanismos responsáveis pelo início do crescimento destes folículos (ativação) ainda não são completamente elucidados. Devido ao grande potencial dos folículos primordiais como material para preservação de espécies animais ameaçadas de extinção ou de alto valor genético, e, ainda, para a preservação da fertilidade em humanos, há um crescente interesse em compreender os mecanismos envolvidos na ativação e posterior crescimento dos oócitos inclusos nestes folículos. Além disso, cerca de 99,9% dos folículos ovarianos sofrem atresia, o que reduz significativamente o potencial reprodutivo das fêmeas. Neste contexto, com o intuito de evitar a grande perda folicular que ocorre *in vivo*, a biotécnica de MOIFOPA permite a recuperação de dezenas a milhares de folículos primordiais a partir de um único ovário. Tais folículos poderiam ser criopreservados e/ou cultivados *in vitro*.

Sabendo-se do grande valor econômico que a espécie caprina representa para o Nordeste brasileiro, é de extrema importância o desenvolvimento de um sistema de cultivo *in vitro* capaz de ativar os folículos primordiais caprinos e assegurar seu posterior crescimento, otimizando o aproveitamento do potencial oocitário desses animais e incrementando a eficiência da reprodução animal.

Além disso, o desenvolvimento de um sistema de cultivo *in vitro* eficiente poderá fornecer subsídios para uma melhor compreensão acerca dos fatores que regulam a foliculogênese inicial (fase pré-antral). Alguns estudos têm investigado o efeito de diversos hormônios (FSH, LH, etc) e fatores de crescimento (como KL, GDF-9, FGF-2, etc) no cultivo *in vitro* de folículos pré-antrais de animais de laboratórios e animais domésticos. Entretanto, os efeitos de diferentes concentrações de FSH, FGF-2 e IAA, bem como o efeito da interação entre essas substâncias ainda não foram avaliados no cultivo *in vitro* de folículos pré-antrais caprinos. Além disso, não são conhecidos os efeitos de um cultivo de longa duração sobre as características estruturais e ultra-estruturais de folículos pré-antrais caprinos. Para este fim, além da técnica de histologia clássica, será empregada neste trabalho, a MET para determinar a qualidade de folículos pré-antrais caprinos cultivados *in vitro* e, conseqüentemente, melhor avaliar a eficiência dos meios de cultivo testados.

4. HIPÓTESES CIENTÍFICAS

Diante do exposto, formularam-se as seguintes hipóteses científicas:

1) IAA, FGF-2 e FSH podem manter a viabilidade folicular, influenciar positivamente a ativação de folículos primordiais e o posterior crescimento *in vitro* de folículos pré-antrais caprinos;

2) Folículos pré-antrais caprinos podem crescer *in vitro*, mantendo a viabilidade, após cultivo de longa duração.

5. OBJETIVOS

5.1. FASE I: Cultivo *in vitro* de folículos pré-antrais caprinos utilizando diferentes concentrações de IAA, FGF-2 e FSH.

Objetivo Geral

- Estudar o efeito do IAA, FGF-2 e FSH isoladamente sobre o cultivo de folículos pré-antrais caprinos *in vitro*.

Objetivos Específicos

- Estabelecer as curvas dose-resposta de IAA, FGF-2 e FSH tendo como parâmetros a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos;
- Analisar morfológica e ultra-estruturalmente os folículos pré-antrais caprinos cultivados *in vitro* com FSH, FGF-2 e IAA.

5.2. FASE II: Avaliação da interação entre FSH e FGF-2 no cultivo *in vitro* de folículos pré-antrais caprinos.

Objetivo Geral

- Cultivar *in vitro* folículos pré-antrais caprinos utilizando FSH e FGF-2 isoladamente ou em associação nas melhores concentrações determinadas na Fase I.

Objetivos Específicos

- Verificar o efeito do FSH e FGF-2 isoladamente ou em associação, sobre a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos;
- Analisar morfológica e ultra-estruturalmente os folículos pré-antrais caprinos cultivados *in vitro* com FSH e FGF-2, isoladamente ou em associação, nas melhores concentrações determinadas na Fase I.

5.3. FASE III: Desenvolvimento *in vitro* de folículos pré-antrais caprinos após cultivo de longa duração.

Objetivo Geral

- Avaliar as taxas de sobrevivência e desenvolvimento *in vitro* de folículos pré-antrais caprinos após cultivo de longa duração (14, 21 ou 28 dias), utilizando FSH e FGF-2 nas melhores concentrações determinadas na Fase II.

Objetivos Específicos

- Verificar o efeito do FSH e FGF-2 sobre a ativação e crescimento *in vitro* de folículos primordiais caprinos após cultivo de longa duração (28 dias).

- Analisar as características morfológicas de folículos pré-antrais caprinos após cultivo de longa duração.

Capítulo I

Técnicas para avaliação da qualidade de folículos ovarianos pré-antrais cultivados *in vitro*

RESUMO

As técnicas de avaliação da qualidade de folículos pré-antrais antes e após o cultivo *in vitro* permitem o monitoramento das alterações morfológicas ocorridas *in vitro*, sendo, portanto, de grande importância para a melhoria dos sistemas de crescimento *in vitro* de folículos pré-antrais. A presente revisão mostra as diferentes técnicas utilizadas para avaliar a qualidade folicular antes e após o cultivo *in vitro*, tais como histologia clássica, microscopia eletrônica de transmissão, sistemas de detecção de apoptose, marcadores de viabilidade folicular e de proliferação de células da granulosa, bem como análise dos produtos de secreção folicular. A importância da utilização concomitante de diferentes técnicas também é discutida nesta revisão.

Capítulo I

Técnicas para avaliação da qualidade de folículos ovarianos pré-antrais cultivados *in vitro*

(*Techniques for quality evaluation of ovarian preantral follicles cultured in vitro*)

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Resumo

As técnicas de avaliação da qualidade de folículos pré-antrais antes e após o cultivo *in vitro* permitem o monitoramento das alterações ocorridas *in vitro*, sendo, portanto, de grande importância para a melhoria dos sistemas de crescimento *in vitro* de folículos pré-antrais. A presente revisão mostra as diferentes técnicas utilizadas para avaliar a qualidade folicular antes e após o cultivo *in vitro*, tais como histologia clássica, microscopia eletrônica de transmissão, sistemas de detecção de apoptose, marcadores de viabilidade folicular e de proliferação de células da granulosa, bem como análise dos produtos de secreção folicular. A importância da utilização concomitante de diferentes técnicas também é discutida nesta revisão.

Palavras-chave: Folículos pré-antrais, cultivo *in vitro*, degeneração, morfologia, viabilidade

Abstract

Techniques to evaluate preantral follicle quality before and after *in vitro* culture allow the accompaniment of changes occurring *in vitro*, being important to improve the systems of preantral follicles growth *in vitro*. The present review shows different techniques to evaluate follicular quality before and after *in vitro* culture, such as histology, transmission electron microscopy, systems to detect apoptosis, markers for follicle viability and proliferation of granulosa cells as well as analysis of products of follicular secretion. The importance of the concomitant utilization of different techniques is also discussed in this review.

Keywords: Preantral follicles, *In vitro* culture, Follicular quality.

1. Introdução

O folículo ovariano é a unidade básica estrutural e funcional do ovário mamífero que fornece o ambiente necessário para o crescimento e a maturação oocitária (Gosden et al., 1993). Apesar da grande população de folículos primordiais presentes no ovário mamífero, a maioria (99,9%) morre por atresia e somente poucos folículos conseguem chegar à ovulação (Mao et al., 2002). A atresia pode ocorrer por via degenerativa e/ou apoptótica (Figueiredo et

al., 1999). As características estruturais e os eventos moleculares da apoptose (morte celular programada) diferenciam este tipo de morte celular da necrose, na qual um grupo de células morre simultaneamente (Tilly, 1996), enquanto a apoptose sempre ocorre em uma única célula circundada por células normais (Kuan & Passaro, 1998).

Conforme o exposto acima, considerando-se o fato de que a quase totalidade dos oócitos será eliminada pelo processo de atresia, caso eles permaneçam no interior dos ovários, a biotécnica de Manipulação de Oócitos Inclusos em Folículos Pré-Antrais (MOIFOPA) vem sendo desenvolvida com o objetivo de recuperar um grande número de oócitos inclusos nestes folículos e, posteriormente, cultivá-los *in vitro* até sua completa maturação, prevenindo-os assim da atresia. Esta biotécnica poderá fornecer milhares de oócitos maturos, que podem ser utilizados, para a multiplicação de animais de alto valor genético ou em via de extinção (Figueiredo et al., 1999).

Nesse sentido, o desenvolvimento de um sistema que permita o crescimento *in vitro* de folículos pré-antrais e que resulte em oócitos aptos a serem maturados e fecundados *in vitro* seria de grande importância tanto para a compreensão da foliculogênese na fase inicial, como para a preservação a longo prazo de células germinativas femininas (Mao et al., 2002). Vários trabalhos têm sido realizados no sentido de promover o crescimento de folículos pré-antrais *in vitro* (camundongos: Eppig & Schroeder, 1989; ratas: Cain et al., 1995; suínos: Wu et al., 2001; bovinos: Gutierrez et al., 2000; ovinos: Cecconi et al., 1999; caprinos: Huanmin & Yong, 2000; felinos: Jewgenow & Stolte, 1996 e humanos: Roy & Treacy, 1993). Entretanto, as condições necessárias para o completo desenvolvimento *in vitro* de folículos pré-antrais de animais domésticos e humanos ainda não foram estabelecidas. Isto se deve principalmente à falta de informação sobre a regulação do crescimento folicular e oocitário na fase pré-antral. Nesse sentido, a utilização de diferentes técnicas que permitam o monitoramento da qualidade e da viabilidade folicular antes e após o cultivo é importante para a melhoria dos sistemas de crescimento *in vitro* de folículos pré-antrais.

Nesta revisão, serão abordadas técnicas utilizadas para analisar a morfologia, a ultra-estrutura e a viabilidade de folículos pré-antrais cultivados *in vitro*, além dos marcadores de atividade proliferativa celular.

2. Técnicas para avaliação de folículos pré-antrais pós-cultivo *in vitro*

Várias técnicas são disponíveis para avaliar a morfologia e a ultra-estrutura dos folículos ovarianos pré-antrais. Com o auxílio destas técnicas, pode-se analisar a ativação, o crescimento e a viabilidade folicular ao longo do período de cultivo *in vitro*. Em geral, os estudos realizados sobre cultivo utilizam as técnicas em conjunto (no mínimo duas) a fim de se obter uma maior precisão sobre a qualidade do folículo.

2.1. Técnicas utilizadas para detectar alteração ou degeneração folicular

2.1.1. Histologia Clássica

A histologia é um método clássico que avalia a morfologia do citoplasma e do núcleo. A histologia clássica (HC) é uma técnica importante para avaliação do cultivo *in vitro* de folículos pré-antrais, pois permite verificar o número e a mudança na morfologia das células da granulosa de pavimentosa para cúbica, por ocasião da ativação folicular, além de permitir analisar a integridade morfológica do oócito e das células da granulosa. Entretanto, a HC não permite avaliar a integridade das organelas citoplasmáticas. Gosden (2000) observou que a HC é relativamente pouco precisa se realizada imediatamente após a descongelação do tecido ovariano, pois algumas alterações das organelas podem se manifestar somente algumas horas após este processo. Por outro lado, a HC apresenta uma grande vantagem, que é a de possibilitar que um grande número de folículos seja avaliado, o que a torna uma técnica importante quando se deseja realizar uma análise quantitativa.

A HC pode ser realizada tanto em folículos isolados como naqueles inclusos no córtex ovariano. Alguns autores mostraram que, na análise histológica, as alterações indicativas de atresia em folículos pré-antrais ocorrem primariamente no oócito, sendo a picnose nuclear o primeiro sinal de atresia (Jorio *et al.*, 1991; Wood *et al.*, 1997). O processo de HC compreende as seguintes etapas: fixação, desidratação, diafanização ou clarificação, infiltração, inclusão, microtomia e coloração das lâminas. Em geral, nos protocolos padrão de HC, os fixadores mais utilizados são: paraformaldeído a 4% (Silva et al., 2004a, b), bouin (Cushman et al., 2001; Nilsson & Skinner, 2002) e carnoy (Matos et al., 2004). As colorações mais comumente empregadas são: hematoxilina-eosina (HE) (Vendola et al., 1999; Nilsson & Skinner, 2002) e Ácido Periódico de Schiff-hematoxilina (PAS-hematoxilina) (Cushman et al., 2001; SILVA et al., 2004a, b – Fig. 1). Embora demande um pouco mais de tempo, a coloração PAS-hematoxilina apresenta a vantagem de permitir a visualização da zona

pelúcida e da membrana basal, além dos demais componentes foliculares, como a membrana basal, as células da granulosa, o citoplasma e o núcleo do oócito. Além disso, existem vários trabalhos em que os folículos foram inclusos em resina e, por fim, corados com azul de toluidina (Abir et al., 1997, Wandji et al., 1996; Yu & Roy, 1999) ou Giemsa (Hemamalini et al., 2003).

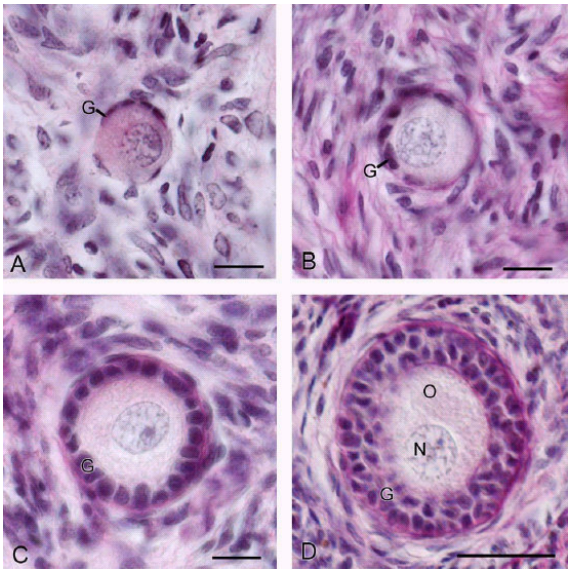

Figura. 1. Estrutura histológica de folículos pré-antrais caprinos após coloração com Ácido Periódico de Schiff-hematoxilina, mostrando: (A) folículo primordial (oócito circundado por uma camada de células da granulosa pavimentosas), (B) transição (oócito circundado por uma camada de células da granulosa pavimentosas e cúbicas), (C) primário (oócito circundado por uma camada de células da granulosa cúbicas) e (D) secundário (oócito circundado por duas ou mais camadas de células da granulosa cúbicas). O: oócito; N: núcleo; G: células da granulosa. Barras; A, B e C = 12,5 μm e D = 25 μm. Fonte: SILVA et al. (2004a).

2.1.2. Microscopia Eletrônica de Transmissão (MET)

A MET é considerada uma boa técnica para avaliação das organelas celulares e das mudanças ultra-estruturais (Salehnia et al., 2002) ocorridas durante a atresia folicular, sendo, portanto, um método mais preciso e eficiente do que a HC para avaliar os folículos pré-antrais após o cultivo *in vitro*. A MET também é eficiente logo após a descongelação do tecido ovariano, pois é possível detectar alterações súbitas nas organelas, tais como o aumento de volume das mitocôndrias (Gosden, 2000). Alguns autores destacaram a

importância da análise ultra-estrutural após conservação *in vitro* de folículos pré-antrais caprinos e ovinos, mostrando que folículos considerados normais após avaliação histológica poderiam apresentar alterações degenerativas na sua ultra-estrutura, tanto após o isolamento (Lucci et al., 1999), como após resfriamento (Silva et al., 2000; Carvalho et al., 2001; Lucci et al., 2004a; Matos et al., 2004) e criopreservação (Rodrigues et al., 2004; Lucci et al., 2004b).

No que se refere à utilização da MET para avaliar folículos pré-antrais após o cultivo, Eppig (1977) observou que oócitos de camundongos cultivados *in vitro* apresentavam aparência morfológica normal quando observados em microscopia ótica. Entretanto, a análise ultra-estrutural mostrou que existiam sinais de degeneração citoplasmática no oócito e nas células da granulosa. Além disso, este autor mostrou que após quatro dias de cultivo, a degeneração do oócito foi observada morfologicamente como um aumento na granulação do citoplasma, o que, na análise ultra-estrutural, foi visto como turgidez das mitocôndrias. Em outro estudo, após a análise ultra-estrutural de folículos pré-antrais de hamster cultivados por quatro dias, observou-se um aumento no número de mitocôndrias tanto do oócito quanto das células da granulosa, além de mitocôndrias arrendondadas e numerosos retículos endoplasmáticos rugosos no ooplasma (Yu & Roy, 1999). Com a progressão do período de cultivo para 16 dias, a análise ultra-estrutural revelou que as células da granulosa de folículos normais encontravam-se bem organizadas e possuíam mitocôndrias redondas e alongadas, com crista bem desenvolvida. Este aumento gradual no número e maturação das mitocôndrias durante os 16 dias de cultivo sugere que ocorreu desenvolvimento do oócito durante o período de cultivo. Da mesma forma, o desenvolvimento do retículo endoplasmático rugoso tanto no oócito como nas células da granulosa também sugere um aumento considerável na síntese de proteína, que é uma característica do processo de desenvolvimento celular (Yu & Roy, 1999). A análise ultra-estrutural de folículos secundários de ratas cultivados por seis dias mostrou que as células da granulosa e da teca normais possuíam retículo endoplasmático liso e mitocôndrias bem desenvolvidas (Zhao et al., 2000). Além disso, o oócito parecia estar em contato com as células da granulosa através das junções comunicantes do tipo “gap”. Entretanto, nos folículos cultivados na ausência do soro, foram observadas alta condensação do material nuclear e presença de vacúolos e/ou gotas lipídicas nas células da granulosa, sugerindo a ocorrência de processo degenerativo. Ademais, as microvilosidades do oócito também não estavam bem desenvolvidas (Zhao et al., 2000). Sadeu et al. (2006) observaram que havia folículos ultra-estruturalmente normais após 35 dias de cultivo *in vitro* de

fragmentos de córtex ovariano de fetos humanos. Estes autores observaram que folículos pré-antrais degenerados apresentavam inicialmente alterações ultra-estruturais no oócito. Recentemente, Matos et al., (2007) utilizaram a técnica de MET para confirmar a integridade ultra-estrutural de folículos pré-antrais caprinos após 7 dias de cultivo *in vitro* em meio suplementado com o Hormônio Folículo Estimulante (Fig. 2).

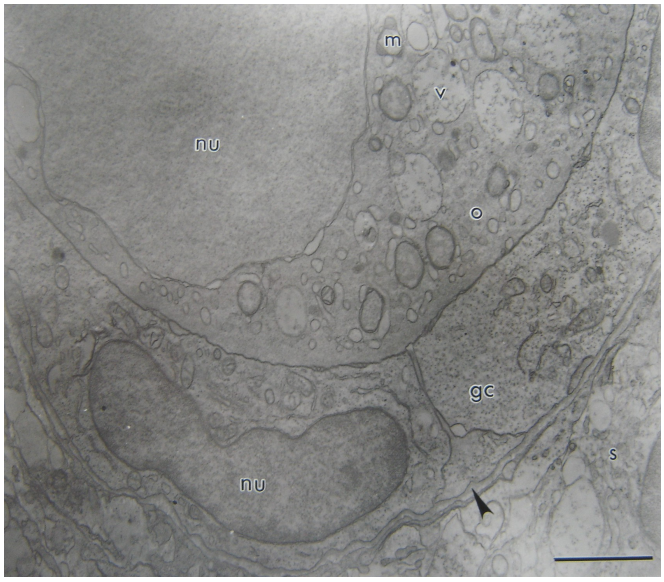

Figura. 2. Micrografia eletrônica de um folículo pré-antral caprino após 7 dias de cultivo *in vitro* em meio suplementado com Hormônio Folículo Estimulante. Notar que o folículo está circundado tanto por células pavimentosas (seta menor), como por células cuboidais (seta maior). Fonte: Matos et al., (2007).

2.2. Técnicas utilizadas para detectar a viabilidade folicular (corantes vitais) e a apoptose

2.2.1. Azul de Trypan

A coloração com azul de Trypan tem sido utilizada por vários pesquisadores para avaliar a viabilidade de folículos isolados após cultivo *in vitro* em diferentes espécies (felinos: Jewgenow, 1996; bovinos: Saha et al., 2000; caprinos: Santos et al., 2006). Este corante avalia a integridade da membrana celular. Nos folículos não viáveis, com membranas danificadas, ocorre penetração do corante, que pode ser visualizada em microscópio invertido (Jewgenow et al., 1998), constituindo uma maneira rápida de analisar a viabilidade de

folículos cultivados (Jewgenow & Goritz, 1995). Amorim et al. (2003) mostraram que a técnica de coloração com Azul de Trypan pode ser utilizada de forma equivalente à histologia clássica, mostrando resultados similares quanto à viabilidade.

2.2.2. Hoescht

O corante Hoescht penetra em células vivas e tem sido largamente empregado em citologia com a finalidade de corar cromossomos. Com a utilização deste corante, avalia-se também a integridade da membrana celular (Saha et al., 2000) e dos componentes nucleares (aparência da vesícula germinativa) (Jewgenow, 1998). Motlik & Fulka (1976) mostraram que a coloração de pequenos folículos pré-antrais com Hoechst permite a visualização ao microscópio de fluorescência de células da granulosa individualmente, determinando, assim, seu número e sua morfologia. Em felinos, observou-se uma mudança na aparência morfológica dos folículos isolados após cinco dias de cultivo em M199 suplementado com soro fetal bovino. Mais de 75% dos folículos estavam circundados por menos de 20 células da granulosa e mais de 30% eram praticamente desnudos. Além disso, foi observado que a cromatina normal (estrutura com aparência reticular) foi substituída por uma cromatina degenerada (estrutura com aparência circular ou com distribuição compacta) (Jewgenow, 1996).

2.2.3. TUNEL

A técnica de TUNEL (terminal deoxynucleotidil tranferase-mediated deoxyuridine triphosphate biotin nick end-labeling) utiliza uma enzima (transferase deoxynucleotidil terminal) para adicionar nucleotídeos aos fragmentos das fitas de DNA quebradas nas células apoptóticas. Esta técnica é utilizada para avaliar a fragmentação do DNA em secções histológicas (Tilly, 1996), permitindo a avaliação histológica, bem como a detecção do grau de apoptose (Pedersen et al., 2003). A coloração do núcleo das células apoptóticas aparece mais escura ou marrom, enquanto as células normais ficam com uma coloração mais clara (Liu et al., 2003).

Utilizando-se a técnica de TUNEL, Mao et al. (2002) observaram que no início do cultivo, a percentagem de células da granulosa apoptóticas em folículos frescos era 0,1%. Após quatro dias de cultivo, essa percentagem aumentou significativamente para 3,4%. Em

bovinos, também se observou que não havia células da granulosa coradas por TUNEL em folículos isolados frescos ou em folículos cultivados na presença de ácido ascórbico (Thomas et al., 2001). Por outro lado, havia um número significativamente maior de células da granulosa apoptóticas em folículos cultivados em meio na ausência do ácido ascórbico.

Além do TUNEL, existem outros tipos de marcadores para detecção da apoptose, que realizam a marcação das extremidades 3' dos fragmentos de DNA. Vários trabalhos têm utilizado um *kit ApopTag Plus* para detectar apoptose após cultivo *in vitro* de folículos pré-antrais em ratas (McGee et al., 1997; 2001) e vacas (Cushman et al., 2001). Nesta técnica, os folículos normais não apresentam fluorescência ao serem analisados, enquanto os folículos atrésicos mostram reação ao anticorpo marcado com fluoresceína (Cushman et al., 2001). Em ratas, a adição do fator de crescimento transformante-β ao meio de cultivo contendo FSH resultou em um aumento na coloração de folículos isolados (140 – 150 μm) após 72 h de cultivo, o que significa aumento na fragmentação de DNA e, conseqüentemente, aumento na ocorrência de apoptose (McGee et al., 2001). Pedersen et al. (2003) mostraram que existe uma correlação entre a técnica de marcação da extremidade 3' dos fragmentos de DNA e a HC para análise das células da granulosa apoptóticas, exceto para os folículos em estágio avançado de atresia. Nestes folículos, existem poucas células da granulosa apoptóticas para serem detectadas pela análise do DNA, o que torna a técnica de marcação da extremidade 3' menos sensível do que a HC. Por outro lado, a marcação das extremidades do DNA visualiza especificamente a extremidade 3' da fita de DNA fragmentada, o que torna essa técnica mais sensível para detecção da apoptose do que a coloração com brometo de etídio (descrito posteriormente), que cora o DNA como um todo (Pedersen et al., 2003).

2.2.4. Brometo de etídio e Iodeto de propídio

O brometo de etídio é comumente utilizado como um corante de ácidos nucléicos. Ele se intercala nas moléculas dos ácidos nucléicos e, quando exposto à luz ultravioleta, emite uma cor vermelho-alaranjado (Silva et al., 2001). Quando uma célula encontra-se sob estresse, alguns dos sistemas de transporte ativo são afetados, seguindo-se a despolarização da membrana citoplasmática e, mais tarde, a sua permeabilização e posterior morte celular (Hewitt & Nebe-Von-Caron, 2001). O corante fluorescente brometo de etídio consegue atravessar uma membrana polarizada, mas só se liga às cadeias de DNA quando a célula possui um sistema de transporte afetado, pois um sistema de transporte ativo expulsaria o

corante da célula (Midgley, 1987). Por ser um agente que intercala na molécula de DNA, o brometo de etídio é um poderoso mutagênico. Assim, a incorporação de brometo de etídio em organismos vivos pode causar mutações.

Recentemente, um estudo em que foi realizada a análise da atresia folicular em eqüinos mostrou que em 25 dos 26 folículos (96%), houve uma correlação entre a técnica de marcação das extremidades 3' dos fragmentos de DNA (utilizando [^{32}P] dideoxy-ATP) e a coloração com brometo de etídio no que se refere à classificação das células da granulosa em apoptóticas ou não apoptóticas (Pedersen et al., 2003). Entretanto, esta última coloração requer uma maior quantidade de DNA quando comparada à técnica de marcação das extremidades 3' dos fragmentos de DNA (Pedersen et al., 2003), além da alta toxicididade (potente cancerígeno) apresentada pelo brometo de etídio.

O iodeto de propídio também é um agente que intercala na molécula de DNA (corante de ácidos nucléicos) e emite uma fluorescência amarela e vermelha. O iodeto de propídio não penetra na membrana de células viáveis. A exclusão do iodeto de propídio é assim um método eficiente para detecção da integridade de membrana (Silva et al., 2001). Entretanto, a melhor forma de entender o mecanismo da exclusão dos corantes é a utilização de corantes em misturas, ou seja, a associação de dois ou mais corantes. Assim como o brometo de etídio, o iodeto de propídio é um forte agente mutagênico. A coloração com iodeto de propídio foi utilizada para analisar a vesícula germinativa no oócito de folículos pré-antrais bovinos cultivados por 12 dias (Thomas et al., 2001).

2.3. Sais de Tetrazolium

A técnica do kit de proliferação celular com sais de tetrazolium é baseada na habilidade das células vivas em metabolizar o sal tetrazolium amarelo para cristais azuis, que podem ser solubilizados e quantificados por espectrofotometria (McGee et al., 1997). No sentido de demonstrar que as alterações no diâmetro folicular representavam um aumento no número de células, McGee et al. (2001) realizaram uma análise do número de células viáveis nos folículos ao final do cultivo (72 h) e observaram que o tratamento com FSH aumentou este número em 56% comparado aos folículos não cultivados.

2.4. Eletroforese e autoradiografia

Após eletroforese do DNA extraído de folículos suínos, Liu et al. (2003) observaram que quando o folículo estava atrésico, uma marcação de DNA era visível, o que indicava a ocorrência da fragmentação do DNA em células da granulosa de folículos atrésicos. Murray et al. (2001) mostraram que os folículos de camundongos cultivados por seis dias em meio controle sem soro apresentavam altos níveis de apoptose, que foi mensurada através do grau de marcação das bandas de DNA. Quando o ácido ascórbico foi adicionado ao meio de cultivo, houve uma redução significativa da incidência de fragmentação de DNA comparado ao meio controle. A fim de monitorar o desenvolvimento folicular *in vitro*, McGee et al. (1997) avaliaram a apoptose de folículos pré-antrais utilizando um sistema de cultivo de 24 h e observaram uma mínima fragmentação do DNA no início do cultivo (0 h). Entretanto, os autores relataram um aumento significativo na fragmentação do DNA internucleossomal ao final do cultivo.

2.5. Marcadores de atividade proliferativa celular

Conforme foi citado anteriormente, após a ativação dos folículos primordiais ocorrem importantes mudanças no oócito e nas células da granulosa. No entanto, a detecção do início de crescimento de folículos primordiais depende da disponibilidade de marcadores sensíveis (Wandji *et al.,* 1996). Dentre os marcadores mais utilizados, pode-se citar: bromo-deoxiuridina (BrdU), antígeno nuclear de proliferação celular (PCNA) e timidina- H^3, os quais estão descritos separadamente, a seguir.

2.5.1. Bromo-deoxiuridina (BrdU)

Para estimar a atividade metabólica das células foliculares, pode-se utilizar marcadores não-radioativos e, dentre estes, um dos mais utilizados é o BrdU. O BrdU é um marcador para a síntese de DNA *in vitro*. A incorporação de BrdU ao DNA das células na fase S do ciclo celular pode ser utilizado como um indicador de atividade proliferativa (Jewgenow, 1998). Para a detecção imunocitoquímica da incorporação de BrdU, realiza-se um protocolo padrão de imunocitoquímica (Jewgenow, 1996, 1998). Jewgenow (1998) mostrou que o procedimento com BrdU pode resultar em diferentes tipos de células marcadas, como oócitos e células da granulosa. Além disso, este estudo também mostrou que aproximadamente 20 a 80% dos folículos pré-antrais felinos (dependendo do tratamento)

estavam marcados após 6 h de cultivo na presença de BrdU, enquanto de 1-3 células da granulosa por folículo encontravam-se. A marcação com BrdU tanto no oócito como nas células da granulosa indica atividade celular vital e evidencia um técnica rápida e eficiente para avaliar as condições de cultivo de folículos pré-antrais. Em ovinos, o fator de crescimento epidermal (EGF) e o fator de crescimento semelhante à insulina-II (IGF-II), bem como o FSH induziram uma forte marcação com BrdU em folículos pré-antrais (61-100 μm de diâmetro) após 48 e 96 h de cultivo *in vitro* (Hemamalini et al., 2003).

Sabe-se que os oócitos mamíferos sintetizam DNA no ovário embrionário. A replicação final do DNA ocorre durante o pré-leptóteno, no início da divisão meiótica. Logo após o nascimento, todos os oócitos estão no estágio de diplóteno da primeira divisão meiótica (Wassarman, 1988). Experimentos de marcação *in vivo* sugerem que a incorporação de BrdU em oócitos deste estágio pode ser explicada pela intensa renovação do DNA durante o crescimento oocitário (Jewgenow, 1998).

2.5.2. Antígeno Nuclear de Proliferação Celular (PCNA)

O antígeno nuclear de proliferação celular (PCNA) é um marcador expresso por células em crescimento e proliferação (Wandji et al., 1996). Após 2, 4 e 7 dias de cultivo *in vitro* de fragmentos ovarianos de fetos bovinos, observou-se um aumento na expressão de PCNA tanto no oócito como nas células da granulosa de folículos primários (Wandji et al., 1996). A expressão do PCNA no oócito pode ser devida ao fato de que o PCNA é uma proteína auxiliar da DNA polimerase delta, que está envolvida no processo de reparo do DNA (Downey et al., 1990). Assim, existe a possibilidade de que a DNA polimerase seja ativada em oócitos em crescimento para reparar o dano do material genético durante a transcrição (Wandji et al., 1996). Em bovinos, houve uma redução na percentagem de folículos primordiais e um aumento dos folículos primários após estímulo exógeno de estradiol (Cushman et al., 2001). Na espécie caprina, Silva et al. (2004a) observaram que em fragmentos de córtex ovariano não cultivados, somente 5,5% dos folículos primordiais possuíam células da granulosa positivas para PCNA, enquanto 46,1% dos folículos em desenvolvimento (transição, primários e secundários) possuíam pelo menos uma célula da granulosa marcada pelo PCNA. Estes autores também observaram que após o cultivo (5 dias) de tecido ovariano caprino, não houve um aumento significativo das percentagens de folículos primordiais e em desenvolvimento com células da granulosa positivas para PCNA,

sugerindo que a atividade proliferativa das células da granulosa não foi alterada durante o cultivo (Fig. 3). O cultivo de fragmentos ovarianos de fetos babuínos revelou que, após 2 ou 4 dias, houve uma intensa coloração por PCNA nas células da granulosa e no oócito da maioria dos folículos em crescimento (Wandji et al., 1997).

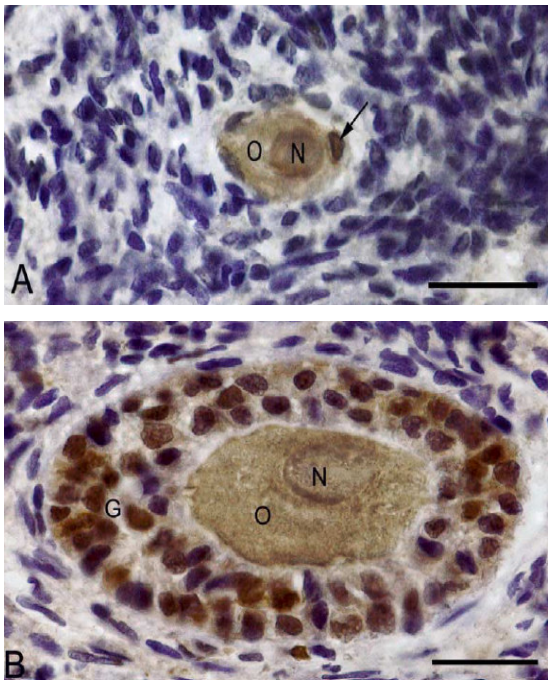

Figura 3. Secção de tecido ovariano após cultivo *in vitro*, mostrando: (A) um folículo primordial e (B) secundário com células da granulosa positivas para PCNA, coradas em marrom. O: oócito; N: núcleo do oócito; G: células da granulosa; seta: células positivas para PCNA em folículo primordial; barras: A: 25 µm, B: 12,5 µm. Fonte: SILVA et al. (2004a).

2.5.3. Timidina triciada (Timidina-H^3)

No sentido de determinar se o crescimento folicular foi devido à proliferação celular, alguns trabalhos utilizaram a timidina-H^3. Este marcador radioativo é adicionado ao meio de cultivo ao final do mesmo, a fim de se realizar a contagem de células da granulosa marcadas e não marcadas. Geralmente, o índice de marcação é computado como a percentagem de células que incorporam a timidina-H^3. Este marcador foi utilizado com sucesso na detecção do início do crescimento de folículos primordiais bovinos (Braw-Tal & Yossefi, 1997). Além disso, após o cultivo por 6 dias de folículos pré-antrais bovinos, observou-se que o fator de

crescimento fibroblástico básico (bFGF) aumentou significativamente o índice de marcação das células da granulosa (Wandji et al., 1996). Nilsson & Skinner (2002) utilizaram a timidina-H^3 como um indicador da proliferação das células da granulosa e da teca de ratas e, após 14 dias de cultivo com o fator de crescimento de diferenciação-9 (GDF-9), não observaram diferença significativa na proliferação destas células. Os autores concluíram que o GDF-9 não estimulou diretamente a atividade proliferativa das células da granulosa e da teca. Yu & Roy (1999) associaram as técnicas de microscopia eletrônica de transmissão à marcação com timidina-H^3 para verificar o crescimento de folículos primordiais de hamster durante 16 dias de cultivo. Estes autores observaram que as células da granulosa de folículos primários e secundários incorporaram timidina-H^3, indicando que estes folículos estavam em crescimento.

2.6. Produtos de secreção

O crescimento e a diferenciação folicular durante o cultivo *in vitro* podem ser refletidos pela secreção de esteróides (Liu et al., 2001). Considerando seu pequeno tamanho, os folículos secretam relativamente grandes quantidades de esteróides, tais como o estrógeno e a progesterona. Outras substâncias produzidas pelos folículos cultivados *in vitro* são a inibina e ativina (Smith et al., 1994), além de produtos da atividade metabólica, como dióxido de carbono (Eppig, 1977) e lactato (Boland et al., 1994).

Quando os folículos são cultivados individualmente, os produtos da secreção folicular são dificilmente detectados devido ao pequeno número de células presentes, mesmo quando há pouco volume de meio de cultivo *in vitro*. Para detectar esses produtos de secreção folicular, podem ser utilizadas análises de alta sensibilidade, entretanto, análises das concentrações de RNAm indicando a expressão gênica ou imunohistoquímica utilizando anticorpos para proteínas específicas são as técnicas mais indicadas (Hartshorne, 1997). Em geral, para a realização do radioimunoensaio, ao final do período de cultivo, o meio é coletado e armazenado a –20 ^{o}C até o momento da análise.

Vários estudos têm realizado análise hormonal após cultivo *in vitro* de folículos pré-antrais. Em camundongos, após cultivo de 4 dias de folículos isolados (100 – 105 μm), observou-se que a adição de FSH e ativina ao meio de cultivo aumentou significativamente a secreção de estrógeno e inibina (Liu et al., 1998). Além disso, a produção de estrógeno é um indicador da proliferação e diferenciação das células da granulosa. Liu et al. (2002)

cultivaram folículos pré-antrais isolados (100 – 120 μm) de camundongas e observaram que, do dia 4 ao 10 de cultivo *in vitro*, à medida que as células da granulosa proliferavam a produção de estrógeno aumentava. Estes mesmos autores mostraram que a atresia das células da granulosa está geralmente associada com a queda na produção folicular de estrógeno (Liu et al., 2002). Nesta mesma espécie, foi relatado que folículos pré-antrais somente foram capazes de produzir estrógeno após a adição de FSH ao meio de cultivo, pois o FSH induziu a diferenciação das células da granulosa e a atividade da aromatase destas células (Adriaens et al., 2004). Esta relação entre a produção de estrógeno e o FSH também foi demonstrada durante cultivo *in vitro* de folículos pré-antrais ovinos (Cecconi et al., 1999). Em bovinos, foi relatado um aumento na secreção de estrógeno após 5 dias de cultivo de folículos isolados (147 – 170 μm). Essa concentração atingiu o máximo após 7 dias e permaneceu nos mesmos níveis até o final do cultivo (dia 13) (Itoh et al., 2002). Nessa mesma espécie, observou-se um aumento na secreção de estrógeno pelos folículos cultivados por 12 dias em meio controle, na presença ou não do ácido ascórbico (Thomas et al., 2001).

Com relação à produção de progesterona, Adriaens et al. (2004) observaram que a secreção deste hormônio foi baixa (< 1 μg/L) até o 12^{o} dia de cultivo *in vitro* de folículos isolados de camundongas e aumentou significativamente 18 h após o início da ovulação. Após cultivo de 12 dias de folículos pré-antrais isolados de camundongas, Liu et al. (2001) mostraram que a concentração basal de progesterona permaneceu em 0,5 ng/ml até o dia 10 de cultivo. Entretanto, a partir do dia 10, foi observado um aumento dos níveis de progesterona (1,3 ng/ml), que foi atribuído à luteinização das células da granulosa.

O papel da inibina na regulação ovariana foi demonstrado em vários estudos. Dentre outras ações, a inibina influencia na produção de estrógeno pelas células da granulosa de ratas (Ying et al., 1986) e age na regulação da maturação oocitária (Smitz & Cortvrindt, 1998). Em camundongos, a produção de inibina por folículos pré-antrais aumentou progressivamente a partir do dia 4 até o dia 12 de cultivo (Cortvrindt et al., 1997). Na mesma espécie, mensurações dos níveis de inibina A e B secretadas após cultivo folicular por 8 dias indicaram que concentrações crescentes foram produzidas ao longo do cultivo (Newton et al., 2002). Nesse mesmo estudo, os autores mostraram que os níveis de ativina secretada pelos folículos também aumentaram progressivamente durante o cultivo. Estes resultados foram acompanhados pelo aumento do diâmetro folicular de 115 μm (dia 1) para 389 μm (dia 8 de cultivo). Além disso, Magoffin & Jakimiuk (1997) realizaram um estudo sobre fluido folicular e mostraram que a concentração de inibina A aumenta com o aumento do tamanho

folicular. Em ratas, após análise pela técnica de Western blot, observou-se um aumento dos níveis de inibina de folículos cultivados por 72 h em meio contendo FSH + fator de crescimento queratinócito (McGee et al., 1999) ou FSH + ativina (McGee et al., 2001), quando comparado ao controle.

3. Considerações finais

As técnicas de avaliação do cultivo *in vitro* permitem o monitoramento da situação (qualidade e atividade) folicular antes e após o cultivo, sendo de grande importância para a melhoria dos sistemas de crescimento *in vitro* de folículos pré-antrais. A análise morfológica e quantitativa (HC) dos folículos, a análise ultra-estrutural (MET) e da viabilidade folicular (azul de trypan, Hoescht), a detecção de apoptose (TUNEL), os marcadores que detectam o início do crescimento de folículos pré-antrais (BrdU, PCNA e timidina-H^3), bem como a análise dos produtos de secreção folicular, são exemplos das diferentes técnicas que podem ser utilizadas para avaliar a qualidade do folículo após o cultivo *in vitro*.

Após o desenvolvimento *in vitro*, é necessária a manutenção da qualidade folicular para que se tenham oócitos aptos a serem maturados e fecundados *in vitro*, ou que possam ser utilizados em outras biotécnicas relacionadas à reprodução animal. Dessa forma, quanto mais parâmetros e técnicas forem utilizados em conjunto para avaliar os folículos pré-antrais após o cultivo *in vitro*, mais precisa será a interpretação da qualidade e da viabilidade folicular.

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Capítulo II

Características histológicas e ultra-estruturais de folículos pré-antrais caprinos após cultivo in vitro na presença ou ausência de Ácido 3-Indol-Acético

Resumo

O objetivo do presente estudo foi avaliar os efeitos do Ácido 3-Indol-Acético (IAA) sobre a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos utilizando critérios histológicos e ultra-estruturais. Fragmentos de córtex ovariano caprino foram cultivados por 1 ou 5 dias em Meio Essencial Mínimo (MEM^+ - meio controle) suplementado com diferentes concentrações de IAA (10, 20, 40 ou 100 ng/mL). Pequenos fragmentos de tecido ovariano não cultivados e cultivados foram processados para a histologia clássica e análise ultra-estrutural. O crescimento folicular e oocitário foi avaliado antes e após o cultivo nos vários meios testados. Os resultados mostraram uma percentagem superior de folículos normais no MEM^+ ou MEM^+ suplementado com IAA (20 ng/mL) quando comparado aos outros tratamentos. O IAA (20 ou 40 ng/mL) aumentou a ativação folicular após 5 dias. Na presença de 10 ou 20 ng/mL de IAA, o diâmetro folicular aumentou após 5 dias de cultivo. Entretanto, estudos ultra-estruturais não confirmaram a manutenção da integridade morfológica de folículos caprinos cultivados por 1 ou 5 dias em MEM^+ suplementado com 20 ng/mL de IAA, mostrando alterações degenerativas no oócito e nas células da granulosa. Em conclusão, a integridade ultra-estrutural de folículos pré-antrais caprinos pode ser mantida com sucesso após cultivo in vitro em MEM^+ sem a adição de IAA. Além disso, a análise ultra-estrutural é necessária para julgar a morfologia de folículos pré-antrais cultivados, uma vez que o IAA afetou negativamente a integridade ultra-estrutural de folículos ativados in vitro, o que não foi detectado pela histologia clássica.

Capítulo II

Histological and ultrastructural features of caprine preantral follicles after in vitro culture in the presence or absence of Indole-3-acetic-acid

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Abstract

The aim of the present study was to evaluate the effects of Indole-3-acetic acid (IAA) on survival, activation and growth of caprine preantral follicles using histological and ultrastructural criteria. Pieces of caprine ovarian cortex were cultured for 1 or 5 days in Minimum Essential Medium (MEM^+ - control medium) supplemented with different concentrations of IAA (10, 20, 40 or 100 ng/mL). Small fragments from non-cultured ovarian tissue and from those cultured for 1 or 5 days in a specific medium were processed for classical histology and ultrastructural analysis. Follicular and oocyte growth were evaluated before and after 1 or 5 days of culture in the various media tested. The results showed a higher percentage of histologically normal follicles in MEM^+ alone or MEM^+ supplemented with IAA (20 ng/mL) than other treatments. IAA at 20 and 40 ng/mL increased the proportion of primordial follicles that entered the growth phase after 5 days. In the presence of 10 and 20 ng/mL of IAA, the follicles increased in diameter after 5 days culture. Ultrastructural studies, however, did not confirm maintenance of the morphological integrity of caprine follicles cultured for 1 or 5 days in MEM^+ supplemented with 20 ng/mL of IAA as was demonstrated by histological studies, but in contrast showed distinct degenerative changes in both oocytes and granulosa cells. In conclusion, ultrastructural integrity of caprine preantral follicles can be successfully maintained after in vitro culture for 5 days in MEM^+ without addition of IAA. In addition, ultrastructural analysis is necessary to judge the morphology of cultured preantral follicles since IAA negatively affects the ultrastructural composition of in vitro activated follicles, which was not detected by classical histology.

Keywords: Caprine, Preantral Follicles, Culture, Indol-3-Acetic Acid, Ultrastructure.

1. Introduction

Several studies have been addressed to evaluate the initiation of follicle growth by culturing ovarian cortex *in vitro* in media supplemented with different hormones and growth factors (bovine: Wandji et al., 1996; Cushman et al., 2002; caprine: Silva et al., 2004; baboon: Fortune et al., 1998; murine: Parrot & Skinner, 1999 and human: Hovatta et al.,

1997; Wright et al., 1999; Hreinsson et al., 2002). Furthermore, attention has been paid to the presence of growth factors, their receptors and hormone receptors in the various preantral follicle compartments to understand the mechanisms underlaying the activation of primordial follicles and further follicle growth and differentiation (for review, see Fortune, 2003; van den Hurk & Zhao, 2005). There are many oocyte-, granulosa- and in later follicle stages also theca-derived factors or other factors and hormones that may be involved in the regulation of follicle development. However, it is unknown if indol-3-acetic acid (IAA) could regulate preantral follicle activation and growth.

The auxin IAA was discovered in plants (Went & Thimann, 1937) as a hormone that controls processes like growth, movements or tropism that lead to cellular expansion and division (Becker & Hedrich, 2002), and is, for example, an important ingredient of coconut water (Toniolli et al., 1996). In animals, IAA is obtained from a diet rich in vegetable stems or by synthesis from tryptophan (Gordon et al., 1972; Mills et al., 1991) and it is present in cerebrospinal fluid (Hu & Dryhurst, 1997), blood (Martinez et al., 1983) and in several organs like lungs, kidneys, liver and brain (Tusell et al., 1984). IAA from coconut water was shown to be effective in promoting spermatozoal survival after in vitro preservation (Toniolli et al., 1996). In addition, IAA was used successfully to preserve caprine preantral follicles during transportation (Ferreira et al., 2001), while in a concentration of 40 ng/mL, IAA prevented cultured ovine preantral follicles from degeneration, but only when it was administered to the culture medium in a mixture with FSH or EGF (Andrade et al., 2005). The latter data were based on histological studies only. However, several authors have emphasized the importance of TEM after in vitro culture of preantral follicles, since it gives close insight into the ultrastructural characteristics of follicles, allowing a better evaluation of their quality (Van den Hurk et al., 1998; Salehnia et al., 2002).

The aim of the present study was to investigate whether IAA benefits the survival, activation and further growth of in vitro cultured caprine preantral follicles. To achieve these goals, both histological and ultrastructural studies were performed to investigate and compare the morphology of non-cultured follicles with those cultured for 1 or 5 days in absence or presence of different concentrations (10, 20, 40 or 100 ng/ml) of IAA. In addition, follicular and oocyte diameters were evaluated before and after 1 or 5 days of culture in the various media tested.

2. Materials and Methods

2.1. Source of ovaries

Ovaries (n=10) from five adult non-pregnant mixed-breed goats were collected at a local slaughterhouse (Fortaleza, Brazil). The animals were cyclic and in good body condition. The ovaries were washed and transported in 0.9% saline solution to the laboratory in thermo flasks with water at 20°C.

2.2. Experimental protocol

Both ovaries from each animal were stripped of surrounded fat tissue and ligaments, and cut in half, where after the medulla, large antral follicles and corpora lutea were removed. Following this, the ovarian cortex was divided in 11 fragments of approximately 3 x 3 mm (1 mm thick). A small part (1 mm^3) of one fragment was taken away randomly and subsequently fixed for ultrastructural examination while the remainder fragment was fixed for classic histological studies (non-cultured controls). The other fragments of ovarian cortex were individually *in vitro* cultured in 24-well culture plate with 1 mL of culture medium for 1 or 5 days at 39°C with 5% CO_2 in air. The basic control medium was Minimum Essential Medium (Cultilab, Rio de Janeiro, Brazil) supplemented with ITS (insulin 6.25 µg/mL, transferrin 6.25 µg/mL, and selenium 6.25 ng/mL), 0.23 mM pyruvate; 2 mM glutamine; 2 mM hypoxanthine; 1.25 mg/mL BSA, 100 µg/mL penicillin, 100 µg/mL streptomycin (Vetec, Rio de Janeiro, Brazil) and 0.25 µg/mL fungizone (MEM^+). This control medium (MEM^+) was supplemented with different concentrations of Indol-3-Acetic Acid (10, 20, 40 or 100 ng/mL) (Vetec, Rio de Janeiro, Brazil). All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Every two days, the culture medium was replaced by same volume fresh. Each treatment was repeated five times, thus using the ovaries of five different animals.

2.2. Histological analysis and assessment of *in vitro* follicle growth

To evaluate the morphology of the caprine follicles, a small part (1 mm^3) from each

non-cultured ovarian tissue fragment and those that were cultured for 1 or 5 days was randomly chosen and removed for TEM studies, while the remainder of the fragments was fixed in Carnoy for 12 h for Light Microscopic (LM) studies. Briefly, for LM analysis, after fixation, the tissue fragments were dehydrated in a graded series of ethanol solutions, clarified with xylene and embedded in paraffin wax. For each piece of ovarian cortex, 7 μm sections were mounted on slides, stained with periodic acid Schiff and hematoxylin (PAS staining system, Sigma, Inc., St. Louis, MO, USA), and examined by light microscopy (Zeiss, Germany) at 100X and 400X magnification.

Preantral follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte), or growing follicles i.e., intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells) as described by Silva et al. (2004). These follicles were classified individually as histologically normal intact when an intact oocyte was present, i.e. an oocyte without a pyknotic nucleus, surrounded by granulosa cells which are well organized in one or more layers and that have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte which have a pyknotic nucleus, and/or is enveloped by disorganized granulosa cells, which have detached from the basement membrane. From each medium and each culture period, approximately 150 follicles were randomly evaluated.

To evaluate follicular activation and growth, only intact follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and growing follicles were calculated at day 0 (controls) and after 1 or 5 days of culture in the various media tested. Major and minor axes of each oocyte and follicle were measured under a microscope with an ocular micrometer. The averages of the minor and major axes were reported as oocyte and follicle diameters, respectively. These values were used to assess the effect of the hormonal treatment on follicular growth.

2.3. Ultrastructural analysis

For ultrastructural analysis, 11 small pieces of ovarian cortex were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer, pH 7.2. After washing the ovarian pieces with sodium cacodylate buffer, they were post-fixed in 1%

osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently, the samples were dehydrated in a graded series of acetone and embedded in Spurr´s epoxy resin. Firstly, semi-thin sections (3 μm) were cut on an ultramicrotome (Reichert Supernova, German) for LM studies and stained with toluidine blue. Subsequently, follicles classified as histologically normal in semi-thin toluidin blue stained sections were submitted to ultrastructural analysis. For that purpose, thin sections (70 nm) were cut and then contrasted with uranyl acetate and lead citrate, and examined using a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope, operating at 80 kV.

2.4. Statistical analysis

The percentages of histologically normal follicles, as well as of primordial and growing follicles in non-cultured and cultured tissue were compared by Chi-square test (StatView for Windows). Mean diameters of oocytes and follicles obtained after the various treatments and after the different culture periods were analyzed by ANOVA and Fisher's test (StatView for Windows, SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effect of media and culture periods on the percentage of histologically normal follicles

The effects of IAA supplementation on follicle survival after 1 and 5 days of culture were shown in Table 1. There was a significant decrease ($P < 0.05$) in the percentages of histologically normal follicles (Fig. 1) after 1 and 5 days of culture compared to non-cultured follicles. However, no considerable effect of IAA concentration was observed after 1 day of culture. With the increase of the culture period from 1 to 5 days, a significant ($P < 0.05$) decrease in the percentage of histologically normal follicles was observed in medium containing 10 ng/mL IAA. After 5 days culture, the addition of 10, 40 and 100 ng/mL IAA significantly ($P < 0.05$) reduced the percentage of histologically normal follicles when compared with MEM^+ alone or MEM^+ supplemented with 20 ng/mL IAA.

Table 1. Percentages of histologically normal follicles in non-cultured tissues and in tissues cultured for 1 or 5 days in MEM^+ supplemented with 0, 10, 20, 40 or 100 ng/mL IAA. Per treatment 150 follicles were evaluated.

Non-cultured (Day 0)	87,3	
Cultured	Day 1	Day 5
MEM^+	64.1* ^{A,a}	63.4* ^{A,a}
IAA 10	66.7* ^{A,a}	54.7* ^{B,b}
IAA 20	64.1* ^{A,a}	64.0* ^{A,a}
IAA 40	56.0* ^{A,a}	51.1* ^{A,b}
IAA 100	59.8* ^{A,a}	59.5* ^{A,b}

* $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (begin control/D0).

(A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$).

(a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

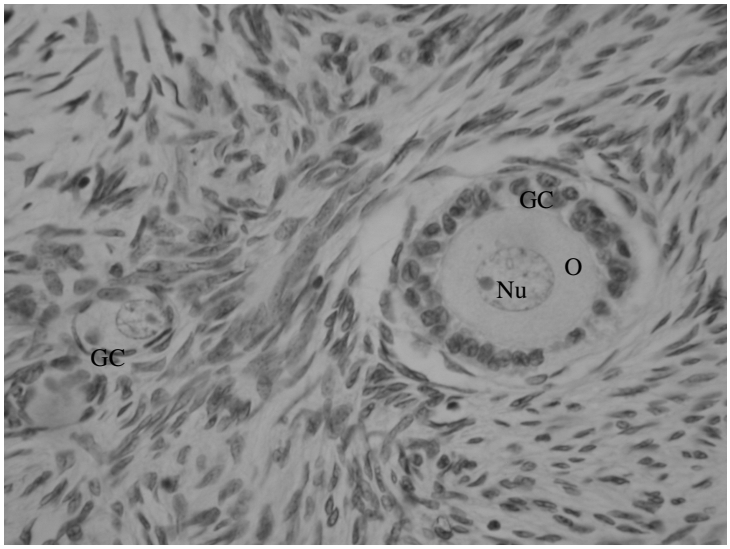

Fig. 1. Histological section of a non-cultured ovarian tissue fragment, showing normal primordial and primary follicles. Periodic acid Schiff-hematoxylin staining. O: oocyte; NU: oocyte nucleus; GC: granulosa cells (400 x).

3.2. Goat primordial follicle activation and growth during in vitro culture

The percentages of primordial and growing follicles in ovarian cortical tissue before and after 1 and 5 days of culture are shown in Table 2. The percentages of primordial and growing follicles in non-cultured cortex tissues were 81.7% and 18.3 %, respectively. Compared to non-cultured and 1 day-cultured tissues, all of 5 day-cultured tissues showed a strong reduction in the proportion of primordial follicles ($P < 0.05$) and an increased proportion of growing follicles ($P < 0.05$).

Table 2. Percentages of primordial and growing follicles (intermediate, primary and secondary) in non-cultured tissues and in tissues cultured for 1 or 5 days in MEM^+ supplemented with various concentrations of IAA. Per treatment 150 follicles were evaluated.

	Primordial follicles		Growing follicles	
Non-cultured (Day 0)	81,7		18,3	
Cultured	Day 1	Day 5	Day 1	Day 5
MEM^+	70,3 ^{A,a}	43,4 $^{* B,a}$	29,7 ^{A,a}	56,6 $^{* B,a}$
IAA 10	71,4 ^{A,a}	46,3 $^{* B,a}$	28,6 ^{A,a}	53,7 $^{* B,a}$
IAA 20	80,9 ^{A,a}	42,5 $^{* B,a,b}$	19,1 ^{A,a}	57,5 $^{* B,a,b}$
IAA 40	75,0 ^{A,a}	27,4 $^{* B,b}$	25,0 ^{A,a}	72,6 $^{* B,b}$
IAA 100	69,2 ^{A,a}	44,3 $^{* B,a}$	30,8 ^{A,a}	55,7 $^{* B,a}$

* $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (begin control/D0).
(A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$).
(a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

When compared to the 5 day-cultured tissues in control MEM^+, addition of 40 ng/mL of IAA to MEM^+ significantly increased ($P < 0.05$) the percentage of growing follicles. No

significant effect of IAA on growing follicles was observed after 1 day culture.

Compared to non-cultured tissue, a significant increase in oocyte diameter was observed, when tissues were cultured for 5 days in a medium with 10 ng/mL IAA (Table 3; $P < 0.05$). Likewise, follicle diameter significantly increased when tissues were cultured for 5 days in presence of 10, 20 and 40 ng/mL IAA, while both oocyte and follicle diameters increased ($P < 0.05$) in media containing 10 and 20 ng/ml IAA.

Table 3. Oocyte and follicle diameters (mean ± S.D.) in tissues non-cultured or cultured for 1 or 5 days in MEM^+ supplemented with various concentrations of IAA. Per treatment 150 follicles were evaluated.

Table 3.

	Oocyte diameter (µm)		Follicle diameter (µm)	
Non-cultured (Day 0)	40.7 ± 2.2		52.2 ± 2.2	
Cultured	Day 1	Day 5	Day 1	Day 5
MEM^+	39.7 ± 1.2 ^{A,a}	41.9 ± 1.0 ^{A,a}	53.9 ± 2.0 ^{A,a}	57.8 ± 1.5 ^{A,a}
IAA 10	43.6 ± 1.5 ^{A,a}	52.9 ± 2.4* ^{B,b}	57.6 ± 1.8 ^{A,a}	79.8 ± 5.4* ^{B,b}
IAA 20	43.5 ± 1.1 ^{A,a}	47.5 ± 2.9 ^{B,a,b}	47.0 ± 1.4 ^{A,a}	74.9 ± 5.9* ^{B,b}
IAA 40	39.7 ± 1.7 ^{A,a}	41.9 ± 2.2 ^{A,a}	55.7 ± 1.9 ^{A,a}	67.5 ± 6.2* ^{B,a}
IAA 100	43.0 ± 1.1 ^{A,a}	43.9 ± 2.4 ^{A,a}	57.9 ± 1.3 ^{A,a}	59.8 ± 5.3 ^{A,a}

* $P < 0.05$, significantly different from non-cultured ovarian cortical tissues (begin controls)

(A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$).

(a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

3.3. Ultrastructural analysis of goat preantral follicles cultured in vitro with 20 ng/mL of IAA

Culture with 20 ng/mL of IAA gave the best results for follicle activation and growth after 5 days. Then, it was decided to perform TEM studies on ovarian fragments that have been treated for 1 and 5 days with 20 ng/mL of IAA, as well as in non-cultured fragments and fragments cultured for 5 days in MEM^+ alone. Regardless of the stage of follicular development, the ultrastructure of histologically normal follicles from the control group and from those cultured in MEM^+ alone for 5 days appeared similar. These follicles exhibited sparse vesicles spread throughout the ooplasm. Moreover, the homogeneous cytoplasm contained numerous rounded mitochondria with peripheral cristae and continuous mitochondrial membranes, although there were occasional elongated forms with parallel cristae. Golgi complexes were rarely observed. Both smooth and rough endoplasmic reticulum were present, either as isolated aggregations or as complex associations with mitochondria and vesicles (Fig. 2A). The oocyte nucleus had uncondensed chromatin and the nucleolus could generally be observed. In all developmental stages, granulosa cells were small, with a greater nuclear-to-cytoplasm ratio as compared with typical normal cell structures. The nuclei were irregularly shaped, with loose chromatin in the center and small aggregates of condensed chromatin in the periphery. Well-developed rough endoplasmic reticulum and mitochondria with well-developed lamellar cristae were the most evident organelles observed in granulosa cells. Abundant gap junctions were observed between granulosa cells as well as between granulosa cells and the oocyte (Fig. 2B).

When cultured in IAA for 1 day, the histology of follicles seemed to be well preserved in semi-thin sections stained with toluidin blue. However, TEM studies revealed some discrete changes in their ultrastructure, which are indicative of initial degeneration. Such follicles generally had an oocyte with large numbers of vesicles spread throughout the ooplasm. Cytoplasmic organelles were more randomly observed. In addition, initial signs of endoplasmic reticulum proliferation and damage to mitochondrial membranes and cristae were observed. The oocyte nucleus appeared misshapen and retracted, and had a wavy membrane (Fig. 2C). Granulosa cells looked swollen, with a low density of cytoplasmic organelles. Furthermore, granulosa cells showed less contact with each other and exhibited obvious fewer gap junctions.

Although the histological images of follicles cultured in 20 ng/mL of IAA for 1 and 5 days were comparable, they showed remarkable differences at the ultrastructural level.

Besides the progression of the changes described above, the follicles cultured for 5 days presented a substantial irregularity of the follicular, oocyte and nuclear outlines. As degeneration progresses, the ooplasm in these follicles was extremely vacuolated, with the vacuoles often being fused thus producing a larger vacuolated area, and in some oocytes the nuclear membrane was broken. Furthermore, some granulosa cells were fragmented or had disappeared, leaving an empty space in the follicular granulosa. Frequently, the connection between the oocyte and granulosa cells had disappeared, and organelles were no longer identifiable (Fig. 2D).

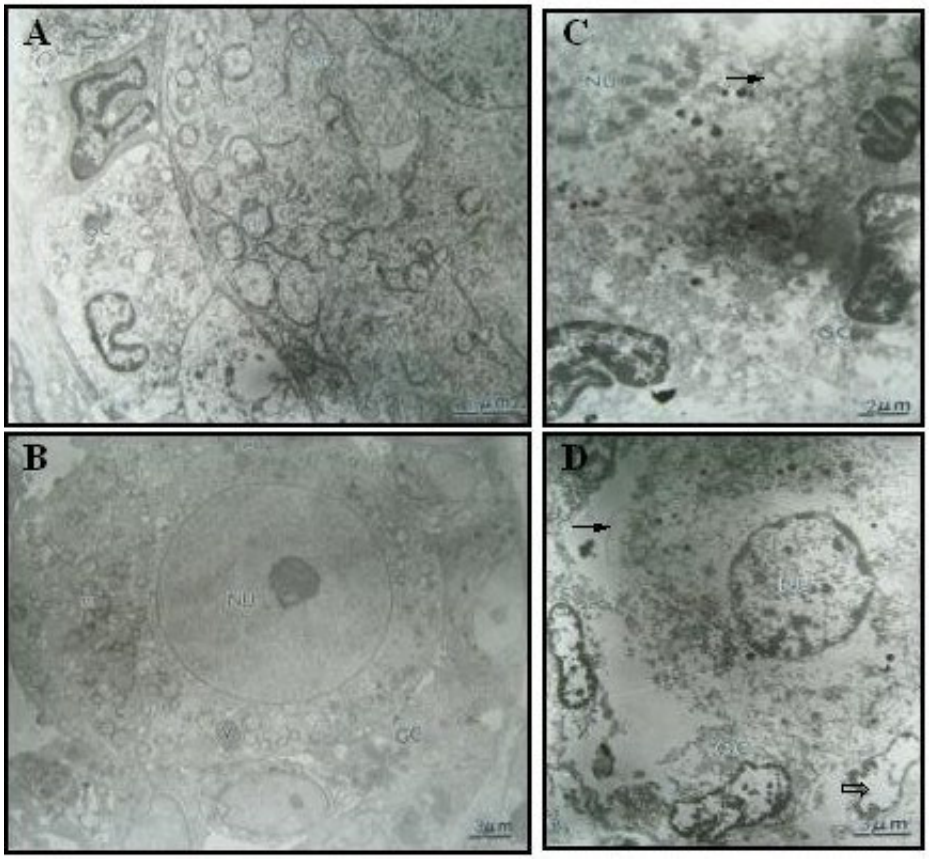

Figure 2. Electron micrographs of caprine preantral follicles before (5200 x) (A) and after culture in MEM$^+$ for 5 days (cultured control) (3240 x) (B), in IAA (20 ng/mL) for 1 day (5600 x) (C) and 5 days (3240 x) (D). In non-cultured follicles and in follicles cultured in MEM$^+$, note the homogeneous cytoplasm with numerous rounded mitochondria (2A e B). Note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration (solid arrow) (2C). Note the empty space in degenerated granulosa cells after in vitro culture with IAA (open arrow) (2D). NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicles.

4. Discussion

The present study described ultrastructural changes in caprine preantral follicles after their in vitro culture for 1 and 5 days. It furthermore reported a dosis-dependent in vitro effect of IAA on the activation of primordial follicles and preantral follicle growth. After 5 days of culture, IAA at a concentration of 20 ng/mL appeared to maintain preantral follicle survival, based on histological parameters, and to stimulate both primordial follicle activation and preantral follicle growth, whereas the follicular ultrastructure was negatively affected. In addition, a lower concentration of IAA (10 ng/mL) was not effective in maintaining follicular survival after 5 days of culture. There are only few papers dealing with IAA effects in animals. In males, Cardoso et al. (2002) demonstrated that canine spermatozoa could be well preserved in presence of 20 ng/ml IAA, which appeared effective in our studies. However, 40 ng/mL IAA was shown to be effective in promoting porcine spermatozoal survival after their in vitro preservation (Toniolli et al., 1996). In females, effects of IAA were previously described on goat preantral follicle preservation (Ferreira et al., 2001) and sheep in vitro cultured preantral follicles, (Andrade et al., 2005). In these studies, positive effects were obtained after LM evaluation of follicles, judged by classical histology only. Andrade et al. (2005) showed that, in combination with EGF or FSH, IAA at a concentration of 40 ng/mL prevented the degeneration of preantral follicles. However, several authors have emphasized the importance of TEM studies of follicles after their in vitro culture, since it gives close insight into the ultrastructural characteristics of follicles, by which could help to avoid too optimistic opinions about the quality of cultured follicles based on light microscopic investigations (van den Hurk et al., 1998; Salehnia et al., 2002; Matos et al. 2004). Our current findings thus confirm this latter view.

The present TEM studies revealed obvious differences in ultrastructural quality of follicles cultured in MEM^+ for 5 days when compared to those in medium supplemented with IAA (20 ng/mL) for 1 or 5 days. Although they had comparable histological images, follicles cultured in MEM^+ maintained their ultrastructural integrity, while those cultured with IAA showed various signs of initial degeneration after 1-day culture, and exhibited more clear degenerative features, like extreme ooplasm vacuolization after 5-days culture. Cytoplasmic vacuoles are characteristic signs of degeneration in oocytes (Silva et al., 2000), granulosa (Hay et al., 1976) and cumulus cells (Assey et al., 1994) during degeneration and may represent endoplasmic reticulum swelling (Tassel & Kennedy, 1980) or altered mitochondrial

structure (Fuku et al., 1995). In goat preantral follicles, mitochondria showing extensive swelling and disappearance of their cristae, and volume-increased endoplasmic reticulum were previously indicated as the first signs of degeneration (Silva et al., 2001). In plants, the auxin IAA increased cell wall plasticity and cell water uptake, and changed cellular permeability, respiratory patterns, and acid nucleic metabolism (Galston & Purves, 1960). However, the molecular mechanisms of auxin action in animal cells are not clear.

Melo et al. (2004) indicated that the induction of cell death by IAA is related to the activity of peroxidase, which lead to the formation of toxic metabolites and oxygen reactive species (ORS), such as H_2O_2 e O_2^-. When cellular antioxidative defense is limited, these ORS may cause oxidation of key molecules that normally release proteases, lipases, and nucleases from mitochondria (Fiers et al., 1999). In our study, IAA might have increased the production of toxic ORS, thus causing the observed higher rate of degeneration.

After 5-days culture, addition of 20 ng/mL of IAA to the medium resulted in a significant increase in activation rate of primordial follicles and in oocyte and follicle diameters. Our TEM findings suggest that this light microscopically observed phenomena are due to follicular necrosis, characterized by cell swelling as a result of increased cellular vacuolization (Barros et al., 2001). Based on findings of Andrade et al. (2005), IAA might activate early folliculogenesis after its possible binding to certain growth factor(s) present in the ovarian tissue. On the other hand, interaction between IAA and local growth factors could have stimulated primordial follicle activation.

When compared with 1 day of culture, oocyte and follicular diameters had increased after 5 days of culture in the presence of 10 or 20 ng/mL of IAA. Similar results were obtained with pea culture in which IAA at a low concentration (5 μM) stimulated growth of pea stem segments, while little growth occurred in the absence of IAA (Moore et al., 1983). More studies are necessary to determine the molecular mechanism of IAA action on the growth of caprine preantral follicles. It is of interest to note here that hydroxy acetic indols are synthesized by the pineal gland and influence reproduction by effecting on gonadal steroidogenesis (Öcal-Irez et al.,1989). IAA thus may function similarly to pineal indols for affecting folliculogenesis. However, like the molecular mechanisms of IAA action on the activation and growth of caprine preantral follicles, the way in which IAA and pineal indols can influence these follicles has to be further investigated.

In conclusion, the ultrastructural integrity of caprine preantral follicles can be successfully maintained after *in vitro* culture for 5 days in MEM^+ without addition of IAA. In

addition, this study showed that IAA is not effective in maintaining the ultrastructural morphology of follicles during in vitro culture. The findings prove that TEM studies are indispensable to judge the morphological status of follicles and for investigating preantral follicle development, since positive effects of compounds on follicle activation and growth observed after routine histological studies could be the result of a negative effect, caused by induced degeneration which only ultrastructurally can be visualized. Further studies will be needed to elucidate the mechanism through which IAA and pineal indols act on ovarian tissue.

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Capítulo III

Efeitos do Fator de Crescimento Fibroblástico-2 sobre o cultivo in vitro de folículos pré-antrais caprinos

Resumo

Os objetivos do presente estudo foram avaliar os efeitos do fator de crescimento fibroblástico-2 (FGF-2) sobre a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos utilizando estudos histológicos e ultra-estruturais. Fragmentos de córtex ovariano caprinos foram cultivados por 1 ou 5 dias em Meio Essencial Mínimo (MEM - meio controle) suplementado ou não com diferentes concentrações de FGF-2 (10, 50, 100 ng/mL). Os fragmentos de tecido ovariano não cultivado (controle) e aqueles cultivados por 1 ou 5 dias foram processados para microscopia eletrônica de transmissão (MET) ou histologia clássica para avaliar a morfologia folicular e para calcular as percentagens de folículos normais. Além disso, foram investigados os efeitos do FGF-2 sobre os diâmetros oocitário e folicular de folículos pré-antrais cultivados. Nossos resultados mostraram que, apesar de as percentagens de folículos normais serem inferiores em fragmentos cultivados do que em não cultivados, não houve diferença entre os tratamentos no dia 1 ou 5 de cultivo. Após 1 e 5 dias de cultivo, um aumento significativo de folículos em crescimento foi observado no meio suplementado com 50 ng/mL de FGF-2. Este tratamento também resultou em um aumento nos diâmetros oocitário e folicular após 5 dias. A MET mostrou que a integridade ultra-estrutural de folículos caprinos foi mantida durante 5 dias de cultivo na presença de 50 ng/mL de FGF-2. Em conclusão, este estudo demonstrou que, na concentração de 50 ng/mL, o FGF-2 mantém a integridade morfológica de folículos pré-antrais caprinos cultivados por 5 dias e estimula a ativação de folículos primordiais e o crescimento de folículos ativados.

Capítulo III

Effects of Fibroblast Growth Factor-2 on the in vitro culture of caprine preantral follicles

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Keywords: Caprine, Preantral Follicles, Culture, FGF-2.

Abstract

The aims of the present study were to evaluate the effects of fibroblast growth factor-2 (FGF-2) on survival, activation and growth of caprine early-staged (preantral) follicles using histological and ultrastructural studies. Fragments of caprine ovarian cortex were cultured for 1 or 5 days in an enriched minimum essential medium (MEM^+), supplemented or not with different concentrations of FGF-2 (10, 50 or 100 ng/mL). Fragments from non-cultured ovarian tissue (control) and from tissues cultured for 1 or 5 days in a specific medium were processed for transmission electron microscopy (TEM) or classical histology to evaluate the morphological quality of caprine preantral follicles and to calculate the percentages of normal follicles. Additionally, effects of FGF-2 on oocyte and follicle diameter of cultured preantral follicles were investigated. Our results showed that, although the percentages of histologically normal follicles were lower in cultured than in non-cultured ovarian tissue fragments, there were no differences in this regard among treatments neither at day 1 or day 5 of culture. After 1 and 5 days of culture, a significantly higher percentage of growing follicles was observed in the medium supplemented with 50 ng/mL of FGF-2. This FGF-2 treatment furthermore resulted in an increase in diameter of both oocytes and follicles that were cultured for 5 days. TEM showed that the ultrastructural integrity of caprine preantral follicles was maintained during their 5 days culture in presence of 50 ng/mL FGF-2. In conclusion, this study demonstrated that at a concentration of 50 ng/mL FGF-2 not only maintains the morphological integrity of 5 days cultured caprine preantral follicles, but also stimulate the activation of primordial follicles and the growth of activated follicles.

1. Introduction

Successful oocyte maturation, fertilization, and embryonic development depend on a correct, coordinated growth and development of the ovarian follicle (Cecconi et al., 2004). Follicular growth and differentiation is a process characterized by morphological and functional changes, which at the more advanced stages of folliculogenesis is primarily regulated by gonadotrophins and further influenced by intra- and extraovarian factors (Fortune, 2003) At an early stage, however, follicles seem to be regulated mainly by

intraovarian factors among which are fibroblast growth factors (FGFs) (Van den Hurk & Zhao, 2005). Among FGFs, especially FGF-2 (previously called basic FGF) is a potent mitogen and involved in cell differentiation, cell migration and angiogenesis in many tissues, including pituitary, retina, adrenal and ovary (Baird et al., 1986; Gospodarowicz et al., 1986). Immunohistochemical studies have localized FGF-2 in growing follicles and corpora lutea of bovine (Schams et al., 1994) and rat (Asakai et al., 1993) ovaries. Furthermore, FGF-2 receptors or their mRNAs have been demonstrated in growing follicles of cow (Wandji et al., 1992) and rat (Shikone et al., 1992; Asakai et al., 1993, 1995), suggesting that FGF-2 plays a role in follicular activation.

In the mammalian ovary, FGF-2 has been implicated in the regulation of granulosa cell proliferation and angiogenesis in the theca layer and in corpora lutea (Gospodarowicz et al., 1986). Some studies have shown that FGF-2 has a mitogenic effect on granulosa and theca cells of preantral follicles from different species (cow: Wandji et al., 1996; domestic cat: Jewgenow, 1996; chicken: Roberts & Ellis, 1999). In addition, FGF-2 promoted activation and growth of rat primordial and primary follicles (Nilsson et al., 2001). Recently, a study showed that FGF-2 stimulated caprine oocyte survival but had no obvious effect on oocyte growth (Zhou & Zhang, 2005). In contrast, in a concentration of 10 ng/mL, FGF-2 did not alter the morphological health nor influenced primordial follicle activation of bovine primary and secondary follicles in vitro (Derrar et al., 2000). However, in most of these studies performed with cultured preantral follicles FGF-2 was tested in only one concentration, while obtained data were based on histological evaluation of follicles only.

This study focuses on possible influences of different concentrations (0, 10, 50 and 100 ng/mL) of FGF-2 on 1 or 5 days cultured caprine ovarian cortical tissue enclosed early-staged follicles. To this end, before and after their culture, the histological structure and ultrastructure of preantral follicles was evaluated, whereafter oocyte and follicular diameters were measured and follicular survival percentages as well as percentages of primordial and percentages of growing follicles calculated.

2. Materials and Methods

2.1. Experimental protocol

Eight ovaries from four adult non-pregnant mixed-breed goats were collected at a

local abattoir. Then, the ovaries were washed and transported in 0.9% saline solution to the laboratory in a thermos flask with water at 32°C. In the laboratory, the pair of ovaries from each animal was stripped of surrounded fat tissue and ligaments, cut in half and the medulla, large antral follicles and corpora lutea were removed. Following this, the cortex from each ovarian pair was divided in 9 fragments of approximately 3 x 3 mm (1 mm thick), totalizing 36 fragments used in the experiment. A small part of one fragment ($1mm^3$) was taken randomly and immediately fixed for histological and ultrastructural examination (non-cultured control). The other fragments of ovarian cortex were cultured individually in 1 mL of culture medium for 1 or 5 days at 39°C with 5% CO_2 in air using a 24-well culture dish. It is important to note that the side of the fragment that was in contact with the culture dish was randomly selected. The basic control medium was Minimum Essential Medium (Cultilab, Rio de Janeiro, Brazil) supplemented with ITS (insulin 6.25 µg/mL, transferrin 6.25 µg/mL, and selenium 6.25 ng/mL), 0.23 mM pyruvate; 2 mM glutamine; 2 mM hypoxanthine; 1.25 mg/mL BSA, 100 µg/mL penicillin, 100 µg/mL streptomycin (Vetec, Rio de Janeiro, Brazil) and 0.25 µg/mL fungizone (MEM^+). For the treatments, this control medium (MEM^+) was supplemented with different concentrations of FGF-2 (10, 50 or 100 ng/mL). All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Every two days, the culture medium was replaced by fresh medium. Each treatment was repeated four times, thus using the ovaries of four different animals.

2.2. Assessment of *in vitro* caprine preantral follicle growth

To evaluate the morphology of the caprine preantral follicles, a small part ($1\ mm^3$) from each non-cultured ovarian tissue fragment and those that were cultured for 1 or 5 days was randomly removed from any region of the fragment and processed for TEM and the remainder was fixed individually in Carnoy for 12 h. Then, they were dehydrated through an alcohol series, clarified with xylene and embedded in paraffin wax. The wax blocks containing the treatments were completely and serially sectioned (7 µm), stained with periodic acid Schiff and hematoxylin (PAS staining system, Sigma, Inc.,St. Louis, MO, USA), and examined by light microscopy (Zeiss, Germany) at 100X and 400X magnification.

The preantral follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte), or growing follicles i.e., intermediate (one layer of flattened to

cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). Preantral follicles were classified individually as histologically normal when an intact oocyte was present surrounded by granulosa cells that are well organized in layers and have no pyknotic nucleus, as shown in Figure 1. Degenerated follicles were defined as follicles with a retracted oocyte, containing a pyknotic nucleus, and with disorganized granulosa cells, which are detached from the basement membrane. Thirty follicles were counted in each treatment to evaluate follicular morphology and activation. As each treatment was repeated four times, a total of 120 follicles were evaluated for each medium and each culture period.

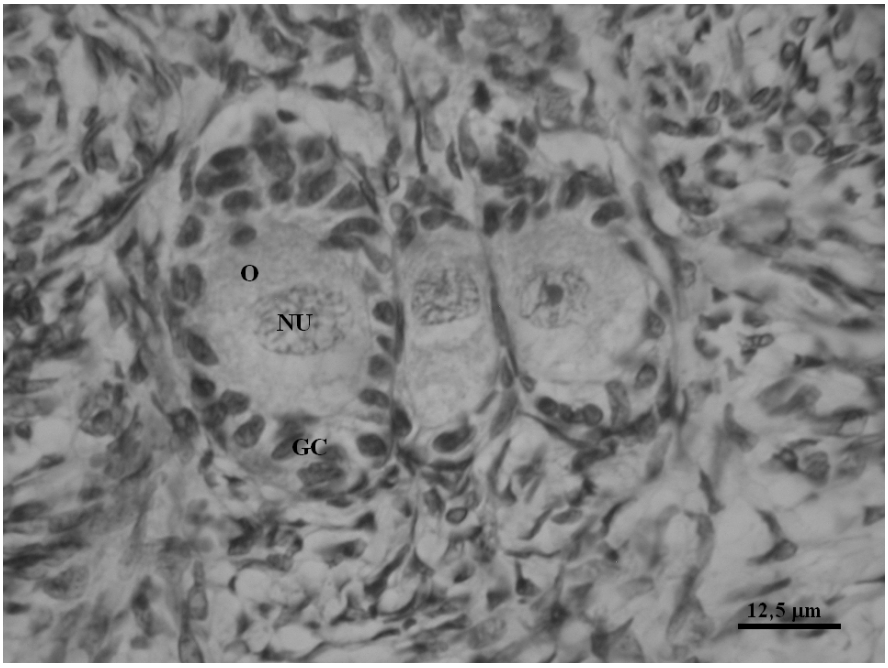

Figure. 1. Histological section of non-cultured tissue after staining with periodic acid Schiff-hematoxylin, showing normal preantral follicles. O: oocyte; NU: oocyte nucleus; GC: granulosa cells.

To evaluate follicular activation and growth, only histologically normal follicles were considered, and the percentages of primordial and growing follicles were calculated at day 0

(control) and after 1 or 5 days of culture in the various media tested. To minimize the possibility of counting more than once, only follicles with a visible oocyte nucleus were recorded. Oocyte and follicular diameter was measured with the aid of an ocular micrometer. Both diameters, from the basement membrane, at right angles to each other in the largest cross-section of each growing oocyte and follicle were measured and averaged. Follicular and oocyte diameter were measured in 20 follicles for each treatment.

2.3. Ultrastructural analysis

To better evaluate follicular quality, from both cultured and non-cultured ovarian tissue fragments, preantral follicles classified as histologically normal in semi-thin toluidin blue stained plastic sections were submitted to ultrastructural analysis. To this end, small pieces of ovarian cortex were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer (pH 7.2), and then washed with sodium cacodylate buffer and postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently, samples were dehydrated in a graded series of acetone and embedded in Spurr´s epoxy resin. Semi-thin sections (3 μm) for light microscopy were made and stained with toluidine blue. After light microscopical tracing of histologically normal follicles in these sections, thin sections (70 nm) were made and contrasted with uranyl acetate and lead citrate, and examined using a Jeol 100 C or 1011 (Jeol, Tokyo, Japan) transmission electron microscope.

2.4. Statistical analysis

Data are expressed as mean ± SEMs. The percentages of surviving follicles at all stages, primordial and growing follicles obtained after 1 or 7 days in the various treatments were subjected to arc-sin transformation before analysis of variance (ANOVA). The data as well as the diameter of oocytes and follicles were analyzed by ANOVA followed by Fisher's protected least significant difference (PLSD) test (StatView for Windows, SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effect of media and culture periods on the percentage of morphologically normal follicles

A total of 1,080 follicles were counted to evaluate follicular morphology, activation and growth. Figure 2 shows the effect of the tested media and culture periods on the percentage of histologically normal follicles in non-cultured (control) ovarian fragments and in tissues cultured for 1 and 5 days. Compared to control values, there was a significant decrease ($P < 0.05$) in the percentage of histologically normal follicles after 1 and 5 days of culture. At day 1 and 5, the percentages of histologically normal follicles were similar among treatments, while these percentages were maintained during the 5 days culture period.

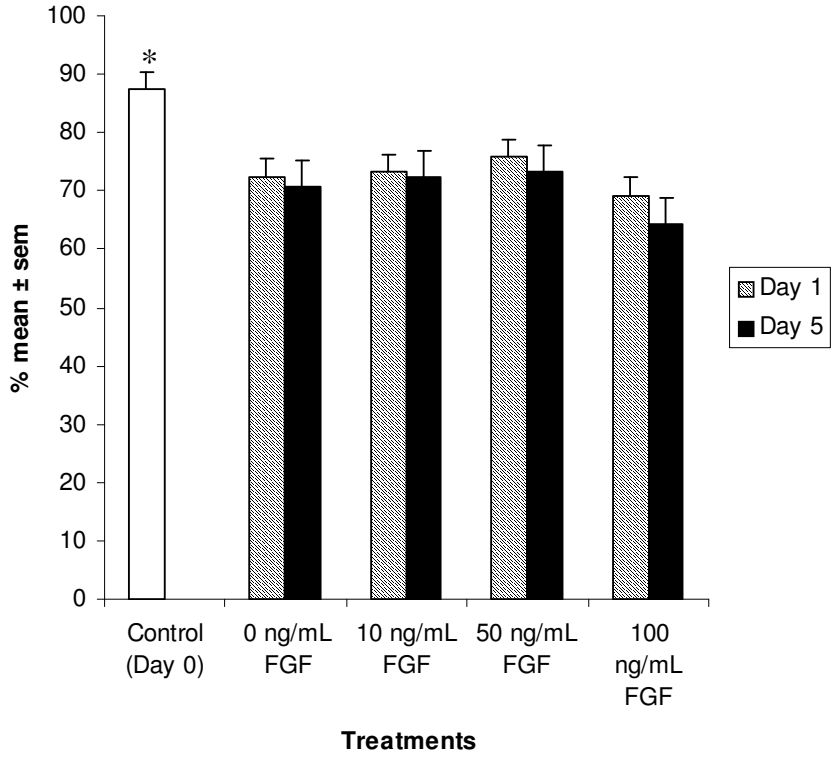

Figure. 2. Percentages (means ± S.E.M) of histologically normal preantral follicles in non-cultured tissue (control) and in tissue cultured for 1 and 5 days in MEM^+ and MEM^+ supplemented with 10, 50 and 100 ng/mL FGF-2. (30 follicles evaluated in each one of four replicates per treatment). * $P < 0.05$, significantly different from cultured ovarian cortical fragments.

3.2. Caprine primordial follicles activation and oocyte and follicle growth during *in vitro* culture

The mean percentages of primordial and growing follicles in ovarian cortical tissue before culture and after 1 and 5 days of culture are shown in Table 1. The percentages of primordial and growing follicles in non-cultured cortex were 83.8% and 16.2%, respectively. Compared to the begin control situation, after 1 day of culture, the proportion of primordial follicles was significantly reduced ($P<0.05$) by all in vitro treatments, whereas the proportion of grown follicles was increased, but only significantly ($P<0.05$) when 50 ng/mL FGF-2 was added to the culture medium. Compared to culture day 1, a further progressive and significant ($P < 0.05$) reduction in the proportion of cultured primordial follicles associated with an increase in the proportion of growing follicles was observed at culture day 5 and in all media tested.

Table 1. Percentages of primordial and growing follicles (intermediate, primary and secondary) (mean ± S.D.) in non-cultured tissues (control) and in tissues cultured for 1 and 5 days in MEM^+ and MEM^+ supplemented with 10, 50 or 100 ng/mL FGF-2.

	Primordial follicles		Growing follicles	
Non-cultured (Day 0)	83.8 ± 6.0^{A}		16.2 ± 1.7^{B}	
Cultured	Day 1	Day 5	Day 1	Day 5
MEM^+	$77.0 \pm 7.4^{*\ A,a}$	$50.6 \pm 9.6^{*\ B,a}$	$23.0 \pm 2.7^{A,a}$	$49.4 \pm 4.3^{*\ B,a}$
FGF 10	$78.4 \pm 4.2^{*\ A,a}$	$48.3 \pm 6.9^{*\ B,a}$	$21.6 \pm 3.2^{A,a}$	$51.7 \pm 8.3^{*\ B,a}$
FGF 50	$60.4 \pm 2.6^{*\ A,a}$	$31.9 \pm 6.9^{*\ B,a}$	$39.6 \pm 8.3^{*A,b}$	$68.1 \pm 11.9^{*\ B,b}$
FGF 100	$67.5 \pm 9.0^{*\ A,a}$	$46.7 \pm 11.8^{*B,a}$	$32.5 \pm 5.0^{A,\ a}$	$50.3 \pm 8.3^{*\ B,a}$

* $P < 0.05$, significantly different from non-cultured ovarian cortical tissue (begin control/D0).

(A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$).

(a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

(30 follicles evaluated in each one of four replicates per treatment).

After 5 days of culture in medium containing 10 or 50 ng/mL of FGF-2, a significant increase in oocyte diameter was seen in unilaminar follicles when compared to non-cultured tissue (Table 2). Treatment with 50 ng/mL of FGF-2 also resulted in a significant ($P < 0.05$) increase in follicular diameter after 5 days of culture when compared to control. A significant ($P < 0.05$) increase in follicle diameter was observed in 50 ng/mL of FGF-2 with the increase of culture period from day 1 to 5. At day 5 of culture, a significantly higher follicular diameter was observed in the medium supplemented with 50 ng/mL of FGF-2 when compared to control and other treatments. Cultured and non-cultured tissue contained variable and relatively low (often zero) numbers of secondary follicles, which were not amenable to statistical comparison.

Table 2. Oocytes and unilaminar follicle diameters (mean ± S.D.) in non-cultured tissue (begin control) and in tissue cultured for 1 and 5 days in MEM^+ and MEM^+ supplemented with various concentrations of FGF-2.

	Oocyte diameter (μm)		Follicle diameter (μm)	
Non-cultured (Day 0)	36.1 ± 6.6		48.4 ± 7.6	
Cultured	Day 1	Day 5	Day 1	Day 5
MEM^+	38.0 ± 9.2 ^{A,a}	38.5 ± 9.4 ^{A,a}	51.0 ± 10.8 ^{A,a}	53.6 ± 11.6 ^{A,a}
FGF 10	39.1 ± 10.1 ^{A,a}	43.6 ± 11.2* ^{A,a}	51.1 ± 11.6 ^{A,a}	55.9 ± 11.8 ^{A,a}
FGF 50	37.8 ± 9.8 ^{A,a}	43.9 ± 11.6* ^{A,a}	52.4 ± 12.5 ^{A,a}	66.3 ± 12.9* ^{B,b}
FGF 100	36.1 ± 8.8 ^{A,a}	40.8 ± 10.4 ^{A,a}	50.4 ± 9.6 ^{A,a}	53.8 ± 11.6 ^{A,a}

* $P < 0.05$, significantly different from non-cultured ovarian cortical tissue (begin control)

(A, B) Different letters in the same row denote significant differences in oocyte or follicle diameter, between culture periods within a given media ($P < 0.05$).

(a, b) Different letters in the same column denote significant differences in oocyte or follicle diameter, between treatments in the same day ($P < 0.05$).

(20 follicles were measured in each treatment)

3.3. Ultrastructural analysis of goat preantral follicles after in vitro culture

Based on the data from our histological studies, we decided to perform TEM studies only on non-cultured follicles (begin controls) and follicles cultured for 5 days in MEM^+ and MEM^+ to which 50 ng/mL FGF-2 was added. On average, five primordial follicles per treatment were evaluated by ultrastructural analysis. Independent of the treatment and independent of the preantral follicle stage histologically normal follicles ultrastructurally showed an healthy oocyte, characterized by sparse vesicles spread throughout an homogeneous cytoplasm, which additionally contained numerous rounded mitochondria with peripheral cristae and continuous mitochondrial membranes, although there were occasional elongated forms with parallel cristae. Golgi complexes were rarely observed. Both smooth and rough endoplasmic reticulum were present, either as isolated aggregations or as complex associations with mitochondria and vesicles. Occasionally, small amounts of zona pelucida material were visible, depending on the plane of section (Fig. 3). The nucleus had uncondensed chromatin and the nucleolus could often be observed. In all preantral follicle stages, granulosa cells were small, with a greater nuclear-to-cytoplasm ratio as compared with typical cell structures. The nuclei were irregularly shaped, with loose chromatin in the inner part, and small peripheral aggregates of condensed chromatin. Well-developed rough endoplasmic reticulum and mitochondria with well-developed lamellar cristae were the most evident organelles observed in granulosa cells (Fig. 4). Many gap junctions were observed between granulosa cells. Furthermore, the oocytes were in contact with granulosa cells through gap junctions, while the follicular granulosa was surrounded by a basal membrane, which was tightly attached to the ovarian stoma. Table 3 shows differences between normal oocyte and granulosa cells ultrastructural features. After 5 days, in all ovarian cortical fragments cultured in FGF-2, an obvious/striking increase was observed in number and size of interfollicular stroma cells (Fig. 5).

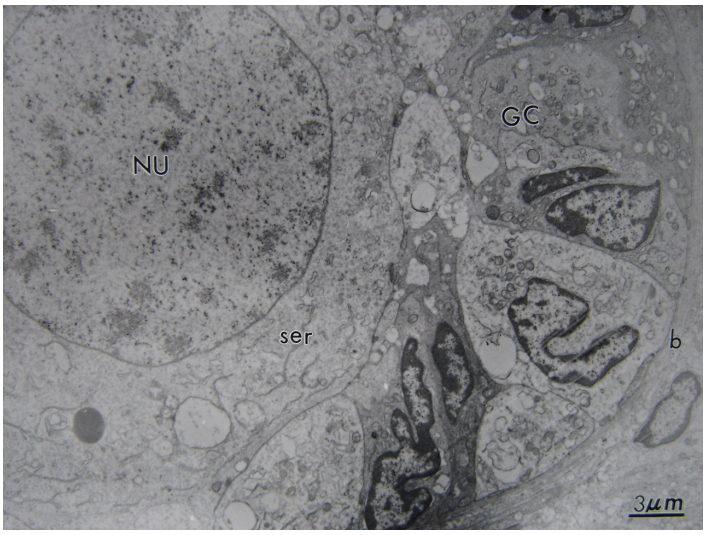

Figure. 3. Electron micrograph of a normal preantral follicle from non-cultured control (3250 x). NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; b: basement membrane.

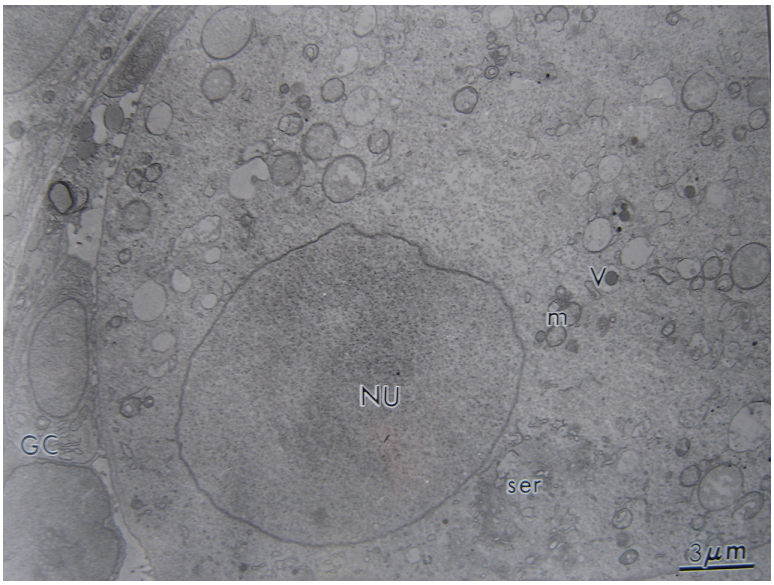

Figure. 4. Electron micrograph of a normal preantral follicle from MEM^{+} (cultured control) (4200 x). NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicles.

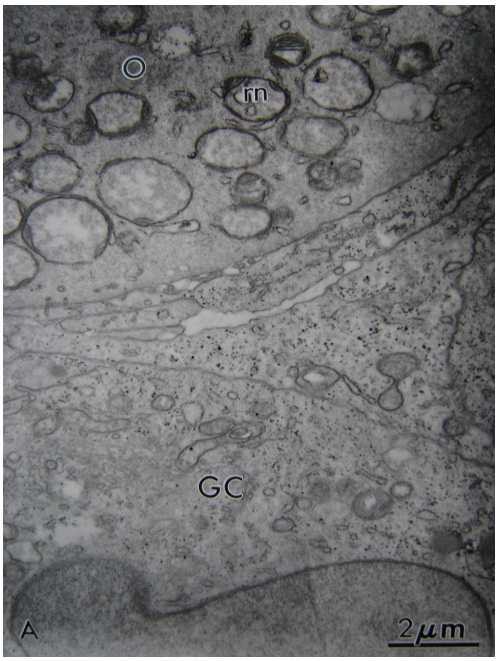

Figure. 5. Electron micrograph of a normal preantral follicle cultured for 5 days in FGF-2 (50 ng/mL) (6500 x). O: oocyte; GC: granulosa cells; m: mitochondria.

Table 3. Ultrastructural features of normal caprine preantral follicles after in vitro culture.

Oocyte	Granulosa cells
Sparse vesicles in the cytoplasm	Great nuclear-to-cytoplasm ratio
Numerous rounded mitochondria	Numerous rounded mitochondria
Golgi complexes rarely observed	Golgi complexes rarely observed
Smooth endoplasmic reticulum isolated or aggregated with mitochondria	Well-developed rough endoplasmic reticulum
Rough endoplasmic reticulum isolated or aggregated with mitochondria	Nucleus irregularly shaped
Uncondensed nucleus chromatin	Nucleus with uncondensed chromatin in the inner part
	Nucleus with peripheral aggregates of condensed chromatin

4. Discussion

The present study reported the importance of FGF-2 addition on the in vitro activation and growth of caprine preantral follicles after 5 days culture. Previous studies with different tissues have clearly established the ability of several tissue-specific growth factors, such as FGF-2, to suppress apoptosis, suggesting that this FGF may function as a survival factor during embryonic and postnatal development (Martin et al., 1991; Nunez et al., 1991). Consistent with the ability of diverse growth factors to block apoptosis in extragonadal tissues, treatment of rat granulosa cells or preovulatory follicles with FGF-2 (30 ng/mL) suppressed the spontaneous onset of apoptotic DNA fragmentation (Tilly et al., 1992). Recently, a study showed that FGF-2 at 50 ng/mL stimulated caprine oocyte survival after culture (Zhou & Zhang, 2005). This was, however not found in our study. Derrar et al. (2000) previously showed that at a concentration of 10 ng/mL, FGF-2 did not alter the survival of cultured bovine primary and secondary follicles. The different effects on follicle survival found, may be due to differences in the composition of the culture media used. For example, in contrast to Zhou & Zhang (2005), we did not include FSH and fetal calf serum in our media, which are known survival factors (Wright et al., 1999) and together with FGF-2 could have evoked a synergistic effect.

Despite the similar rates of histologically normal follicles observed after their culture in MEM^{+} either or not supplemented with FGF-2 in different concentrations, addition of 50 ng/ml of FGF-2 to the medium significantly increased the activation rate of preantral follicles after 1 and 5 days of culture. FGF-2 is expressed by the oocytes of primordial follicles and granulosa and theca cells of growing follicles in cows (Van Wezel et al., 1995; Yamamoto et al., 1997; Nilsson et al., 2001) and corpora lutea of cows (Schams et al., 1994) and rats (Asakai et al., 1993). Furthermore, FGF-2 receptors or their mRNAs have been demonstrated in growing follicles of cows (Wandji et al., 1992) and rats (Shikone et al., 1992; Asakai et al., 1993, 1995). FGF-2 is important in regulating a wide range of ovarian functions including granulosa cell mitosis (Roberts & Ellis, 1999), steroidogenesis (Vernon & Spicer, 1994), differentiation (Anderson & Lee, 1993) and apoptosis (Tilly et al., 1992). Similar to our results, FGF-2 (40 ng/mL) was efficient to stimulate primordial follicle activation in rats (Nilsson et al., 2001). These results suggest that FGF-2 plays a role in follicular activation. In

correspondence with our findings with caprine follicles, FGF-2 did not alter bovine primordial follicle activation at a concentration of 10 ng/mL (Derrar et al., 2000).

In the current study, compared to control values, both oocyte and follicular diameter had increased at day 1 of culture in the presence of 10 or 50 ng/mL FGF-2. After 5 days of culture, however, only the concentration of 50 ng/mL had significantly stimulated the growth of follicles. We infer that during long cultures only a concentration of 50 ng/mL is sufficient to stimulate the growth of follicles by increasing the oocyte diameter and/or the number or size of granulosa cells. In accordance with this, Wandji et al. (1996) showed that at 50 ng/mL FGF-2 increased the diameter and stimulate granulosa cell proliferation in 6 days cultured bovine follicles. Other authors also observed that FGF-2 promotes an increase in follicular diameter and proliferation of granulosa cells (bovine: Nuttinck et al., 1996; domestic cats: Jewgenow, 1996; chicken: Roberts & Ellis, 1999; rats: Nilsson et al., 2001). In addition, FGF-2 was found to stimulate the proliferation of cultured bovine theca and stroma cells (Nilsson et al., 2001). At a concentration of 50 ng/mL, FGF-2 appeared to increase DNA follicular synthesis in cultured mouse preantral follicles (Roy & Greenwald, 1991). These results indicate that FGF-2 may promote ovarian granulosa, stroma, and theca cell proliferation during the development of early-staged follicles (Nilsson et al., 2001). Our ultrastructural studies also showed a striking increase in number and size of interfollicular stroma cells in cultured ovarian cortical tissue. Therefore, we are of opinion that FGF-2 is not only able to directly effect early-staged follicle growth, but also to stimulate adjacent stroma cells to promote somatic cell growth as a preparation for future theca formation.

In the present study, preantral follicles cultured for 5 days in MEM^{+} or MEM^{+} supplemented with FGF (50 ng/mL) appeared ultrastructurally normal, which confirmed our impression, based on routine histological studies, that these follicles were healthy. Several authors have emphasized the importance of TEM after in vitro culture of preantral follicles, since it gives close insight into the ultrastructural characteristics of follicles, allowing a better evaluation of their quality (Van den Hurk et al., 1998; Salehnia et al., 2002). Eppig (1977) for example showed that mouse oocytes cultured in vitro for 4 days were normal at the light microscopic level, but ultrastructurally exhibited large numbers of swollen mitochondria. Furthermore, TEM studies revealed detailed differences in ultrastructural quality of rat preantral follicles cultured for 6 days in serum-containing media when compred with those in serum-free medium (Zhao et al., 2000).

Our findings also showed spontaneous activation of cultured follicles in MEM^{+}. Such activation was previously observed by many authors (caprine: (Silva et al., 2004); bovine: (Wandji et al., 1996; Braw-Tal & Yossefi, 1997; Cushman et al., 2002; Fortune et al., 2000]; baboon: (Wandji et al., 1997; Fortune et al., 1998); humans: (Hovatta et al., 1997). The mechanisms that regulate the primordial to developing follicles transition are not clear. It could be that added insulin is a cause, as has been indicated for the cow (Yang & Fortune, 2002), rat (Kezele et al., 2002) and human (Louhio et al., 2000). Fortune et al. (2000) suggested that an inhibitor of medullary origin regulates follicles activation in vivo and therefore separation of the cortex from the medulla may cause primordial follicles activation in vitro. However, when bovine ovarian cortical pieces were cultured in the presence of medullary tissue, follicle activation was not inhibited (Derrar et al., 2000). Another hypothesis is that in vitro culture appears to induce activation of primordial follicles because the media are richer in nutrients and/or oxygen than ovarian cortex in vivo, since the cortical region of the ovary is poorly vascularized (Van Wezel & Rodgers, 1996). These environmental changes may directly stimulate follicular development or indirectly through the release of intrafollicular stimulatory factors such as bone morphogenetic protein-7, growth differentiation factor-9 and FGF-2 (Fortune, 2003; van den Hurk & Zhao, 2005) or diminish the release of inhibitory factors, such as anti-Müllerian hormone (Durlinger et al., 1999) produced by stroma cells and the larger preantral follicles present in the cultured ovarian tissue.

In conclusion, this study with caprine follicles showed that, in contrast to concentrations of 0, 10 and 100 ng/mL, a concentration of 50 ng/mL FGF-2 is able to promote the activation of primordial follicles and growth of activated preantral follicles that were 5 days in vitro cultured. Although the culture procedure itself resulted in lower percentages of morphologically normal preantral follicles, histological and ultrastructural analyses revealed that follicle survival is not influenced by FGF-2 treatment.

Acknowledgements

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Capítulo IV

Papel essencial do Hormônio Folículo Estimulante na manutenção da viabilidade in vitro de folículos pré-antrais caprinos

Resumo

Os objetivos deste trabalho foram investigar os efeitos do Hormônio Folículo Estimulante (FSH) sobre a sobrevivência, a ativação e o crescimento de folículos primordiais caprinos utilizando estudos histológicos e ultra-estruturais. Fragmentos de córtex ovariano caprinos foram cultivados por 1 ou 7 dias em Meio Essencial Mínimo (MEM – meio controle) suplementado com diferentes concentrações de FSH (0, 10, 50 ou 100 ng/mL). Pequenos fragmentos de tecido ovariano não cultivados e aqueles cultivados por 1 ou 7 dias em um meio específico foram processados para histologia clássica e microscopia eletrônica de transmissão (MET). Além disso, foram avaliados os efeitos do FSH sobre os diâmetros oocitário e folicular de folículos cultivados. Os resultados mostraram que as menores percentagens de folículos normais foram observadas após 7 dias de cultivo no meio controle. Após 1 dia de cultivo, a maior percentagem de folículos em crescimento foi observada no meio suplementado com 50 ng/mL de FSH. Na presença de 10 ou 50 ng/mL de FSH, foi observado um aumento tanto no diâmetro oocitário como folicular no dia 7 de cultivo. A MET mostrou a integridade ultra-estrutural de folículos após 1 dia de cultivo em MEM e após 7 dias em MEM acrescido de 50 ng/mL de FSH, mas, não confirmou a integridade daqueles folículos cultivados por 7 dias em MEM. Em conclusão, este estudo demonstrou que o FSH, na concentração de 50 ng/mL, mantém a integridade morfológica de folículos pré-antrais caprinos cultivado por 7 dias, bem como estimula a ativação de folículos primordiais e o crescimento de folículos ativados.

Capítulo IV

Essential role of Follicle Stimulating Hormone in the maintenance of caprine preantral follicle viability in vitro

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(Inglaterra)

Abstract

The aims of the present study were to investigate the effects of follicle stimulating hormone (FSH) on survival, activation and growth of caprine primordial follicles using histological and ultrastructural studies. Pieces of caprine ovarian cortex were cultured for 1 or 7 days in Minimum Essential Medium (MEM - control medium) supplemented with different concentrations of FSH (0, 10, 50 or 100 ng/mL). Small fragments from non-cultured ovarian tissue and from those cultured for 1 or 7 days in a specific medium were processed for classical histology and transmission electron microscopy (TEM). Additionally, effects of FSH on oocyte and follicle diameter of cultured follicles were evaluated. The results showed that the lowest percentage of normal follicles was observed after 7 days of culture in control medium. After 1 day of culture, a higher percentage of growing follicles was observed in the medium supplemented with 50 ng/mL of FSH. In the presence of 10 and 50 ng/mL of FSH, an increase in diameter of both oocyte and follicle on day 7 of culture was observed. TEM showed ultrastructural integrity of follicles after 1 day of culture in MEM and after 7 days in MEM plus 50 ng/mL FSH, but did not confirm the integrity of those follicles cultured for 7 days in MEM. In conclusion, this study demonstrated that FSH at concentration of 50 ng/mL not only maintains the morphological integrity of 7 days cultured caprine preantral follicles, but also stimulate the activation of primordial follicles and the growth of activated follicles.

Keywords: Caprine, Primordial Follicles, Culture, FSH, Activation.

1. Introduction

Culture systems for primordial follicles are important for studying their oocyte development, especially because these follicles are a large potential source of oocytes that could be used in vitro for embryo production. The factors that control primordial follicle activation and further growth of primary follicles are not well understood. Since the cortical region where the primordial follicles are located is poorly vascularized, the development of these follicles is probably regulated by locally produced growth factors.

Endocrine hormones, like FSH, are known to regulate the production of several growth factors that play a critical role in primordial follicle activation and growth. FSH acts by binding to its receptor expressed on granulosa cells (Ulloa-Aguirre et al., 1995;

O'Shaughnessy et al., 1996) and more recent reports indicate its presence in oocytes, suggesting additional sites of action in the ovary (Meduri et al., 2002). Although FSH receptors are expressed from the primary follicles onward (Oktay et al., 1997), FSH may play an indirect effect on very early follicle development via factors released by larger follicles or ovarian stromal cells. For example, FSH promotes proliferation of granulosa cells via paracrine factors such as IGF-1 and activin (van den Hurk & Zhao, 2005). In addition, FSH regulates expression of kit ligand (KL), growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) in murine follicles (Joyce et al., 1999, Thomas et al., 2005). These factors have been implicated in the activation of primordial follicles (van den Hurk & Zhao, 2005).

Studies in vitro have demonstrated that addition of FSH to culture media promotes preantral follicular growth and antrum formation in many species (mouse: Spears et al., 1998; murine: McGee et al., 1997; human: Wright et al., 1999; bovine: Gutierrez et al., 2000; ovine: Cecconi et al., 1999; Zhou & Zhang, 2005; suine: Mao et al., 2002). Moreover, it is known that FSH inhibits apoptosis in preantral follicles cultured in vitro (mouse: Baker et al., 1997; rat: McGee et al., 1997; human: Roy & Treacy, 1993). In goats, Silva et al. (2004) demonstrated that FSH, at concentration of 100 ng/mL, increased follicle and oocyte diameters after 5 days culture, but no effect of FSH on both primordial to primary follicle transition and viability was observed. Clearly, FSH is known to be the main regulator of follicle development in vivo and in vitro, but a specific role or the possibility of dose-dependent action of FSH in promoting primordial to primary follicle transition and growth have not been tested, being essential to investigate whether FSH play a role in this step of folliculogenesis. In addition, most of the studies investigating primordial follicle activation are based on histological evaluation, being very important to use ultrastructural analysis to confirm follicular viability.

Studies with goats are important to improve our knowledge about the factors that control early folliculogenesis in mammals and to explore possible physiological differences among species. Goats are present on all continents and are commercially seen as highly attractive livestock, since they constitute an important source of products such as meat, milk, fibre and skin. Thus, the aim of the present study was to investigate whether FSH has a beneficial role in the survival, activation and further growth of in vitro cultured goat primordial follicles enclosed in ovarian cortex. To this end, both histological and ultrastructural studies were performed to investigate and compare the morphology of follicles

before and after culture for 1 or 7 days in the absence or presence of FSH at different concentrations (0, 10, 50 or 100 ng/ml).

2. Materials and Methods

2.1. Source of ovaries

Ovaries (n=10) from five adult non-pregnant mixed-breed goats (1-3 years of age) were collected at a local slaughterhouse. The animals were cyclic and in good body condition. Then, the ovaries were washed and transported in 0.9% saline solution to the laboratory in thermo flasks with water at 32°C.

2.2. Experimental protocol

At the laboratory, both ovaries from each animal were stripped of surrounded fat tissue and ligaments, and cut in half, where after the medulla, large antral follicles and corpora lutea were removed. Following this, the ovarian cortex was divided into 11 fragments of approximately 3 x 3 mm (1 mm thick). One fragment was immediately fixed for classic histological studies (non-cultured controls) while a smaller fragment (1 mm^3) was randomly collected and subsequently fixed for ultrastructural examination. The other fragments of ovarian cortex were individually in vitro cultured in 1 mL of culture medium for 1 or 7 days at 39°C with 5% CO_2 in air using a 24-well culture dish. The control medium was Minimum Essential Medium (Cultilab, Rio de Janeiro, Brazil) supplemented with ITS (insulin 6.25 µg/mL, transferrin 6.25 µg/mL, and selenium 6.25 ng/mL), 0.23 mM pyruvate; 2 mM glutamine; 2 mM hypoxanthine; 1.25 mg/mL BSA, 100 µg/mL penicillin, 100 µg/mL streptomycin (Vetec, Rio de Janeiro, Brazil) and 0.25 µg/mL fungizone (MEM^+). This control medium (MEM^+) was supplemented with different concentrations of porcine FSH (10, 50 or 100 ng/mL - provided by Dr. J.F. Beckers, Liège, Belgium). All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Every two days, the culture medium was replaced by fresh medium. Each treatment was repeated five times, thus using the ovaries of five different animals.

2.2. Histological analysis and assessment of in vitro follicle growth

To evaluate the morphology of caprine follicles after 1 or 7 days of culture, a small part (1 mm^3) from each fragment was randomly removed for TEM studies, while the remainder was fixed in Carnoy for 12 h for histological studies. After fixation, the tissue fragments were dehydrated in a graded series of ethanol, clarified with xylene and embedded in paraffin wax. For each piece of ovarian cortex, 7μm sections were mounted on slides, stained with periodic acid Schiff and hematoxylin (PAS staining system, Sigma, Inc., St. Louis, MO, USA), and examined by light microscopy (Zeiss, Germany) at 100X and 400X magnification.

The follicles were classified as described by Hulshof et al. (1994) in primordial (one layer of flattened granulosa cells around the oocyte), or growing follicles i.e., intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). These follicles were classified individually as histologically normal when an intact oocyte was present, i.e. an oocyte without a pyknotic nucleus, surrounded by granulosa cells which are well organized in one or more layers and that have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which have a pyknotic nucleus, and/or are surrounded by disorganized granulosa cells, which are detached from the basement membrane. From each medium and each culture period, approximately 150 follicles were randomly evaluated.

To evaluate follicular activation and growth, only intact follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and growing follicles were calculated at day 0 (controls) and after 1 or 7 days of culture in the various media tested. Major and minor axes of each oocyte and follicle were measured with the aid of an ocular micrometer. The averages of the minor and major axes were reported as oocyte and follicle diameters, respectively. These values were used to assess the effect of the hormonal treatment on follicular growth.

2.3. Ultrastructural analysis

For ultrastructural analysis, small pieces of ovarian cortex were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer, pH 7.2. After

washing the ovarian pieces with sodium cacodylate buffer, they were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently, samples were dehydrated in a graded series of acetone and embedded in Spurr´s epoxy resin. Firstly, semi-thin sections (3 μm) were cut on an ultramicrotome (Reichert Supernova, German) for light microscopy studies and stained with toluidine blue. Subsequently, follicles classified as histologically normal in semi-thin toluidin blue stained sections were submitted to ultrastructural analysis. For that purpose, thin sections (70 nm) were cut and then contrasted with uranyl acetate and lead citrate, and examined using a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope, operating at 80 kV.

2.4. Statistical analysis

Data are expressed as mean ± SEMs. The percentages of surviving follicles at all stages, primordial and growing follicles obtained after 1 or 7 days in the various treatments were subjected to arc-sin transformation before analysis of variance (ANOVA). The data as well as the diameter of oocytes and follicles were analyzed by ANOVA followed by Fisher's protected least significant difference test (PLSD test) (StatView for Windows, SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effect of FSH and culture periods on follicle survival

Figure 1 shows the effect of different concentrations of FSH on follicle survival, i.e. the percentage of histologically normal follicles in ovarian tissues after 1 and 7 days of culture. When compared to non-cultured follicles, a significant decrease ($P < 0.05$) in the percentages of histologically normal follicles (Fig. 2) was observed after 1 and 7 days culture, but no significant effect of FSH on follicle survival was observed. With the increase of the culture period from 1 to 7 days, a significant ($P < 0.05$) decrease in the percentage of normal follicles was observed in control medium (MEM^+), but not in medium with FSH (Fig. 1).

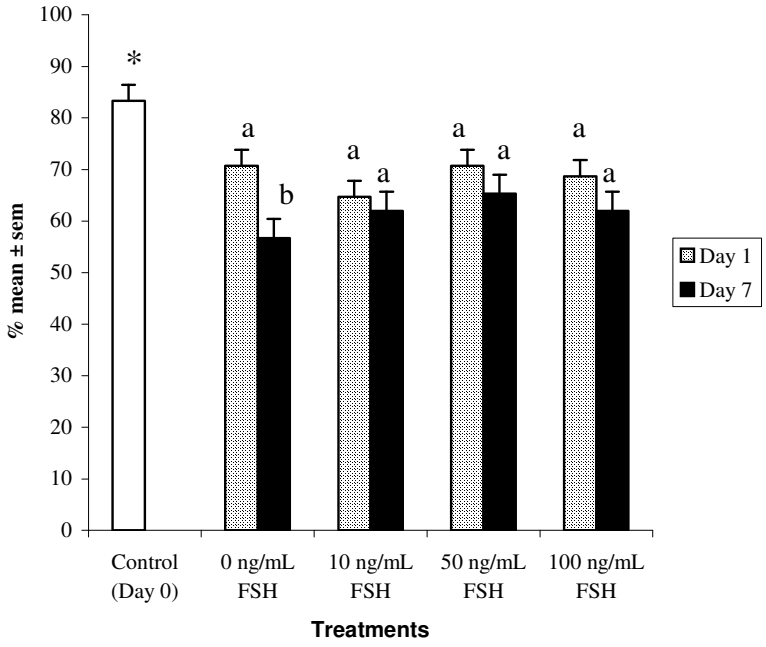

Figure 1. Percentages (means ± S.E.M) of histologically normal preantral follicles in non-cultured tissue (control) and in tissue cultured for 1 and 7 days in MEM^{+} and MEM^{+} supplemented with 10, 50 and 100 ng/mL FSH. (30 follicles evaluated in each one of five replicates per treatment). * $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/D0). (a, b) Different letters denote significant differences between culture periods within the same medium ($P < 0.05$).

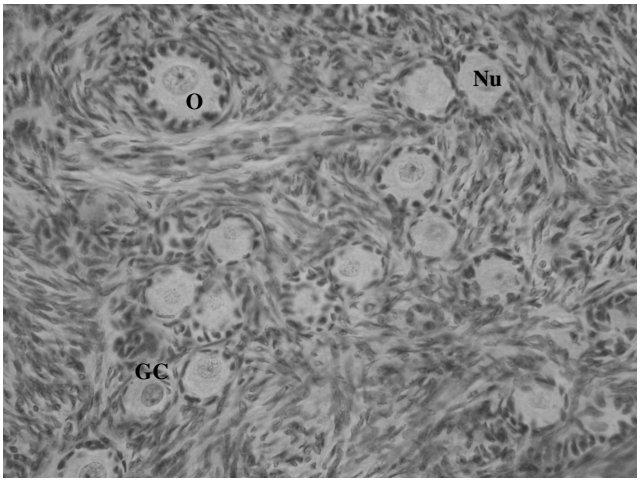

Figure 2. Histological section of non-cultured tissue after staining with periodic acid Schiff-hematoxylin, showing normal primordial and primary follicles. O: oocyte; NU: oocyte nucleus; GC: granulosa cells (400 x).

3.2. Goat primordial follicle activation and growth during in vitro culture

The percentages of primordial and growing follicles in ovarian cortical tissue before and after 1 and 7 days of culture are shown in Table 1. The percentages of primordial and growing follicles in non-cultured cortex were 81.7 % and 18.3 %, respectively. After 1 day of culture, when compared to non-cultured follicles, a reduction ($P<0.05$) of primordial follicles concomitant with a significant increase of growing follicles was observed in all treatments, except when 10 ng/mL FSH was added to the culture medium. Compared to day 1, after 7 days culture, a strong reduction of primordial follicles ($P < 0.05$) was observed, except in tissues cultured in MEM plus 50 ng/mL FSH. Cortical tissues cultured with 10 or 100 ng/mL of FSH had a significant increase ($P < 0.05$) in the percentage of growing follicles after 7 days of culture when compared to day 1. At day 1 of culture, addition of 50 ng/mL of FSH to control medium significantly decreased ($P < 0.05$) the percentage of primordial follicles when compared to non-cultured tissue and other treatments. Furthermore, addition of 50 ng/mL FSH increased significantly ($P < 0.05$) the percentage of growing follicles when compared to other treatments, except when 100 ng/mL was used.

Table 1. Percentages (mean ± S.E.M.) of primordial and growing follicles (intermediate, primary and secondary) in non-cultured tissues and in tissues cultured for 1 or 7 days in MEM^+ (control medium) and MEM^+ supplemented with various concentrations of FSH. Per treatment 150 follicles were evaluated.

	Primordial follicles		Growing follicles	
Non-cultured (Day 0)	81.7 ± 1.2^{A}		18.3 ± 1.2^{B}	
Cultured	Day 1	Day 7	Day 1	Day 7
MEM^+	$71.7 \pm 8.7^{*\,A,a}$	$49.4 \pm 5.0^{*\,B,a}$	$28.3 \pm 4.6^{*\,A,a}$	$50.6 \pm 4.8^{*\,A,a}$
FSH 10	$81.5 \pm 2.7^{A,a}$	$45.2 \pm 5.0^{*\,B,a}$	$18.5 \pm 3.7^{A,a}$	$54.8 \pm 4.4^{*\,B,a}$
FSH 50	$45.3 \pm 4.7^{*\,A,b}$	$49.0 \pm 2.7^{*\,A,a}$	$54.7 \pm 6.2^{*A,b}$	$51.0 \pm 2.8^{*\,A,a}$
FSH 100	$62.2 \pm 2.7^{*\,A,a}$	$38.7 \pm 1.6^{*\,B,a}$	$37.8 \pm 2.9^{*\,A,a,b}$	$61.3 \pm 2.3^{*B,a}$

* $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/Day 0). (A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$). (a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

After 7 days of culture in medium containing 10, 50 or 100 ng/mL of FSH, a significant increase in oocyte diameter was seen in unilaminar follicles, i.e., primordial, intermediate and primary follicles together, when compared to non-cultured tissue (Table 2; $P < 0.05$). In addition, the presence of 10 or 50 ng/mL of FSH promoted a significant ($P < 0.05$) increase in follicular diameter after 7 days of culture when compared to control. A significant increase in follicle diameter was observed with the increase of culture period from day 1 to 7 only in tissues cultured with 50 ng/mL of FSH. At day 7, tissue cultured in presence of 50 ng/mL FSH had the highest follicle diameter ($P < 0.05$). Cultured and non-cultured tissue contained variable and relatively low (often zero) numbers of secondary follicles, which were not amenable to statistical comparison.

Table 2. Oocyte and follicle diameters (mean ± S.E.M.) in non-cultured tissues and in tissues cultured for 1 or 7days in MEM^+ (control medium) and MEM^+ supplemented with various concentrations of FSH. Per treatment 150 follicles were evaluated.

	Oocyte diameter (μm)		Follicle diameter (μm)	
Non-cultured (Day 0)	40.7 ± 2.2		52.2 ± 2.2	
Cultured	Day 1	Day 7	Day 1	Day 7
MEM^+	38.6 ± 1.9 ^{A,a}	39.6 ± 2.0 ^{A,a}	51.6 ± 2.1 ^{A,a}	53.0 ± 2.2 ^{A,a}
FSH 10	40.9 ± 2.3 ^{A,a}	44.0 ± 2.3* ^{A,a}	53.1 ± 2.6 ^{A,a}	59.3 ± 3.4* ^{A,a}
FSH 50	39.7 ± 2.3 ^{A,a}	44.3 ± 3.2* ^{A,a}	54.4 ± 2.9 ^{A,a}	66.6 ± 6.3* ^{B,b}
FSH 100	36.9 ± 2.3 ^{A,a}	41.3 ± 2.1* ^{A,a}	51.0 ± 2.5 ^{A,a}	54.9 ± 2.3 ^{A,a}

* $P < 0.05$, significantly different from non-cultured ovarian cortical tissues (control/Day 0)
(A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$). (a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

3.3. Ultrastructural analysis of goat preantral follicles

Based on histological results, TEM studies were performed in non-cultured follicles

(control) and in follicles cultured for 1 and 7 days in MEM^+ (control) or MEM^+ plus 50 ng/mL FSH. The ultrastructural characteristics of follicles from non-cultured tissue and those cultured in medium without or with 50 ng/mL FSH for 1 day appeared similar, but after 7 days only tissues cultured with FSH had normal follicles. These follicles exhibited sparse vesicles spread throughout the ooplasm. The homogeneous cytoplasm furthermore contained numerous rounded mitochondria with peripheral cristae and continuous mitochondrial membranes, although there were occasional elongated forms with parallel cristae (Fig. 3). Golgi complexes were rarely observed. Both smooth and rough endoplasmic reticulum were present, either as isolated aggregations or as complex associations with mitochondria and vesicles (Fig. 4). The oocyte nucleus had uncondensed chromatin and the nucleolus could generally be observed. In all developmental stages, granulosa cells were small, with a greater nuclear-to-cytoplasm ratio as compared with typical cell structures. The nuclei were irregularly shaped, with loose chromatin in the inner part, and small peripheral aggregates of condensed chromatin. Well-developed rough endoplasmic reticulum and mitochondria with well-developed lamellar cristae were the most evident organelles observed in granulosa cells. Gap junctions were abundantly observed between granulosa cells as well as between granulosa cells and the oocyte (Fig. 5).

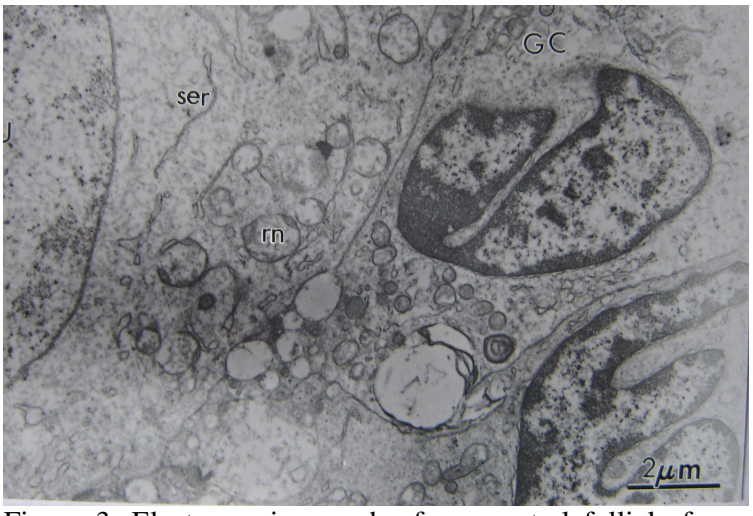

Figure 3. Electron micrograph of a preantral follicle from a non-cultured control ovarian fragment. Note the homogeneous cytoplasm with numerous rounded mitochondria. NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum (6600 x).

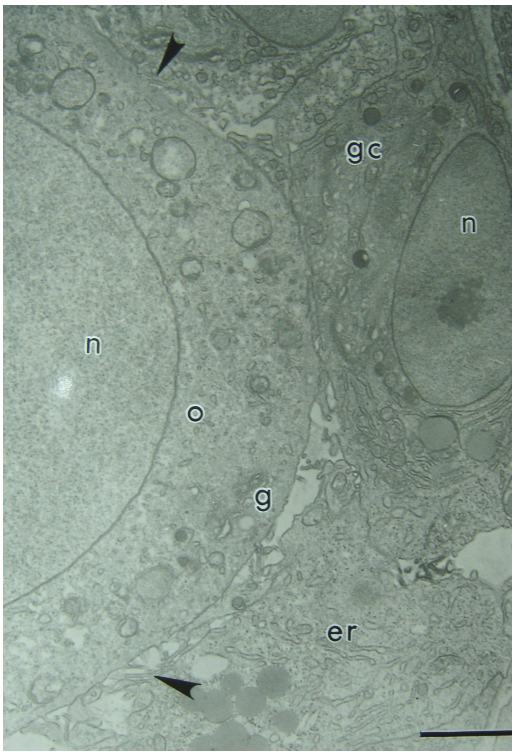

Figure 4. Electron micrograph of a preantral follicle cultured for 1 day in MEM^{+} (cultured control). Note the great nuclear-to-cytoplasm ratio in granulosa cells. n: nucleus; o; oocyte; g: Golgi complex; gc: granulosa cells; er: endoplasmic reticulum; arrowhead: microvillus (4000 x). Bar: 3 μm.

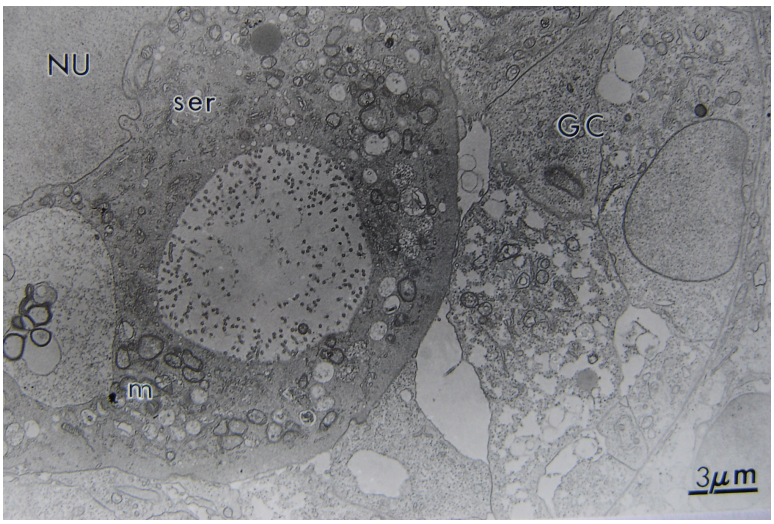

Figure 5. Electron micrograph of a preantral follicle cultured in FSH (50 ng/mL) for 7 days. Note the smooth endoplasmic reticulum and mitochondria, which were the most evident organelles observed in ooplasm. NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicle (3300 x).

Cortical tissues cultured in control medium for 7 days had histologically normal follicles, but TEM studies revealed some changes in their ultrastructure, which are indicative of degeneration. Such follicles showed an oocyte extremely vacuolated, with the vacuoles often being fused and producing a larger vacuolated area. Organelles were more randomly distributed throughout the cytoplasm and signs of endoplasmic reticulum proliferation and damage to mitochondrial membranes and cristae were observed. The oocyte nucleus appeared misshapen and retracted, and had a wavy membrane. Granulosa cells look swollen, while the density of cytoplasmic organelles was low. Frequently, the connection between the oocyte and granulosa cells had disappeared, while organelles were no longer identifiable. Furthermore, granulosa cells showed less contact with each other and exhibited obvious fewer gap junctions (Fig. 6).

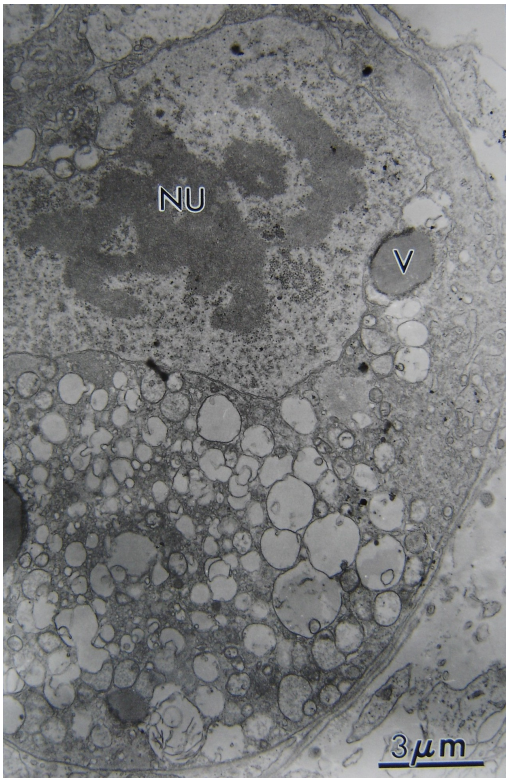

Figure 6. Electron micrograph of a preantral follicle cultured for 7 days in MEM^{+} (cultured control) that were scored as histologically normal at the LM level. Note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration. NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; v: vesicles (3900 x).

4. Discussion

The present study demonstrated the importance of FSH on in vitro activation and growth of caprine primordial follicles in a 7 days culture system. Although very little is known about the regulation of primordial follicle development, FSH seems to be a very effective factor in maintaining follicle viability (Ralph et al., 1996; Wandji et al., 1996; Saha et al., 2000). However, Silva et al. (2004) did not observed a significant effect of FSH on follicle survival after 5 days culture, probably because a high concentration of FSH (100 ng/mL) was used. In our study, addition of 50 ng/mL of FSH to culture medium was very important to maintain the percentage of normal follicles after 7 days culture. Hsueh et al. (1994) and Chun et al. (1994) suggested that the diffusion of several essential chemical and physical factors through the basal membrane could be compromised in the absence of FSH. In addition, cultures without FSH more frequently resulted in extrusion of the oocyte from its original follicular structure (Cortvrindt et al., 1997), which may be caused either by damage or reduction in number of gap junctions (Amsterdam & Rotmensch, 1987; Hsueh et al.; 1994). In addition, FSH inhibited apoptosis in preantral follicles cultured in vitro (mouse: Baker & Spears, 1997; rat: McGee et al., 1997; human: Roy & Treacy, 1993; Wright et al., 1999; suine: Mao et al., 2002).

Addition of 50 ng/ml of FSH to the medium increased the activation rate of primordial follicles as early as day 1 of culture when compared to other treatments. However, after 7 days of culture, follicular activation was similar among all treatments. Ovarian follicular development is known to proceed to primordial and primary stages independently of the action of FSH. This has been observed in mice carrying invalidations of the FSHβ and FSHR genes (Kumar et al., 1997; Dierich et al., 1998) and also in patients with mutations suppressing the function of the FSHR (Beau et al., 1998; Touraine et al., 1999). FSH binds to its receptor expressed on granulosa cells (Ulloa-Aguirre et al., 1995; O'Shaughnessy et al., 1996) and oocytes (Meduri et al., 2002) from the primary follicles onward (Oktay et al., 1997). Recently, after culture of caprine ovarian cortical tissue for 5 days, Silva et al. (2004) showed that FSH (100 ng/mL) did not promote activation of caprine primordial follicles. Previous studies have confirmed that FSH at this concentration did not influence bovine follicular activation (Fortune et al., 1998; Braw-Tal & Yossefi, 1997; Derrar et al., 2000). On the other hand, we demonstrated that a lower concentration of FSH (50 ng/mL) can improve follicular activation and survival. In addition, Joyce et al. (1999) reported that FSH stimulates

Kit ligand mRNA expression in granulosa cells of preantral follicles. Kit ligand (KL) has been shown to be essential for oocyte growth (Nilsson & Skinner, 2001; Eppig, 2001) and primordial follicle activation (Parrot & Skinne, 1999). FSH can also modulate the levels of BMP-15 and GDF-9 in growing follicles (Thomas et al., 2005) and these growth factors are essential for primordial and primary follicle development in mice (Dong et al, 1996) and sheep (Galloway et al., 2000).

In the current study, follicular diameter had the highest increase when cultured in presence of 50 ng/mL for 7 days. Itoh et al. (2002) also demonstrated that 50 ng/mL FSH increased both oocyte and follicular diameters in 13 days cultured bovine follicles. FSH receptor expression has been reported to develop progressively during the transition from primordial to primary to secondary follicle (Oktay et al., 1997). The presence of FSH receptors in these early follicles presumably explains the effect of FSH on oocyte growth in preantral follicles. Since there are FSH receptors in oocytes, it is possible that FSH must act in both cell types to promote follicular growth and development (Méduri et al., 2002). Furthermore, a two-way exchange may occur between the oocyte and granulosa cells and a direct action of FSH on oocytes produces compounds whose diffusion into the granulosa cells is necessary for their proliferation and maturation (Méduri et al., 2002). Other authors observed that FSH promotes an increase in follicular diameter and proliferation of granulosa cells (rats: McGee et al., 1997; mouse: Nayudu and Osborn, 1992; Cortvrindt *et al*., 1996, 1997, 1998; bovine: Wandji et al., 1996; Saha et al., 2000; caprine: Silva et al., 2004; ovine: Cecconi et al., 1999; human: Roy & Treacy, 1993; Wright et al., 1999). In addition, FSH was found to stimulate antrum formation and steroidogenesis in granulosa cells (Boland et al., 1993; Nayudu and Osborn, 1992; Ralph et al., 1995, 1996; Wandji et al., 1996; Gutierrez et al., 2000; Wu et al., 2000; Mao et al., 2002; Abir et al. 1997; Mitchell et al., 2002; Kreeger et al., 2005). Adriaens et al. (2004) reported that omission of FSH during the early preantral stage tends to compromise a maximal oocyte developmental competence.

Several authors have emphasized the importance of TEM after in vitro culture of preantral follicles, since it gives close insight into the ultrastructural characteristics of follicles, allowing a better evaluation of their quality (Van den Hurk et al., 1998; Zhao et al. 2000; Salehnia et al., 2002). In the present study, preantral follicles cultured for 1 day in MEM without or with FSH (50 ng/mL) respectively, appeared ultrastructurally normal, which confirmed the results obtained in the histological studies. However, after 7 days culture, TEM studies revealed differences in ultrastructural quality of follicles cultured without or with FSH

(50 ng/mL). Although they had comparable histological morphology, follicles cultured in the presence FSH maintained their ultrastructural integrity, while those cultured without FSH showed various signs of initial degeneration after 7-day culture, and exhibited more clear degenerative features, like ooplasm vacuolization. Cytoplasmic vacuoles are characteristic signs of degeneration in oocytes (Silva et al., 2000), granulosa (Hay et al., 1976) and cumulus cells (Assey et al., 1994) and may represent endoplasmic reticulum swelling (Tassel & Kennedy, 1980) or altered mitochondrial structure (Fuku et al., 1995). In goat preantral follicles, mitochondria showing extensive swelling and disappearance of their cristae, and endoplasmic reticulum that have increased in volume, were previously indicated as the first signs of degeneration (Silva et al., 2001).

In conclusion, this study with caprine follicles showed that a concentration of 50 ng/mL FSH is able to promote the activation of primordial follicles and growth of activated preantral follicles. Furthermore, these data support the vital role of FSH in maintaining healthy oocyte growth and follicular ultrastructure after 7 days culture. This culture system should be useful for studying the regulation of early follicular growth and development, especially because these follicles represent a large source of oocytes that could be used in vitro for embryo production.

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Capítulo V

Hormônio Folículo Estimulante e Fator de Crescimento Fibroblástico-2 interagem e promovem o desenvolvimento *in vitro* de folículos primordiais caprinos

Resumo

Os objetivos do presente trabalho foram investigar os efeitos da interação entre o Hormônio Folículo Estimulante (FSH) e o Fator de Crescimento Fibroblástico-2 (FGF-2) sobre a sobrevivência, o crescimento folicular inicial e o posterior crescimento de folículos pré-antrais caprinos. Fragmentos de córtex ovariano caprino foram cultivados por 1 ou 7 dias em Meio Essencial Mínimo (MEM) suplementado com FSH, FGF-2 ou FSH + FGF-2. Pequenos fragmentos de tecido ovariano não cultivado e aqueles cultivados por 1 ou 7 dias foram processados para histologia clássica e microscopia eletrônica de transmissão (MET) para verificar a morfologia e o crescimento folicular. Os resultados mostraram que, após 7 dias de cultivo, as maiores percentagens de folículos normais foram observadas em meio suplementado com FSH. Após 7 dias de cultivo, a interação entre FSH e FGF-2 foi mais eficiente para promover a ativação folicular e o crescimento oocitário. A MET confirmou a integridade ultra-estrutural dos folículos após 1 dia de cultivo em MEM e após 7 dias em todos os tratamentos, exceto naqueles folículos cultivados por 7 dias em MEM. Em conclusão, este estudo demonstrou que a interação entre FSH e FGF-2 estimulou a ativação de folículos primordiais e o posterior crescimento de folículos em desenvolvimento. Além disso, estes dados mostraram que o FSH é importante para manter a integridade folicular após 7 dias de cultivo in vitro.

Capítulo IV

Follicle Stimulating Hormone and Fibroblast Growth Factor-2 interact and promote goat primordial follicle development in vitro

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(Austrália)

Abstract

The aims of the present study were to investigate the effects of the interaction between follicle stimulating hormone (FSH) and fibroblast growth factor-2 (FGF-2) on survival, follicular growth initiation and further growth of caprine preantral follicles. Pieces of caprine ovarian cortex were cultured for 1 or 7 days in Minimum Essential Medium (MEM) supplemented with FSH, FGF-2 or FSH + FGF-2. Small fragments from non-cultured ovarian tissue and from those cultured for 1 or 7 days were processed for classical histology and transmission electron microscopy (TEM) to verify follicular morphology and growth. The results showed that, after 7 days culture, the highest percentages of normal follicles were observed in medium supplemented with FSH. After 7 days culture, the interaction between FSH and FGF-2 was most effective to promote the initiation of primordial follicles growth and oocyte growth. TEM showed ultrastructural integrity of follicles after 1 day of culture in MEM and after 7 days in all treatments, except in those follicles cultured for 7 days in MEM. In conclusion, this study demonstrated that the interaction between FSH and FGF-2 stimulate the initiation of primordial follicles growth and the subsequent growth of developing follicles. Furthermore, these data showed that FSH is important to maintain follicular integrity after 7 days culture.

Keywords: Caprine, Primordial Follicles, Culture, FSH, FGF-2.

1. Introduction

Understanding the signals that initiate and control growth of preantral follicles, which include primordial, primary and secondary follicles, would be an important step toward developing a successful in vitro maturation system for primordial follicles. The factors and mechanisms involved in this process are yet to be well defined. Irrespective of gonadotrophin involvement, there is good evidence that intra- and extraovarian factors are implicated in this process (Fortune, 2003).

Follicle stimulating hormone (FSH) is known to regulate the expression of several growth factors, such as kit ligand (KL), which play a critical role in follicular growth initiation (Thomas et al., 2005). FSH acts by binding to its receptor expressed on granulosa cells (O'Shaughnessy et al., 1996) and oocytes (Méduri et al., 2002). Studies in vitro have

demonstrated that addition of FSH to culture media promotes preantral follicular growth and antrum formation in many species (for review, see Van den Hurk and Zhao, 2005). Moreover, it is known that FSH inhibits apoptosis in rat preantral follicles cultured in vitro (McGee et al., 1997). Recently, Matos et al. (2007a) demonstrated that FSH is essential to maintain the morphological integrity of 7 days cultured caprine primordial follicles.

One factor that is involved in paracrine signaling within the follicle is fibroblast growth factor-2 (FGF-2). Studies have localized FGF-2 in ovarian follicles (Nilsson et al., 2001; Ben-Haroush et al., 2005) and corpora lutea (Asakai et al., 1993), while FGF-2 receptors have been demonstrated in growing follicles (Wandji et al., 1992; 1995; Ben-Haroush et al., 2005). Some in vitro studies have shown that FGF-2 promoted growth of primordial and primary follicles (Nilsson et al., 2001) as well as proliferation of granulosa and theca cells (Wandji et al., 1996). In goats, Matos et al. (2007b) recently demonstrated that a concentration of 50 ng/mL FGF-2 is able to activate primordial follicles after 5 days of culture, although follicle survival was not influenced by FGF-2 treatment.

Regarding to FSH and FGF-2 interaction, Shikone et al. (1992) suggest that FSH induces functional receptors for FGF-2 in rat granulosa cells and that FGF-2 may play a role in the process of granulosa cell differentiation under the influence of FSH. In bovine, FSH plus FGF-2 promoted an increase in follicular diameter (Wandji et al., 1995). However, there are apparently no reports showing that the interaction between FSH and FGF-2 promotes primordial to primary follicle transition and growth in caprine, being essential to investigate whether these substances play a role in early folliculogenesis.

The aim of the present study was to investigate whether the interaction between FSH and FGF-2 has a beneficial role in the survival, initiation of primordial follicles growth and further growth of in vitro cultured goat primordial follicles enclosed in ovarian cortex. To this end, both histological and ultrastructural studies were performed to investigate and compare the morphology of follicles before and after culture for 1 or 7 days in the absence or presence of FSH, FGF-2 or the association of FSH and FGF-2.

2. Materials and Methods

2.1. Source of ovaries

The animals used in this study were in good body condition and were non-pregnant and presumed to be undergoing normal estrous cycles as judged by the presence of normal corpora lutea at slaughter. Ovaries (n=8) from four adult non-pregnant mixed-breed goats were collected at a local slaughterhouse. Then, the ovaries were washed and transported in Minimal Essential Medium (MEM) to the laboratory in thermo flasks with water at 32°C.

2.2. Experimental protocol

At the laboratory, both ovaries from each animal were stripped of surrounded fat tissue and ligaments, and cut in half, where after the medulla, large antral follicles and corpora lutea were removed. Following this, the cortex from each ovarian pair was divided into 9 fragments of approximately 3 x 3 mm (1 mm thick), totalizing 36 fragments used in the experiment. One fragment was immediately fixed for classic histological studies (non-cultured controls) while a smaller fragment (1 mm^3) was randomly collected and subsequently fixed for ultrastructural examination. The other fragments of ovarian cortex were individually in vitro cultured in 1 mL of culture medium for 1 or 7 days at 39°C with 5% CO_2 in air using a 24-well culture dish. The control medium was MEM supplemented with ITS (insulin 6.25 μg/mL, transferrin 6.25 μg/mL, and selenium 6.25 ng/mL), 0.23 mM pyruvate; 2 mM glutamine; 2 mM hypoxanthine; 1.25 mg/mL BSA, 100 μg/mL penicillin, 100 μg/mL streptomycin (Vetec, Rio de Janeiro, Brazil) and 0.25 μg/mL fungizone (MEM^+). For the treatments, this control medium (MEM^+) was supplemented with porcine FSH (provided by Dr. J.F. Beckers, Liège, Belgium), FGF-2 (basic, from bovine pituitary glands, MP Biomedicals, Solon, OH, USA) or FSH + FGF-2. Based on our previous studies with caprine preantral follicles, both substances were used at a concentration of 50 ng/mL (Matos et al., 2007a,b). All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Every two days, the culture medium was replaced by fresh medium. Each treatment was repeated four times, thus using the ovaries of four different animals.

2.2. Histological analysis and assessment of in vitro follicular growth

To evaluate the morphology of caprine follicles after 1 or 7 days of culture, a small part (1 mm^3) from each fragment was randomly removed for TEM studies, while the

remainder was fixed in Carnoy for 12 h for histological studies. After fixation, the tissue fragments were dehydrated in a graded series of ethanol, clarified with xylene and embedded in paraffin wax. The wax blocks containing the treatments were completely and serially sectioned (7 μm), stained with periodic acid Schiff and hematoxylin (PAS staining system, Sigma, Inc., St. Louis, MO, USA), and examined by light microscopy (Zeiss, Germany) at 100X and 400X magnification.

The follicles were classified as described by Silva et al. (2004) in primordial (one layer of flattened granulosa cells around the oocyte), or growing follicles i.e., intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). These follicles were classified individually as histologically normal when an intact oocyte was present, i.e. an oocyte without a pyknotic nucleus, surrounded by granulosa cells which are well organized in one or more layers and that have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which have a pyknotic nucleus, and/or are surrounded by disorganized granulosa cells, which are detached from the basement membrane. Thirty follicles were counted in each treatment to evaluate follicular morphology and follicular growth initiation. As each treatment was repeated four times (four replicates), a total of 120 follicles were evaluated for each medium and each culture period.

To evaluate initiation of primordial follicles growth and further growth of developing follicles, only intact follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and growing follicles were calculated at day 0 (controls) and after 1 or 7 days of culture in the various media tested. To minimize the possibility of counting more than once, only follicles with a visible oocyte nucleus were recorded. Oocyte and follicular diameter was measured with the aid of an ocular micrometer. Both diameters, from the basement membrane, at right angles to each other in the largest cross-section of each growing oocyte and follicle were measured and averaged. Follicular and oocyte diameter were measured in 20 follicles for each treatment.

2.3. Ultrastructural analysis

For ultrastructural analysis, small pieces of ovarian cortex were fixed in 2%

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

2.4. Statistical analysis

Data are expressed as mean ± SEMs. The percentages of surviving follicles at all stages, primordial and growing follicles obtained after 1 or 7 days in the various treatments were subjected to arc-sin transformation before analysis of variance (ANOVA). The data of primordial and developing follicles as well as the diameter of oocytes and follicles were analyzed by ANOVA followed by Fisher's protected least significant difference test (PLSD test) (StatView for Windows, SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effect of FSH, FGF-2 and FSH + FGF-2 and culture periods on follicle survival

Figure 1 shows morphologically normal preantral follicles after 7 days culture in the association between FSH and FGF-2. A total of 1,080 follicles were counted to evaluate follicular morphology, initiation of primordial follicles growth and further growth of developing follicles. The effect of FSH, FGF-2 or FSH + FGF-2 on follicle survival, i.e. the percentage of histologically normal follicles in ovarian tissues after 1 and 7 days of culture was shown in Figure 2. When compared to non-cultured follicles (91.8% normal follicles), a

significant decrease ($P < 0.05$) in the percentages of histologically normal follicles was observed after 1 and 7 days culture. No significant effect of FSH or FGF on follicle survival was observed after 1 day of culture. In contrast, after 7 days culture, higher percentages of histologically normal follicles were observed in medium supplemented with FSH (66.7%) when compared to MEM^+ only (56.3%) or MEM^+ supplemented with the interaction FSH + FGF-2 (56.5%). With the increase of the culture period from 1 to 7 days, a significant ($P < 0.05$) decrease in the percentage of normal follicles was observed in all treatments, but not in medium supplemented only with FSH.

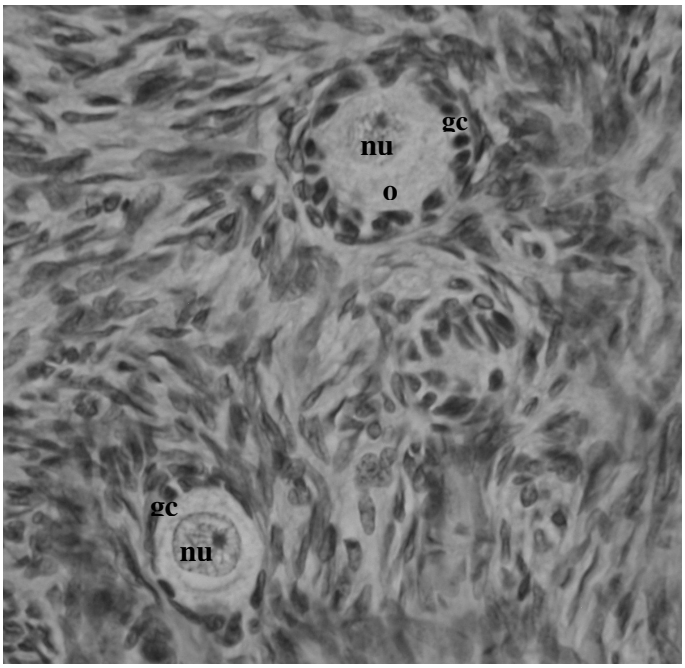

Figure 1. Histological section of tissue cultured for 7 days in the interaction between FSH + FGF-2, showing normal follicles after staining with periodic acid Schiff-hematoxylin. o: oocyte; nu: oocyte nucleus; gc: granulosa cell (400 x).

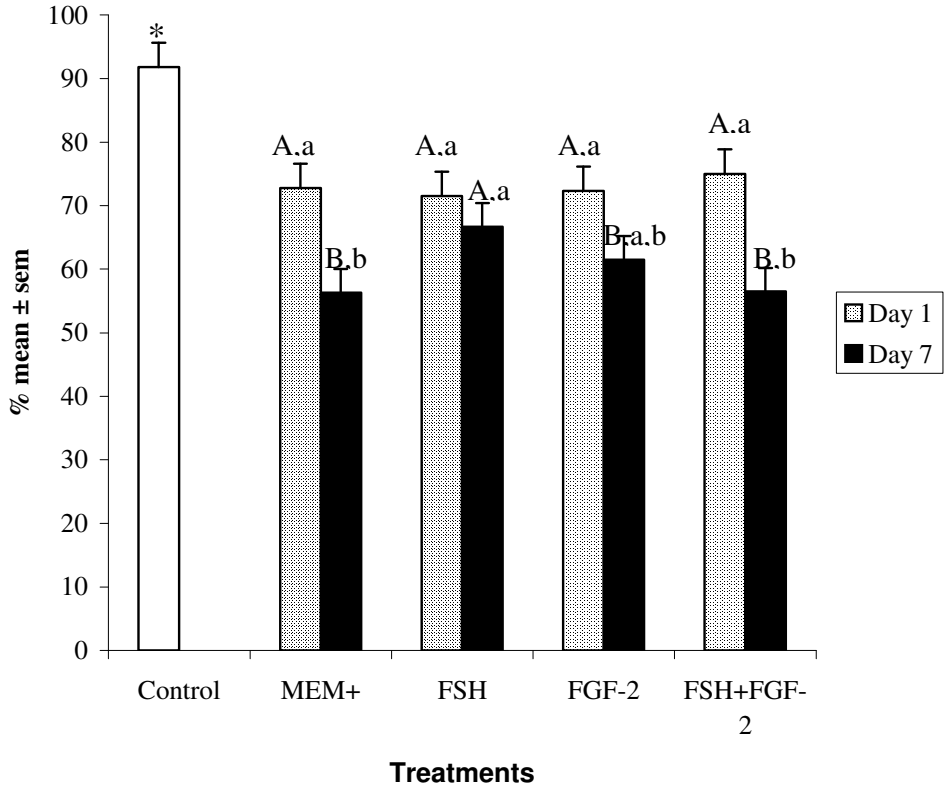

Figure 2. Percentages (means ± S.E.M) of histologically normal preantral follicles in non-cultured tissue (control) and in tissue cultured for 1 and 7 days in MEM^+ and MEM^+ supplemented with FSH, FGF-2 or FSH + FGF-2.

* $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/D0).

(A, B) Different letters denote significant differences between culture periods within the same medium ($P < 0.05$). (a, b) Different letters denote significant differences among treatments in the same period ($P < 0.05$).

3.2. Follicular growth initiation and growth of developing follicles during in vitro culture

The percentages of primordial and growing follicles in ovarian cortical tissue before and after 1 and 7 days of culture are shown in Table 1. The percentages of primordial and growing follicles in non-cultured cortex were 86% and 14%, respectively. After 1 day of culture, when compared to non-cultured follicles, a reduction ($P < 0.05$) of primordial

follicles concomitant with a significant increase of growing follicles was observed in all treatments. Cortical tissues cultured in all treatments had a significant reduction of primordial follicles ($P < 0.05$) associated with a significant increase ($P < 0.05$) in the percentage of growing follicles after 7 days of culture when compared to day 1, except in tissues cultured in MEM^+ plus FSH. Compared to control medium (MEM^+), after 1 day of culture, the proportion of primordial follicles was significantly reduced ($P < 0.05$) whereas the proportion of growing follicles was increased in medium supplemented with FSH or the interaction FSH + FGF-2 ($P < 0.05$). At day 7 of culture, addition of both FSH and FGF-2 to control medium significantly decreased ($P < 0.05$) the percentage of primordial follicles and increase the percentage of growing follicles when compared to non-cultured tissue and other treatments.

Table 1. Percentages (mean ± S.E.M.) of primordial and growing follicles (intermediate, primary and secondary) in non-cultured tissues and in tissues cultured for 1 or 7 days in MEM^+ (control medium) and MEM^+ supplemented with FSH, FGF-2 or FSH + FGF-2. Per treatment 150 follicles were evaluated.

	Primordial follicles		Growing follicles	
Non-cultured (Day 0)	86.0 ± 3.0^{A}		14.0 ± 2.5^{B}	
Cultured	Day 1	Day 7	Day 1	Day 7
MEM^+	$64.3 \pm 2.8^{*\ A,a}$	$45.8 \pm 1.7^{*\ B,a}$	$35.7 \pm 2.8^{*\ A,a}$	$54.2 \pm 1.4^{*\ B,a}$
FSH	$45.0 \pm 3.9^{*\ A,b}$	$40.8 \pm 3.0^{*\ A,a}$	$55.0 \pm 2.4^{*\ A,b}$	$59.2 \pm 1.0^{*\ A,a}$
FGF-2	$56.7 \pm 4.3^{*\ A,a,c}$	$42.3 \pm 3.3^{*\ B,a}$	$43.3 \pm 2.5^{*A,a,c}$	$57.7 \pm 7.5^{*\ B,a}$
FSH + FGF-2	$52.4 \pm 5.7^{*\ A,b,c}$	$26.5 \pm 5.8^{*\ B,b}$	$47.6 \pm 5.8^{*\ A,b,c}$	$73.5 \pm 2.3^{*\ B,b}$

* $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/Day 0).
(A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$). (a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

As early as 1 day of culture, the interaction FSH + FGF-2 significantly ($P < 0.05$) increase oocyte diameter in unilaminar follicles, i.e., primordial, intermediate and primary follicles together, when compared to non-cultured tissue (Table 2). After 7 days of culture in all treatments, except in control medium (MEM^+), a significant increase in oocyte diameter

was observed when compared to non-cultured tissue ($P < 0.05$). In addition, the interaction between FSH + FGF-2 promoted a significant ($P < 0.05$) increase in oocyte diameter after 7 days of culture when compared to other treatments. Furthermore, after 7 days culture, follicle diameter increased significantly ($P < 0.05$) in all treatments when compared to control medium. With the increase of culture period from day 1 to 7, a significant ($P < 0.05$) increase in oocyte (FSH + FGF-2) and follicle (FSH, FGF-2 or FSH + FGF-2) diameters was observed. Cultured and non-cultured tissue contained variable and relatively low (often zero) numbers of secondary follicles, which were not amenable to statistical comparison.

Table 2. Oocytes and unilaminar follicle diameters (mean ± S.E.M.) in non-cultured tissue (begin control) and in tissue cultured for 1 and 7 days in MEM^+ and MEM^+ supplemented with FSH, FGF-2 or FSH + FGF-2.

	Oocyte diameter (μm)		Follicle diameter (μm)	
Non-cultured (Day 0)	35.4 ± 1.4		49.9 ± 1.6	
Cultured	Day 1	Day 7	Day 1	Day 7
MEM^+	38.6 ± 1.7 ^{A,a}	38.8 ± 1.3 ^{A,a}	50.4 ± 2.0 ^{A,a}	52.0 ± 1.5 ^{A,a}
FSH	38.9 ± 2.0 ^{A,a}	43.9 ± 1.9* ^{A,a}	51.8 ± 2.6 ^{A,a}	60.4 ± 1.9* ^{B,b}
FGF-2	36.3 ± 1.8 ^{A,a}	40.8 ± 1.8* ^{A,a}	50.7 ± 2.5 ^{A,a}	59.2 ± 1.9* ^{B,b}
FSH + FGF-2	41.6 ± 2.1* ^{A,a}	50.4 ± 2.5* ^{B,b}	53.9 ± 2.6 ^{A,a}	63.9 ± 2.9* ^{B,b}

* $P < 0.05$, significantly different from non-cultured ovarian cortical tissues (control/Day 0). (A, B) Different letters in the same row denote significant differences between culture periods within the same medium ($P < 0.05$). (a, b) Different letters in the same column denote significant differences among treatments in the same period ($P < 0.05$).

3.3. Ultrastructural analysis of goat preantral follicles

Based on histological results, TEM studies were performed in non-cultured follicles (control) and in follicles cultured for 1 and 7 days in MEM^+ (control medium), MEM^+ plus FSH, FGF-2 or FSH + FGF-2. On average, five primordial follicles per treatment were evaluated by ultrastructural analysis. The ultrastructural characteristics of follicles from non-

cultured tissue and those cultured in medium without or with FSH, FGF-2 or FSH + FGF-2 for 1 day appeared similar, but after 7 days, only tissues cultured with FSH, FGF-2 or FSH + FGF-2 had normal follicles. These follicles exhibited a large oocyte with sparse vesicles spread throughout the ooplasm. Furthermore, the homogeneous cytoplasm contained numerous rounded mitochondria and occasional elongated forms that contained transverse or parallel cristae (Fig. 3A). Golgi complexes were rarely observed. Both smooth and rough endoplasmic reticulum were present, either as isolated aggregations or as complex associations with mitochondria and vesicles. The oocyte nucleus had uncondensed chromatin, which was uniformly distributed throughout the oocyte nucleus, and the nucleolus could generally be observed. In all developmental stages, granulosa cells were small, with a greater nuclear-to-cytoplasm ratio as compared with typical cell structures (Fig. 3B). The nuclei were irregularly shaped, with loose chromatin in the inner part, and small peripheral aggregates of condensed chromatin. Well-developed rough endoplasmic reticulum and mitochondria were the most evident organelles observed in granulosa cells (Fig. 3C). Microvillar extensions of the plasma membranes of the oocyte as well as granulosa cells were visible in the space between these compartments.

Cortical tissues cultured in control medium (MEM^{+}) for 7 days had histologically normal follicles, but TEM studies revealed some changes in their ultrastructure, which are indicative of degeneration. Atretic follicles had an oocyte extremely vacuolated, as well as condensation of both oocyte and granulosa cell chromatin. Organelles were more randomly distributed throughout the cytoplasm and signs of endoplasmic reticulum proliferation and damage to mitochondrial membranes and cristae were observed. The oocyte nucleus appeared misshapen and retracted, and had a wavy membrane. Granulosa cells look swollen, while the density of cytoplasmic organelles was low. Frequently, the connection between the oocyte and granulosa cells had disappeared, while organelles were no longer identifiable. Furthermore, granulosa cells showed less contact with each other and exhibited obvious fewer gap junctions (Fig. 3D).

4. Discussion

The present study demonstrated the importance of FSH and FGF-2 on in vitro viability, follicular growth initiation and further growth of caprine preantral follicles in a 7 days culture system. In our study, addition of FSH alone to culture medium was very

important to maintain the percentage of normal follicles after 7 days culture. Although very little is known about the regulation of primordial follicle development, FSH seems to be a very effective factor in maintaining preantral follicle viability or inhibiting apoptosis after in vitro culture (Baker and Spears, 1997; Saha et al., 2000; Mao et al., 2002). Hsueh et al. (1994) suggested that the diffusion of several essential chemical and physical factors through the basal membrane could be compromised in the absence of FSH. In addition, cultures without FSH more frequently resulted in extrusion of the oocyte from its original follicular structure (Cortvrindt et al., 1997), which may be caused either by damage or reduction in number of gap junctions (Amsterdam and Rotmensch, 1987; Hsueh et al.; 1994). With regard to FGF-2, its receptors are expressed in granulosa cells (Ojeda and Dissen, 1994) and previous studies showed that this factor plays an important role in maintaining granulosa cell viability during follicular development (Trolice et al., 1997). Recently, a study showed that FGF-2 at 50 ng/mL stimulated caprine oocyte survival after culture (Zhou and Zhang, 2005). However, in our study, the association of FSH and FGF-2 did not further increase preantral follicles survival in 7 days culture. Similar results were obtained after 6 days culture of bovine preantral follicles (Wandji et al., 1995).

After 7 days culture, the interaction FSH and FGF-2 was more efficient in promoting primordial to primary follicle transition than FSH or FGF-2 alone. In vivo, ovarian follicular development is known to proceed to primordial and primary stages independently of the action of FSH. This has been observed in mice carrying invalidations of the FSHβ and FSHR genes (Kumar et al., 1997; Dierich et al., 1998). Previous studies have confirmed that FSH alone did not influence follicular growth initiation (Derrar et al., 2000; Silva et al., 2004). On the other hand, recently, we have demonstrated that FSH (50 ng/mL) could improve caprine follicular growth initiation and survival (Matos et al., 2006a). In addition, Joyce et al. (1999) reported that FSH stimulates Kit ligand mRNA expression in granulosa cells of preantral follicles. Kit ligand has been shown to be essential for oocyte growth (Eppig, 2001) and initiation of primordial follicles growth (Parrot and Skinner, 1999). FSH can also modulate the levels of BMP-15 and GDF-9 in growing follicles (Thomas et al., 2005) and these growth factors are essential for primordial and primary follicle development (Dong et al, 1996; Galloway et al., 2000). According to Shikone et al. (1992), FSH stimulates the expression of FGF-2 receptors in granulosa cells, which suggests that FSH could enhance the stimulatory effect of FGF-2 on follicle follicular growth initiation. Since FGF-2 and its receptors are expressed in ovarian follicles (Nilsson et al., 2001; Ben-Haroush et al., 2005), this system is

important in regulating a wide range of ovarian functions including granulosa cell mitosis (Roberts and Ellis, 1999), steroidogenesis (Vernon and Spicer, 1994), differentiation (Anderson and Lee, 1993) and apoptosis (Tilly et al., 1992). Similar to our recent results (Matos et al., 2006b), FGF-2 (40 ng/mL) was efficient to stimulate the initiation of primordial follicles growth in rats (Nilsson et al., 2001).

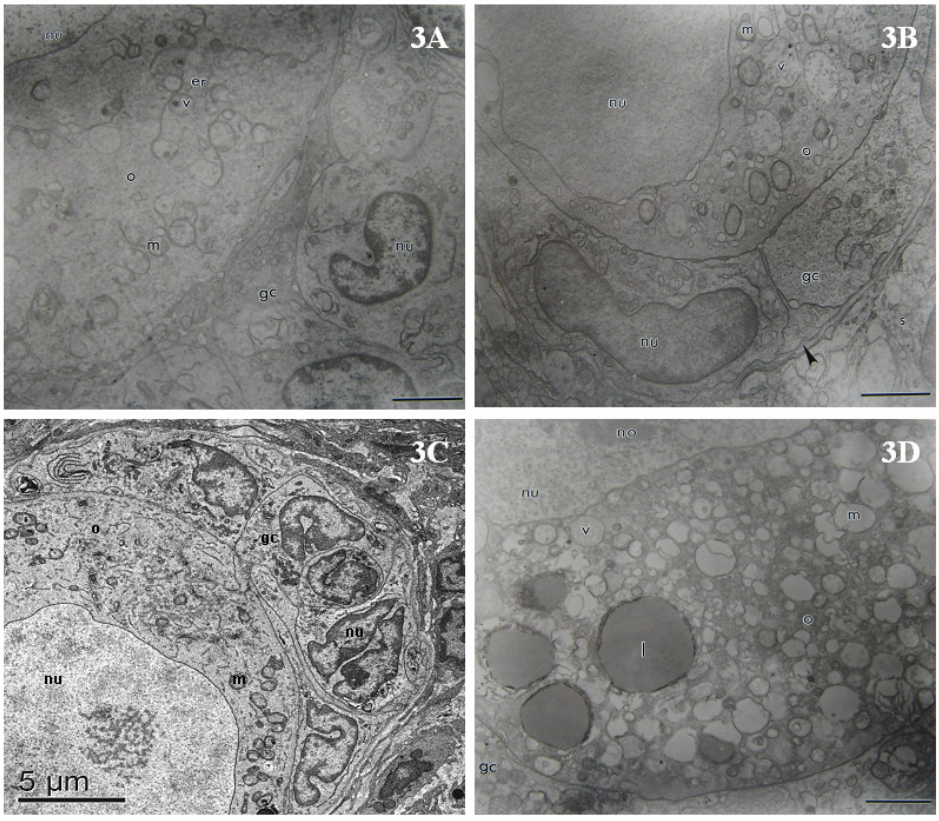

Figure 3. Electron micrographs of caprine preantral follicles before (non-cultured control; 5200 x, Bar: 3 µm) (A) and after culture for 7 days in FSH (5600 x; Bar: 3 µm) (B), FSH + FGF-2 (6000 x; Bar: 5 µm) (C) and MEM^{+} (control medium; 7000 x; Bar: 2 µm) (D). In figures 3A, B and C, note the homogeneous cytoplasm with numerous rounded mitochondria and the basement membrane integrity. In figure 3D, note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration. o: oocyte; nu: nucleus; no: nucleolus; gc: granulosa cell; er: endoplasmic reticulum; m: mitochondria; v: vesicles; l: lipid droplet; s: stroma; arrowhead: basement membrane.

In the current study, oocyte diameter had the highest increase when cultured in presence of both FSH and FGF-2 for 7 days. With similar results, Wandji et al. (1995) also demonstrated that FSH and FGF-2, alone or in combination, were the most effective treatments in increasing the diameter of preantral follicles. Since there are FSH receptors in granulosa cells and oocytes of early follicles, it is possible that FSH may act in both cell types to promote follicular growth and development (Méduri et al., 2002). Furthermore, a two-way exchange may occur between the oocyte and granulosa cells and a direct action of FSH on oocytes produces compounds whose diffusion into the granulosa cells is necessary for their proliferation and maturation (Méduri et al., 2002). Other authors observed that FSH (Saha et al., 2000; Silva et al., 2004) and FGF-2 (Jewgenow, 1996; Nilsson et al., 2001) promotes an increase in follicular diameter and proliferation of granulosa cells. In addition, FGF-2 was found to stimulate the proliferation of cultured bovine theca and stroma cells (Nilsson et al., 2001). At a concentration of 50 ng/mL, FGF-2 appeared to increase DNA follicular synthesis in cultured mouse preantral follicles (Roy and Greenwald, 1991). In our study, we suggest that FSH increased the number of binding sites for FGF-2 after culture compared to that in non-cultured tissue, which could stimulate follicular growth.

Several authors have emphasized the importance of ultrastructural studies after in vitro culture of preantral follicles, since it gives close insight into the electron-microscopical morphology of follicles, allowing a better evaluation of their quality (Van den Hurk et al., 1998; Sadeu et al., 2006). In the present study, caprine preantral follicles cultured for 7 days in medium supplemented with FSH, FGF-2 or both appeared ultrastructurally normal, which confirmed the results obtained in the histological studies. However, follicles cultured without FSH or FGF-2 showed various signs of initial degeneration, like ooplasm vacuolization. Cytoplasmic vacuoles are characteristic signs of degeneration in oocytes (Silva et al., 2000), granulosa (Hay et al., 1976) and cumulus cells (Assey et al., 1994) and may represent endoplasmic reticulum swelling or altered mitochondrial structure (Silva et al., 2001).

In conclusion, this study with caprine follicles showed that the interaction between FSH and FGF-2 is able to promote the initiation of primordial follicles growth and subsequent growth of developing preantral follicles. Furthermore, these data support the vital role of FSH in maintaining healthy oocyte growth and follicular ultrastructure after 7 days culture. A molecular biological study will be useful to determine the expression of FSH and FGF-2 in caprine ovary and its potential role in regulating early follicular growth and development.

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Capítulo VI

Hormônio Folículo Estimulante melhora a sobrevivência, o crescimento e a formação de folículos primários caprinos no cultivo *in vitro* de longa duração

Resumo

O objetivo deste estudo foi investigar os efeitos do Hormônio Folículo Estimulante (FSH) e Fator de Crescimento Fibroblástico-2 (FGF-2) sobre a sobrevivência e o crescimento de folículos primordiais caprinos. Tecidos corticais ovarianos foram cultivados em meio contendo FSH sozinho ou na interação FSH + FGF-2 por 1, 7, 14, 21 ou 28 dias. Para avaliar o intervalo de troca de meio, os tecidos ovarianos foram cultivados com FSH e a troca do meio de cultivo foi realizada a cada 2 (FSH-2d) ou 7 dias (FSH-7d). Tecidos ovarianos não cultivados e cultivados foram fixados para estudos histológicos. Os folículos foram analisados de acordo com o estádio de desenvolvimento e a morfologia. Os resultados mostraram que após o cultivo, exceto no dia 1, as percentagens de folículos normais reduziram em todos os tratamentos quando comparado aos tecidos não cultivados. Após 28 dias, as maiores percentagens de folículos normais foram observadas no meio suplementado com FSH-2d. A suplementação com FSH e a troca de meio a cada 7 dias promoveram a maior ativação folicular após 7 dias de cultivo. Além disso, ambos os tratamentos com FSH aumentaram significativamente a percentagem de folículos primários após 28 dias de cultivo quando comparado à associação entre FSH + FGF-2. Após 28 dias, o meio suplementado com FSH-2d foi o mais eficiente para promover o crescimento oocitário e folicular. Em conclusão, este estudo demonstrou que o FSH estimula a ativação de folículos primordiais caprinos e o crescimento de folículos primários após 28 dias de cultivo. Além disso, a adição de FSH ao meio e a troca deste meio a cada dois dias mantêm a morfologia de folículos caprinos após cultivo de longa duração.

Capítulo VI

Follicle Stimulating Hormone improves survival, growth and formation of caprine primary follicles in long-term culture in vitro

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Molecular, Reproduction and Development

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Abstract

The aim of this study was to investigate the effects of follicle stimulating hormone (FSH) and fibroblast growth factor-2 (FGF-2) on survival and growth of caprine primordial follicles. Ovarian cortical tissues were cultured in media containing FSH alone or the interaction FSH + FGF for 1, 7, 14, 21 or 28 days. To evaluate the interval of medium replacement, the ovarian tissues were cultured with FSH and replacement of culture medium was performed each 2 (FSH-2d) or 7 days (FSH-7d). Non-cultured and cultured ovarian tissue were fixed for histological studies. The follicles were analysed for stage of development and morphology. The results showed that after culture, except on day 1, the percentage of normal follicles had decreased in all treatments when compared to non-cultured tissues. After 28 days, the highest percentage of normal follicles were observed in medium supplemented with FSH-2d. The supplementation with FSH and medium replacement at each 7 days, promoted a higher follicular activation after 7 days culture. In addition, both treatments with FSH significantly increased the percentage of primary follicles after 28 days culture when compared to the association FSH + FGF-2. Furthermore, after 28 days, media supplemented with FSH-2d was most effective to promote oocyte and follicular growth. In conclusion, this study demonstrated that FSH stimulates the activation of caprine primordial follicles and the growth of primary follicles after 28 days of culture. Furthermore, addition of FSH to the medium and refreshment of this medium every other day maintained caprine follicular morphology after long-term culture.

Short tittle: Long-term culture of caprine preantral follicles.

Keywords: Caprine, Primordial Follicle, Long-term culture, FSH, FGF-2.

Introduction

Successful reproductive technologies depend on the production of fully-grown, developmentally competent oocytes in vitro. Since primordial follicles form the largest population of follicles in the ovary, the development of a culture system from this stage of growth can potentially produce mature oocytes and will significantly advance the use of these technologies. In addition, it may make possible the preservation and long-term storage of the

female germ plasm. To date, complete in vitro development from a primordial follicle to a fully mature ovulatory follicle and offspring has been achieved only in mice (Eppig & O'Brien, 1996).

In larger species, early follicular development follows a very lengthy and complex process (Gougeon, 1996). It has been estimated that primary follicles need approximately 120 days to grow to secondary and that a longer time is needed for primordial follicles to develop to primary (Gougeon, 1996). Therefore, an extended culture period may be required for a follicle to develop to the preovulatory stage. Few long-term in vitro culture studies achieved the transition from primordial to primary follicles (ovine: Muruvi et al., 2005; human: Otala et al., 2002; Sadeu et al., 2006) or the development of antral follicles (bovine: Gutierrez et al., 2000; mouse: Mitchell et al., 2002). Interestingly, in most of the in vitro culture systems, independently of the culture period, the medium has been totally or partially replaced by fresh medium every other day (Silva et al., 2004; Muruvi et al., 2005).

The factors and mechanisms involved in follicular activation (transition from primordial to growing follicles) and growth remain essentially unknown. However, there is good evidence that intra- and extraovarian factors are implicated in this process (Fortune, 2003). Follicle stimulating hormone (FSH) receptors are expressed from the primary follicles onward (Oktay et al., 1997) on both granulosa cells (O'Shaughnessy et al., 1996) and oocytes (Méduri et al., 2002). FSH may play an indirect effect on very early follicle development, regulating the expression of several growth factors, such as kit ligand (KL), which play a critical role in follicular activation (van den Hurk and Zhao, 2005). Several in vitro studies have demonstrated that FSH promotes preantral follicular growth and antrum formation in many species (for review, see Van den Hurk and Zhao, 2005). Additionally, it is known that FSH inhibits apoptosis in preantral follicles cultured in vitro (Roy and Treacy, 1993; McGee et al., 1997). Recently, we demonstrated that FSH is essential to maintain the morphological integrity of 7 days cultured caprine primordial follicles enclosed in ovarian tissue (Matos et al., 2007a).

One factor that is involved in paracrine signaling within the follicle is fibroblast growth factor-2 (FGF-2). This factor is a potent mitogen and involved in cell differentiation, cell migration and angiogenesis in many tissues (Baird et al., 1986; Gospodarowicz et al., 1986). It has been localized in ovarian follicles (Nilsson et al., 2001; Ben-Haroush et al., 2005) and corpora lutea (Asakai et al., 1993), while FGF-2 receptors have been demonstrated in growing follicles (Wandji et al., 1992; 1995; Ben-Haroush et al., 2005). Some in vitro

studies have demonstrated that FGF-2 promoted growth of primordial and primary follicles (Nilsson et al., 2001) as well as proliferation of granulosa and theca cells (Wandji et al., 1996). In goats, Matos et al. (2007b) recently showed that FGF-2 is able to activate primordial follicles after 5 days of culture, although follicle survival was not influenced by FGF-2 treatment.

With regard to FSH and FGF-2 interaction, it has been suggested that FSH induces functional receptors for FGF-2 in rat granulosa cells and that FGF-2 may play a role in the process of granulosa cell differentiation under the influence of FSH (Shikone et al., 1992). We recently demonstrated that the interaction between FSH and FGF-2 promoted caprine follicular activation and oocyte growth (Matos et al., 2007c) in a 7 days culture system. Similarly, FSH associated with FGF-2 increased bovine follicular diameter (Wandji et al., 1995). However, there are apparently no reports showing the effects of FSH alone or in association with FGF-2 in the follicular activation and growth after long-term culture of caprine ovarian tissue. Furthermore, no culture system has been reported that can maintain caprine follicular integrity for longer than 14 days (Huanmin & Yong, 2000). Additionally, it is not known if the interval of medium change (two or seven days) could impair follicular development.

The aims of the current study were to 1) establish a culture system that would be able to maintain caprine follicles integrity after long-term culture in vitro; 2) evaluate the effects of FSH alone or in combination with FGF-2 on survival, activation and further growth after long-term culture of caprine primordial follicles enclosed in ovarian tissue; 3) evaluate the effects of the interval of medium change on the follicular survival and development.

Materials and Methods

Source of caprine ovarian tissue

All reagents used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. The animals used in this study were in good body condition and were non-pregnant. They presumed to be undergoing normal estrous cycles as judged by the presence of normal corpora lutea at slaughter. Ovaries (n=8) from four adult non-pregnant mixed-breed goats were collected at a local slaughterhouse (Fortaleza,

Brazil). Then, the ovaries were washed and transported in Minimal Essential Medium (MEM) to the laboratory in thermo flasks with water at 32°C.

Long-term tissue culture conditions

Both ovaries from each animal were stripped of surrounded fat tissue and ligaments, and cut in half, where large antral follicles, corpora lutea and medulla were removed. Following this, the cortex from each ovarian pair was divided into 16 fragments of approximately 3 x 3 mm (1 mm thick). One fragment was taken away randomly and subsequently fixed for classic histological studies (non-cultured control). The other fragments of ovarian cortex were individually in vitro cultured in 24-well culture plate with 1 mL of culture medium for 1, 7, 14, 21 or 28 days at 39°C with 5% CO_2 in air. The culture medium consisted of MEM supplemented with ITS (insulin 6.25 μg/mL, transferrin 6.25 μg/mL, and selenium 6.25 ng/mL), 0.23 mM pyruvate; 2 mM glutamine; 2 mM hypoxanthine; 1.25 mg/mL BSA, 100 μg/mL penicillin, 100 μg/mL streptomycin (Vetec, Rio de Janeiro, Brazil) and 0.25 μg/mL fungizone. For the treatments, this medium was supplemented with FSH-2d, FSH + FGF-2 (with replacement of culture medium at each two days) or FSH-7d (with replacement of culture medium at each seven days). Based on our previous studies with caprine preantral follicles culture, both substances were used at a concentration of 50 ng/mL (Matos et al., 2007a, 2007b). Porcine FSH was provided by Dr. J.F. Beckers (Liège, Belgium) and FGF-2 (basic, from bovine pituitary glands) was purchased from MP Biomedicals (OH, USA). Each treatment was repeated four times, thus using the ovaries of four different animals.

Assessment of follicular development and survival

Before (control) and after 1, 7, 14, 21 or 28 days of culture, fragments from each treatment were fixed in Carnoy, dehydrated through an ethanol series, clarified with xylene and embedded in paraffin wax. The wax blocks were completely and serially sectioned (7 μm), stained with periodic acid Schiff and hematoxylin (PAS staining system, Sigma, Inc., St. Louis, MO, USA), and analysed by light microscopy (Zeiss, Germany) at 100X and 400X magnification.

The follicles were classified as described by Silva et al. (2004) in primordial (one

layer of flattened granulosa cells around the oocyte), or growing follicles i.e., intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). These follicles were classified individually as histologically normal when an intact oocyte was present, i.e. an oocyte without a pyknotic nucleus, surrounded by granulosa cells which are well organized in one or more layers and that have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which have a pyknotic nucleus, and/or are surrounded by disorganized granulosa cells, which are detached from the basement membrane.

To minimize the possibility of counting more than once, only follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and growing follicles were calculated at day 0 (controls) and after 1, 7, 14, 21 or 28 days of culture in the various media tested. Fifty follicles were counted in each treatment (each ovarian fragment) to evaluate follicular morphology and activation. Each treatment was repeated four times and a total of 200 follicles were evaluated for each medium and culture period.

Oocyte and follicular diameter were measured with the aid of an ocular micrometer. Both diameters, from the basement membrane, at right angles to each other in the largest cross-section of each growing oocyte and follicle were measured and averaged. Follicular and oocyte diameter were measured in 20 follicles for each treatment.

Statistical analysis

Data are expressed as mean ± SEMs. The percentages of surviving follicles at all stages, primordial and growing follicles obtained after 1, 7, 14, 21 or 28 days in the various treatments were subjected to arc-sin transformation before analysis of variance (ANOVA). The data of primordial and growing follicles as well as the diameter of oocytes and follicles were analyzed by ANOVA followed by Fisher's protected least significant difference test (PLSD test) (StatView for Windows, SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when $P < 0.05$.

Results

Effect of treatments and culture periods on follicle survival

A total of 3,200 follicles were counted to evaluate follicular morphology, activation and growth of developing follicles. Follicles were observed in both the central and peripheral areas of the cultured fragments. Primordial follicles were the most abundant type found in non-cultured ovarian tissue (control - Fig. 1A). After 7, 14 or 21 days of culture, intermediate follicles were the predominant stages in all treatments (Fig. 1B). On the other hand, analysis of ovarian sections from day 28 of culture in FSH treatment revealed the presence of numerous primary (Fig. 1C) and some secondary follicles (Fig. 1D).

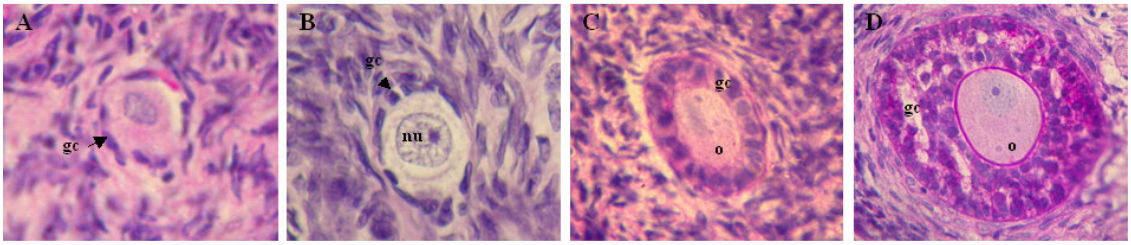

Figure 1. Histological sections of caprine ovarian tissue, showing normal follicles after staining with periodic acid Schiff-hematoxylin. (A) Primordial follicle from non-cultured tissue. Note the single layer of flattened granulosa cells around the oocyte. (B) Intermediate follicles were the most common follicular stage observed after 7 days of culture in all treatments. Note the presence of both flattened and cuboidal granulosa cells around the oocyte. (C) After 14 days, treatment with FSH + FGF-2 promoted an increase in the percentage of primary follicles. (D) Secondary follicles found after 28 days of culture in FSH treatments. o: oocyte; nu: oocyte nucleus; gc: granulosa cell (400 x).

The effect of different treatments on follicle survival, i.e. the percentage of histologically normal follicles in ovarian tissues after culture was shown in Figure 2. Except on day 1, there was a significant decrease ($P < 0.05$) in the percentages of histologically normal follicles after all culture periods compared to non-cultured follicles (78.5%). No significant effect of any treatment on follicle survival was observed after 1 day of culture. In contrast, after 14 (63%) and 28 (60.5%) days of culture, higher percentages ($P < 0.05$) of

histologically normal follicles were observed in medium supplemented with FSH-2d (with replacement of culture medium at each two days) when compared to the interaction between FSH and FGF (46.5%) or FSH-7d (44.5%) (with replacement of culture medium at each seven days). Interestingly, comparing treatments with FSH only, after all culture periods (except day 1), the highest percentages of normal follicles where always observed when the medium was replaced every 2 days instead of every 7 days.

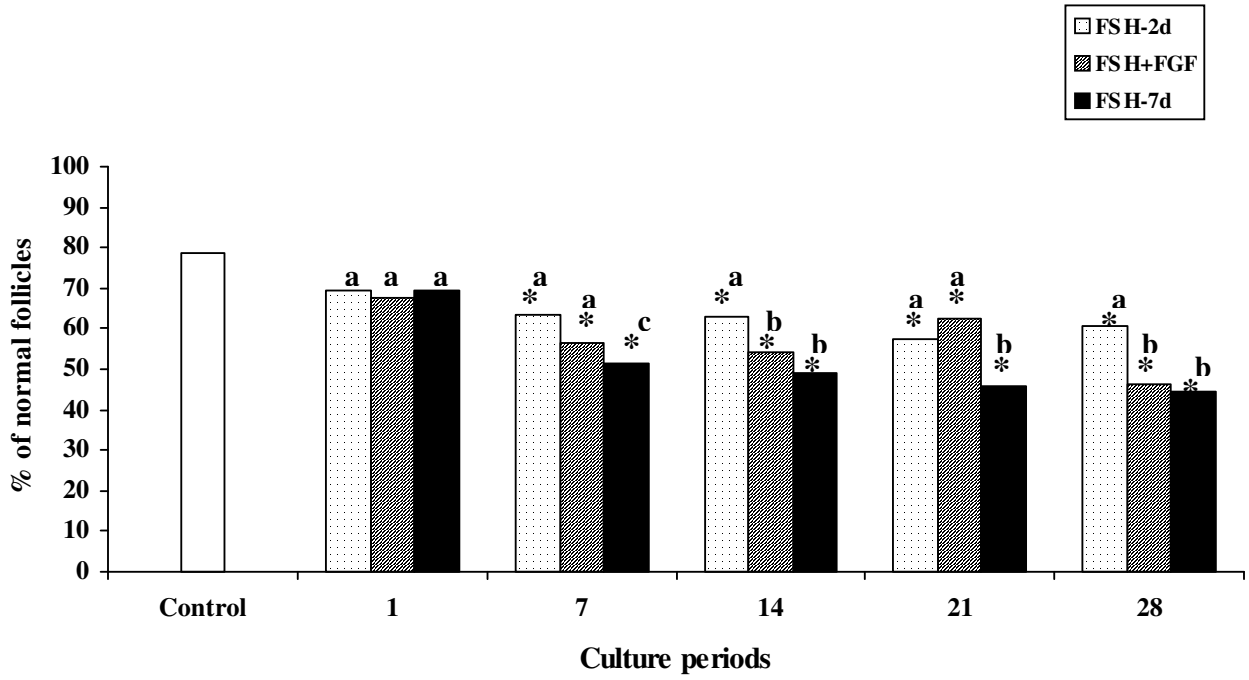

Figure 2. Percentages (means ± S.E.M) of histologically normal preantral follicles in non-cultured tissue (control) and in tissue cultured for 1, 7, 14, 21 or 28 days in medium supplemented with FSH-2d, FSH + FGF-2 or FSH-7d. * $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/D0). (a, b) Different letters denote significant differences among treatments in the same period ($P < 0.05$).

Activation and growth of developing follicles during long-term culture in vitro

Figure 3 shows the percentages of primordial and growing follicles in ovarian cortical tissue before and after culture. In fresh tissues, the percentages of primordial, intermediate,

primary and secondary follicles were 76.4%, 18.1%, 3% and 2.5%, respectively. After culture, in all treatments tested, the proportion of primordial follicles was reduced significantly (Fig. 3A, $P < 0.05$) when compared to control, as a result of a coincident increase in the proportion of intermediate follicles (Fig. 3B, $P < 0.05$). Cortical tissues cultured in FSH-7d and FSH-2d showed a significant reduction of primordial follicles (Fig. 3A, $P < 0.05$) associated with a significant increase in the percentage of intermediate follicles (Fig. 3B, $P < 0.05$) after 7 and 14 days of culture, respectively, when compared to day 1. FSH together with FGF-2 increased the proportion of primary follicles during the 14-day culture period ($P < 0.05$). In addition, after 28 days of culture, there were significantly more primary follicles in FSH cultures (both medium refreshment at each 2 or 7 days) than in control or treatment with FSH + FGF-2 (Fig. 3C, $P < 0.05$). Furthermore, FSH-2d significantly increased ($P < 0.05$) the percentage of secondary follicles when compared to the association FSH + FGF-2 after 28 days of culture (Fig. 3D, $P < 0.05$).

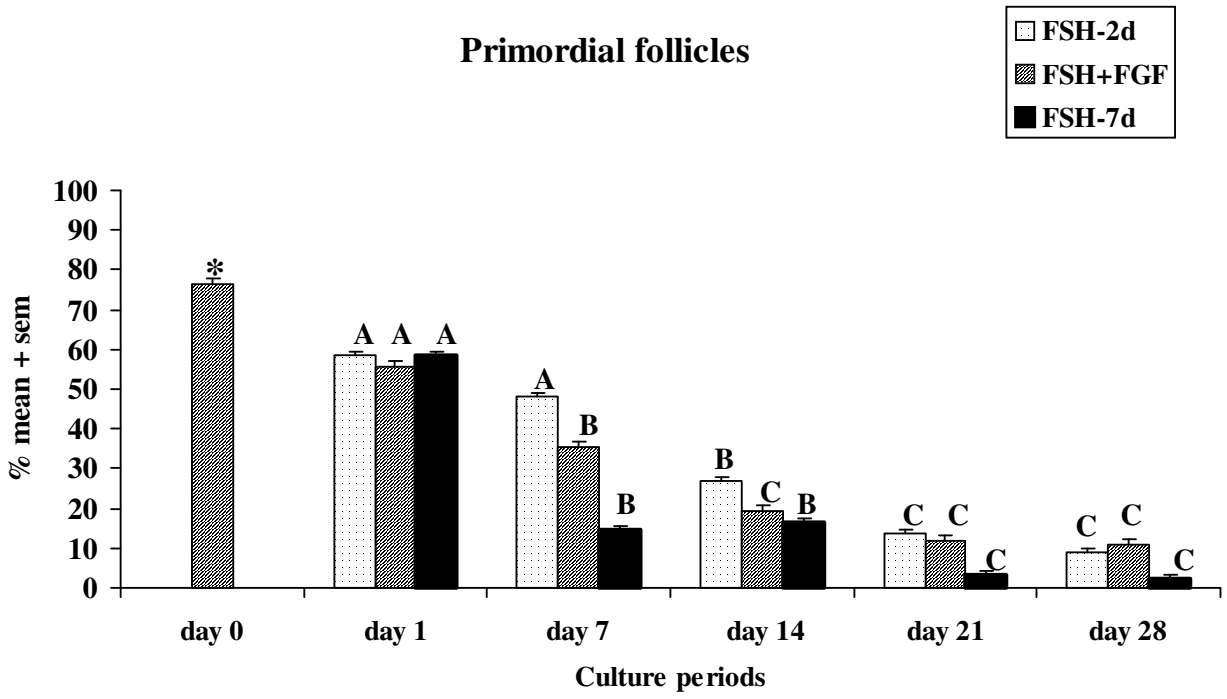

Fig. 3A

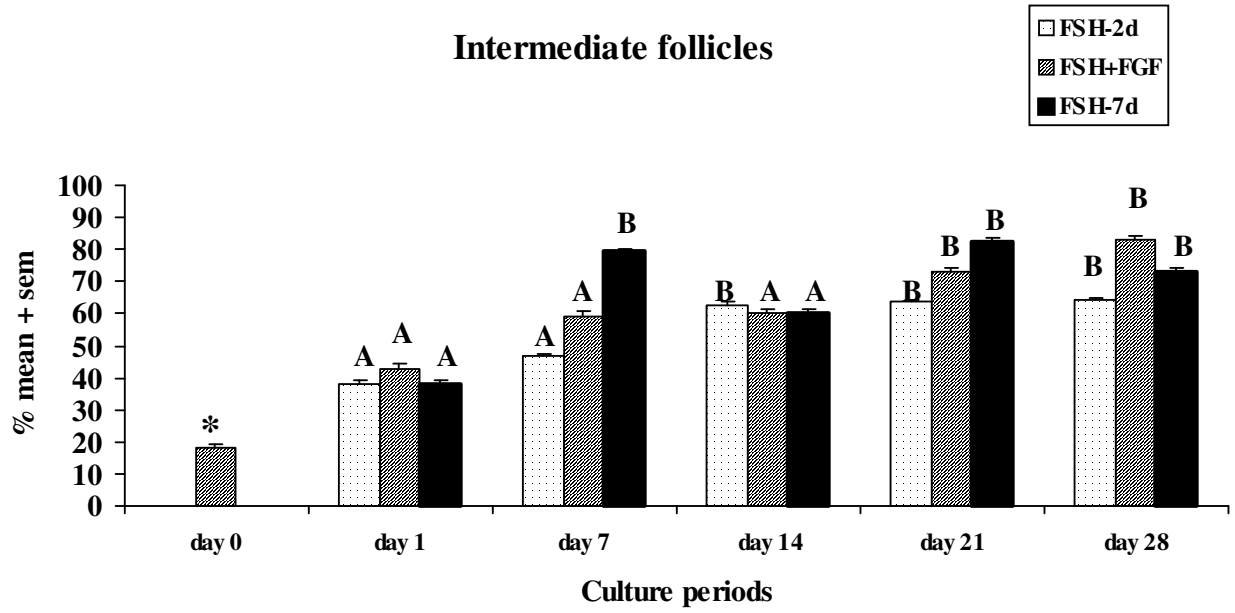
Intermediate follicles
FSH-2d
FSH+FGF
FSH-7d
% mean + sem
100
90
80
70
60
50
40
30
20
10
0
day 0
day 1
day 7
day 14
day 21
day 28
Culture periods
A
B
*

Fig. 3B

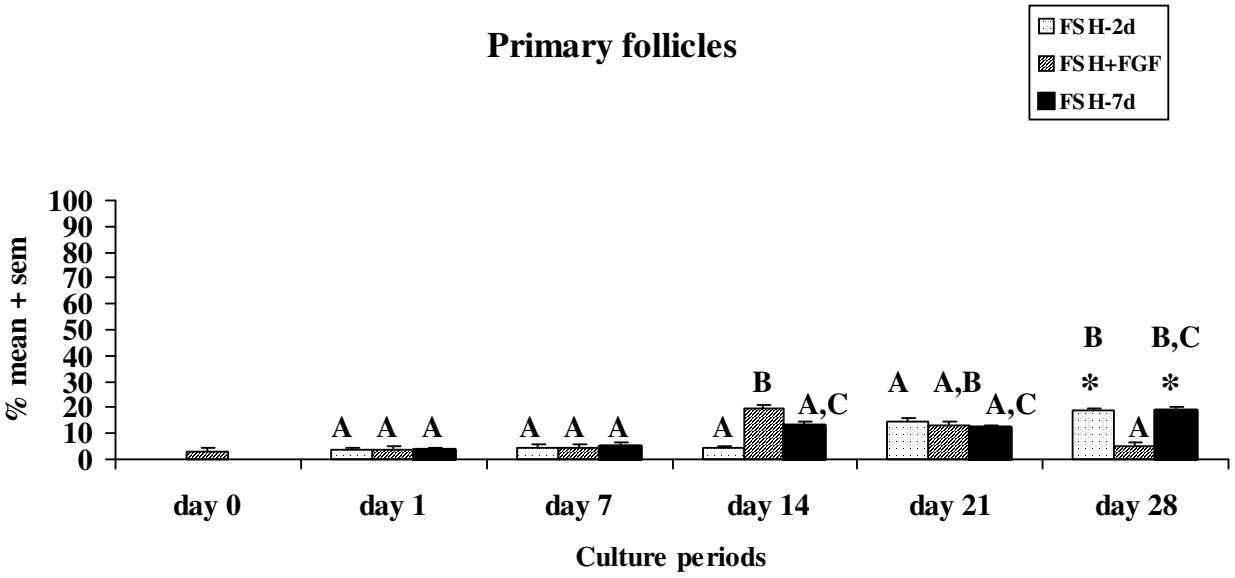
Primary follicles
FSH-2d
FSH+FGF
FSH-7d
% mean + sem
100
90
80
70
60
50
40
30
20
10
0
day 0
day 1
day 7
day 14
day 21
day 28
Culture periods
A
A,B
A,C
B
B,C
*

Fig. 3C

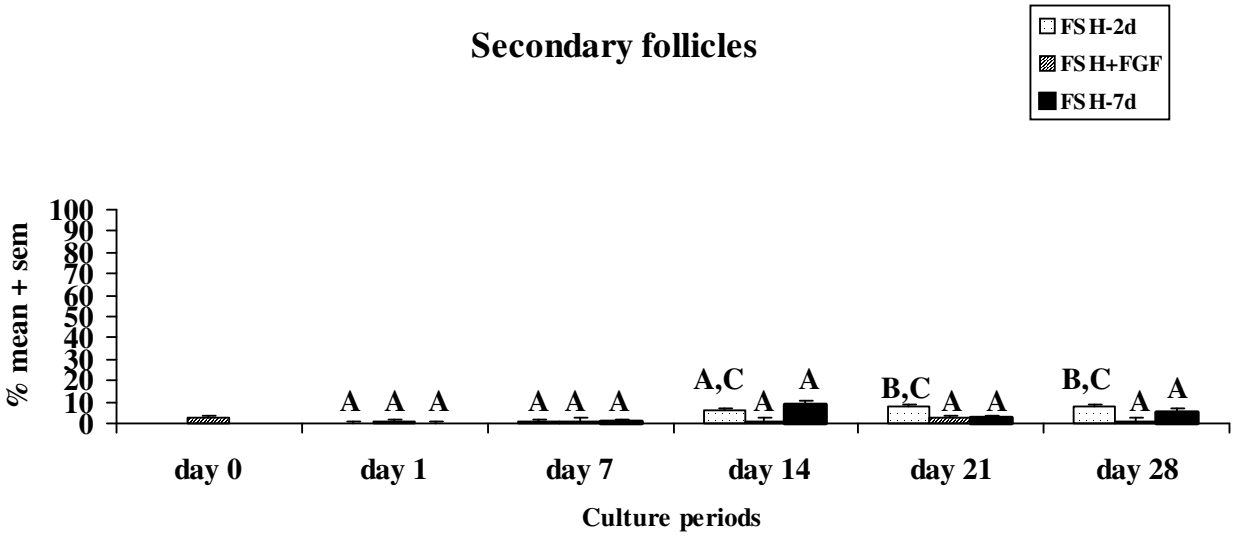

Fig. 3D

Figure 3. Percentages (means ± S.E.M) of (A) primordial, (B) intermediate, (C) primary and (D) secondary follicles in non-cultured tissue (control) and in tissue cultured for 1, 7, 14, 21 and 28 days in FSH-2d, FSH + FGF-2 or FSH-7d. * $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/D0). (A, B) Different letters denote significant differences between culture periods within the same medium ($P < 0.05$).

As early as day 1 of culture, a significant ($P < 0.05$) increase in oocyte (FSH + FGF-2, Fig. 4A) and follicle (both treatments with FSH, Fig. 4B) diameters were observed when compared to non-cultured tissue. After 28 days of culture, all treatments promoted a significant increase in both oocyte and follicle diameter when compared to control (Fig. 4A and 4B, $P < 0.05$). In addition, after 21 days of culture, treatment with FSH-2d had the smallest oocyte and follicle diameters. In contrast, after 28 days of culture, FSH-2d had a positive effect on follicle growth, increasing significantly ($P < 0.05$) both oocyte and follicle diameters when compared to other treatments.

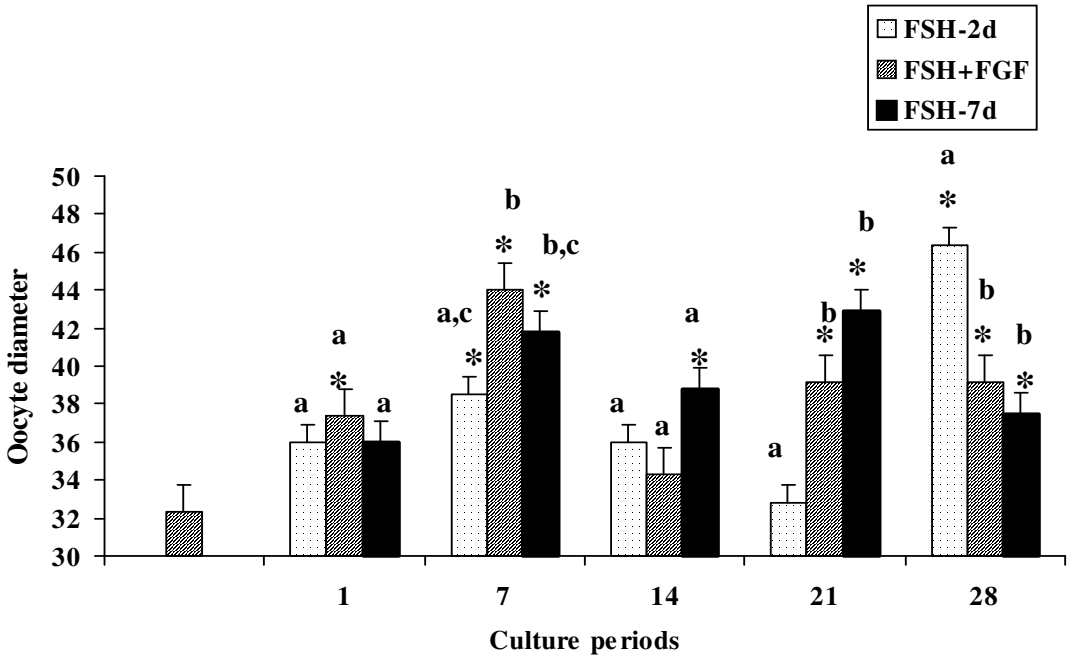

Fig. 4A

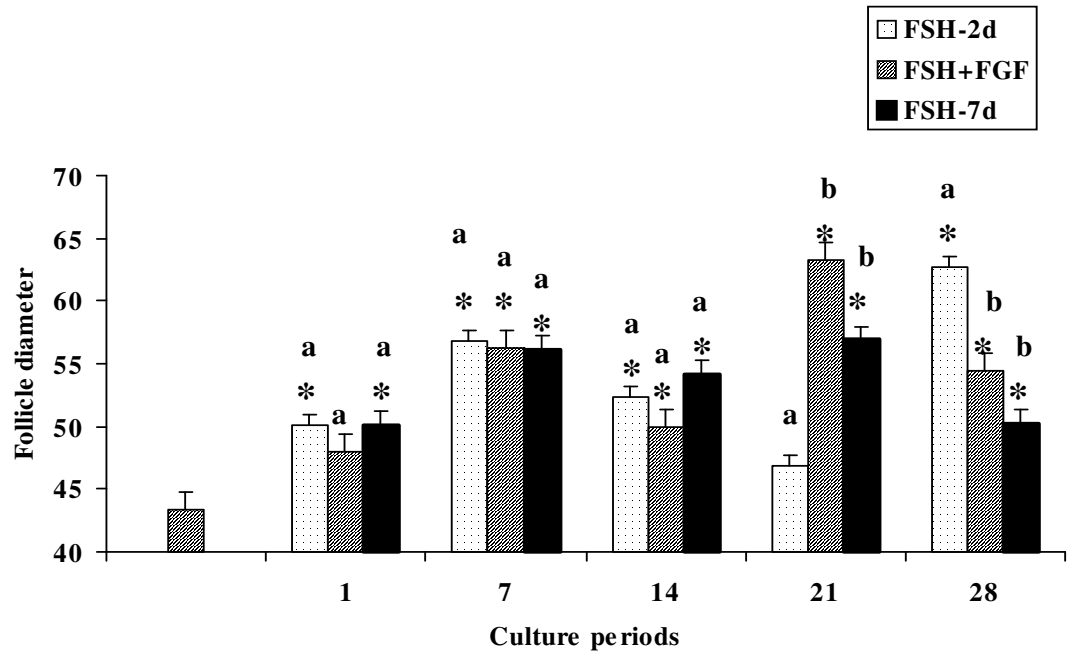

Fig. 4B

Figure 4. Mean oocyte (A) and follicle (B) diameter in µm (± S.E.M) on days 1, 7, 14, 21 and 28 of in vitro culture. * $P < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/D0). (a, b) Values with different letters denote significant differences among treatments in the same period ($P < 0.05$).

Discussion

This study shows the development of a culture system for caprine ovarian tissue that supports follicle growth and survival in vitro for up to 28 days based on histological evaluation. To our knowledge, this is the first report of primary follicles development after long-term culture of caprine ovarian cortical slices. Follicular viability and development after long-term culture was also achieved in many species (Gutierrez et al., 2000; Muruvi et al., 2005). However, no culture system has been reported that can maintain caprine follicular integrity for longer than 14 days (Huanmin and Yong, 2000).

Addition of FSH alone to culture medium was important to maintain the percentage of normal follicles after long-term in vitro culture. Once FSH was added, follicular survival exceeded 60% after 28 days of culture. These results confirm earlier studies in which FSH maintained preantral follicle viability or inhibited apoptosis after long-term culture (Cortvrindt et al., 1997; Wright et al., 1999). It is suggested that the absence of FSH in the medium could compromise the diffusion of several essential chemical and physical factors through the basal membrane (Hsueh et al., 1994). In addition, cultures without FSH more frequently resulted in extrusion of the oocyte from its original follicular structure (Cortvrindt et al., 1997), which may be caused either by damage or reduction in number of gap junctions (Amsterdam and Rotmensch, 1987; Hsueh et al.; 1994). Interestingly, the proportion of degenerated follicles increased in the cultures in which the medium supplemented with FSH was replenished every 7 days. We suggest that caprine follicles enclosed in cortical tissue require the metabolic support given periodically by the additional components of the culture medium to maintain their viability.

In the first week of culture, addition of FSH to culture medium without refreshment of this medium was more efficient in promoting follicular activation. Probably, up to 7 days, follicles cultured in ovarian tissue produced paracrine factors in addition to the medium components, which stimulated a rapid primordial follicle activation. In contrast, the higher degeneration observed in this treatment after 7 days could be a consequence of improper follicular growth (Mhawi et al., 1991). It is known that after activation, organelle multiplication and an increase of the uptake of nutrient occurs. Some follicles could die after activation due to an inadequate environment to continue their normal development (Rüsse, 1983). In vivo, ovarian follicular development is known to proceed to primordial and primary

stages independently of the action of FSH. This has been observed in mice carrying invalidations of the FSHβ and FSHR genes (Kumar et al., 1997; Dierich et al., 1998). Recently, we have demonstrated that FSH could improve caprine follicular growth initiation and survival (Matos et al., 2006a). In addition, Joyce et al. (1999) reported that FSH stimulates Kit ligand mRNA expression in granulosa cells of preantral follicles. Kit ligand has been shown to be essential for oocyte growth (Eppig, 2001) and initiation of primordial follicles growth (Parrot and Skinner, 1999).

After 14 days of culture, medium supplemented with FSH + FGF-2 stimulated primary follicle development. However, with the increase of culture period, this interaction did not promote a further primary follicle growth. In addition, primary follicles grew after 28 days of culture with FSH, independently of the frequency of medium replenishment. According to Shikone et al. (1992), FSH stimulates the expression of FGF-2 receptors in granulosa cells, which suggests that FSH could enhance the stimulatory effect of FGF-2 on primary follicular growth up to 14 days of culture. It might be that after two weeks of culture, the follicles increased their metabolism and consequently the need for additional nutrients in the medium. Furthermore, FSH can modulate the levels of GDF-9 in growing follicles and this growth factor is essential for primary follicle development (Thomas et al., 2005).

Follicular growth is demonstrated by the increase in oocyte as well as follicular diameter after 28 days of culture with FSH-2d (replenishment of the medium at each 2 days). Follicles cultured in medium supplemented with FSH and replaced each 7 days appeared healthy, but they were smaller, probably by the lack of nutrients in the medium. Since there are FSH receptors in granulosa cells and oocytes of early follicles, it is possible that FSH may act in both cell types to promote follicular growth and development (Méduri et al., 2002). Additionally, a two-way exchange may occur between the oocyte and granulosa cells and a direct action of FSH on oocytes produces compounds whose diffusion into the granulosa cells is necessary for their proliferation and maturation (Méduri et al., 2002). Previous observations have showed FSH promotes an increase in both oocyte (Itoh et al., 2002) and follicular diameter (Wright et al. 1999) after long-term culture.

In conclusion, in this study, the development of a culture system for caprine primordial follicles that supports oocyte and follicle growth as well as long-term follicle survival has been achieved. Under these culture conditions, FSH stimulated caprine primary follicles growth and maintained follicular morphology after 28 days.

Acknowledgements

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6. CONCLUSÕES GERAIS

- O IAA não é eficiente para manter a integridade ultra-estrutural de folículos pré-antrais caprinos após o cultivo *in vitro*. Entretanto, esta integridade pode ser mantida com sucesso após 5 dias de cultivo *in vitro* em MEM sem a adição de IAA.

- O FGF-2 e o FSH isoladamente, ambos na concentração de 50 ng/mL, promovem a ativação de folículos primordiais caprinos e o crescimento de folículos pré-antrais ativados, mantendo a integridade ultra-estrutural destes folículos após 5 e 7 dias de cultivo, respectivamente.

- A associação entre FSH e FGF-2 é mais eficiente para promover o início do crescimento de folículos primordiais caprinos e o posterior crescimento folicular do que o FSH ou FGF-2 isoladamente após 7 dias de cultivo *in vitro*. Entretanto, após esse mesmo período de cultivo, a adição de FSH ao meio é importante para a manutenção da sobrevivência e da integridade ultra-estrutural de folículos pré-antrais caprinos.

- Após cultivo *in vitro* de longa duração (28 dias), o FSH estimula o crescimento de folículos primários e mantém a morfologia folicular.

7. PERSPECTIVAS

- As fortes evidências do envolvimento do FSH e do FGF-2, isoladamente ou em associação, no controle da foliculogênese inicial em caprinos, demonstradas neste trabalho, abrem novas possibilidades para a realização de vários estudos *in vitro* na espécie caprina. Desta forma, sistemas de cultivo *in vitro* podem ser desenvolvidos visando à otimização do crescimento folicular e posterior produção de oócitos maturos a partir de oócitos inclusos em folículos caprinos nos estádios iniciais de desenvolvimento.

- Tendo em vista os bons resultados obtidos com o FSH neste trabalho, a sua interação com outros hormônios e/ou fatores de crescimento pode ser útil para melhorar os sistemas de cultivo *in vitro* de folículos pré-antrais caprinos, otimizando a posterior obtenção de oócitos maturos nesta espécie. Além disso, torna-se importante verificar o papel do FSH na expressão destes hormônios e/ou fatores de crescimento no ovário caprino.

- O sistema de cultivo *in vitro* de longa duração apresentado neste trabalho pode ser de grande importância para analisar o efeito da adição de substâncias em diferentes momentos do cultivo ou do desenvolvimento folicular e ainda, para testar a utilização de um meio seqüencial no cultivo *in vitro* de folículos pré-antrais caprinos.

8. REFERÊNCIAS GERAIS

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