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FACULDADE DE VETERINÁRIA
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JAMILY BEZERRA BRUNO

CULTIVO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS CAPRINOS UTILIZANDO ANGIOTENSINA II (ANG II), FATOR DE CRESCIMENTO DO ENDOTÉLIO VASCULAR (VEGF) E PEPTÍDEO INTESTINAL VASOATIVO (VIP)

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

Área de Concentração: Reprodução e Sanidade Animal.

Linha de pesquisa: Reprodução e sanidade de pequenos ruminantes.

Orientador: Prof. Dr. José Ricardo de Figueiredo

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Aos meus pais,

ao meu marido,

à minha filha,

dedico.

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Resumo

Os objetivos do presente estudo foram avaliar o efeito de diferentes concentrações da angiotensina II (ANG II), fator de crescimento do endotélio vascular (VEGF) e peptídeo intestinal vasoativo (VIP) no desenvolvimento *in vitro* de folículos pré-antrais caprinos e verificar a localização do receptor 2 do VEGF (VEGFR-2) e a expressão dos receptores da ANG II (AGTR1 e AGTR2) em ovários caprinos. Para investigar a localização do VEGFR-2 e expressão do AGTR1 e AGTR2 no ovário caprino, foram empregadas as técnicas de imunohistoquímica e PCR em tempo real, respectivamente. Para avaliar a eficiência das substâncias supracitadas sobre a sobrevivência e o crescimento folicular, fragmentos de córtex ovariano foram cultivados *in vitro* por um ou sete dias em meio essencial mínimo suplementado (MEM^{+}) adicionado de diferentes concentrações das referidas substâncias. Antes e após o período de cultivo, os fragmentos de córtex ovariano foram fixados e processados para histologia clássica. Os folículos foram classificados quanto ao grau de desenvolvimento em primordiais ou em desenvolvimento, e quanto à sobrevivência em normais ou degenerados. O diâmetro folicular foi avaliado antes e após o cultivo. As técnicas de microscopia de fluorescência e microscopia eletrônica de transmissão (MET) foram utilizadas para melhor avaliar a viabilidade e morfologia desses folículos, respectivamente. Após definição das melhores concentrações de VIP, foi realizado um último experimento a fim de quantificar a expressão dos RNAm para o VIP em ovários caprinos através da técnica de PCR em tempo real, e avaliar a influência do VIP isoladamente ou em associação ao FSH sobre a sobrevivência e o desenvolvimento de folículos secundários caprinos isolados, bem como sobre a expressão do RNAm para o VIP e receptor de FSH (FSH-R) nesses folículos cultivados por seis dias. Os resultados do cultivo *in vitro* mostraram que após sete dias, 10 ng/mL de ANG II promoveu a manutenção da viabilidade e ultraestrutura de folículos pré-antrais caprinos. Os RNAm para AGTR1 e AGTR2 foram detectados em todas categorias e tipos foliculares estudados. No que se refere ao VEGF, as concentrações de 10 e 200 ng/mL foram mais eficientes para promover o crescimento e manter a viabilidade, respectivamente. Além disso, o receptor VEGFR-2 foi encontrado em oócitos de folículos em todos os estágios de desenvolvimento e em células da granulosa de folículos em crescimento. O VIP foi mais efetivo em promover o crescimento e manutenção da ultraestrutura folicular após sete dias de

cultivo quando utilizado na concentração de 10 ng/mL. A presença de RNAm para o VIP foi demonstrada em todas categorias foliculares em ovários de cabras, mostrando um aumento em seus níveis durante a transição de folículo primordial para secundário. No entanto, a utilização de VIP e/ou FSH não afetou o desenvolvimento de folículos secundários e não alterou os níveis de RNAm para FSH-R embora tenha reduzido os níveis de RNAm para o VIP após cultivo *in vitro* de folículos pré-antrais por seis dias. Diante disso, concluiu-se que ANG, VEGF e VIP apresentam importantes funções no desenvolvimento *in vitro* de folículos pré-antrais caprinos.

Palavras-chave: ANG II. VIP. VEGF. Pré-antral. Cultivo *in vitro*.

Abstract

The aims of this study were to evaluate the effect of different concentrations of angiotensin II (ANG II), vascular endothelial growth factor (VEGF) and vasoactive intestinal peptide (VIP) on the caprine preantral follicle development and to verify the localization of VEGF receptor 2 (VEGFR-2) and the expression of ANG II receptors (AGTR1 and AGTR2) in goat ovaries. In order to investigate the localization of VEGFR-2 and the expression of AGTR1 and AGTR2 in goat ovaries, it was used the immunohistochemistry and real-time PCR techniques, respectively. To evaluate the effect of the substances mentioned above on the survival and follicular growth, fragments of ovarian cortex were cultured *in vitro* for one or seven days in minimal essential medium (MEM^{+}) supplemented with different concentrations of these substances. Before and after the culture period, fragments of ovarian cortex were fixed and processed for histology. Follicles were classified according to their development as primordial or developing, and for survival in normal or degenerated. The follicular diameter was measured before and after culture. The fluorescence microscopy and transmission electron microscopy (TEM) techniques were used to better evaluate the viability and morphology of these follicles, respectively. After defining the best concentrations of VIP, a last experiment was conducted to quantify the expression of VIP mRNA in goat ovaries by real-time PCR, and evaluate the influence of VIP alone or in combination with FSH on the survival and development of isolated secondary follicles and on the expression of VIP and FSH receptor (FSH-R) mRNA in these follicles cultured for six days. The results of *in vitro* culture showed that after seven days, 10 ng/mL ANG II promoted the maintenance of viability and ultrastructure of goat preantral follicles. The AGTR1 and AGTR2 mRNA were detected in all categories and follicular types studied. With regard to VEGF, the concentrations of 10 and 200 ng/mL were more effective to promote follicular growth and to maintain the viability, respectively. Furthermore, the VEGFR-2 receptor was found in oocytes of follicles at all stages of development and in granulosa cells of growing follicles. VIP was more effective to promote the growth and the maintenance of follicular ultrastructure after seven days of culture when it was used at 10 ng/mL. The results of real-time PCR demonstrated the presence of VIP mRNA in all follicular categories of goat ovaries, showing an increase in VIP mRNA levels during the transition from primary to secondary follicle. However, the use of VIP and/or FSH

did not affect the development of secondary follicles and did not alter the FSH-R mRNA levels, although it reduced the VIP mRNA levels after *in vitro* culture of preantral follicles for six days. According to the results, it was concluded that ANG, VEGF and VIP have important roles in the *in vitro* development of caprine preantral follicles.

Keywords: ANG II. VIP. VEGF. Preantral. *In vitro* culture.

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LISTA DE ABREVIATURAS E SIGLAS

AC	: Adenylate cyclase (Adenilato ciclase)
AGTR 1	: Receptor 1 para ANG
AGTR 2	: Receptor 2 para ANG
AMH	: Anti-müllerian hormone (Hormônio anti-mülleriano)
ANG II	: Angiotensin II (Angiotensina II)
ANPT	: Angiopoietin (Angiopoietina)
ANOVA	: Analysis of variance (Análise de variância)
as	: antisense (anti senso)
ATP	: Adenosine-5'-triphosphate (Adenosina-5'- trifosfato)
bFGF	: Basic fibroblast growth factor (Fator de crescimento fibroblástico básico)
BMP-4, -7, -15:	: Bone morphogenetic protein-4, -7, -15 (Proteína morfogenética do osso-4, -7, -15)
BSA	: Bovine serum albumin (albumina sérica bovina)
Ca^{++}	: Íon cálcio
cAMP	:Cyclic adenosine-3',5'-monophosphate (Adenosina-3',5'-monofosfato cíclico)
CAPES	: Coordenação de Aperfeiçoamento do Pessoal de Nível Superior
cc	: células do cúmulus
cDNA	: DNA complementar
CG	: Células da granulosa
CGP	: Primordial germ cells (Células germinativas primordiais)
c-Kit	: Receptor para kit ligand
CL	: Corpo lúteo
CNPq	: Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	: Dióxido de Carbono
COCs	: Complexos cumulus-oócitos
CPqAM	: Centro de Pesquisa Aggeu Magalhães
CREB	: Element binding protein
CT	: Threshold cycle

d	: degenerated (degenerado)
DAB	: Diaminobenzidina
DNA	: Deoxyribonucleic acid (Ácido desoxirribonucléico)
dNTP	: Deoxy-nucleotide-triphophates (Desoxinucleotídeo trifosfato)
EGF	: Epidermal growth factor (Fator de crescimento epidermal)
EG-VEGF	:Endocrine gland-derived vascular endotelial growth factor (Fator de crescimento do endotélio vascular derivado da glândula endócrina)
er	: endoplasmic reticulum (retículo endoplasmático)
ET-1	: Endothelin-1 (Endotelina-1)
FGF-2 , -7, -10	: Fibroblast growth factor-2, -7, -10 (Fator de crescimento fibroblástico-2, -7, -10)
FGFR2	: Receptor 2 para FGF
Fig α	: Factor in the germline alpha (Fator de linha germinal α)
Fig.	: Figura
FINEP	: Financiadora de Estudos e Projetos
FIOCRUZ	: Fundação Oswaldo Cruz
FSH	: Follicle stimulating hormone (Hormônio folículo estimulante)
FSHR	: Receptor de FSH
FSHr	: FSH recombinante
g	: gravidade
G	: Proteína G
GAPDH	:Glyceraldehydes-2-phophate dehydrogenase (Gliceraldeído-2-fosfato desidrogenase)
gc	: granulosa cell (células da granulosa)
GDF-9	: Growth differentiation factor-9 (Fator de crescimento e diferenciação-9)
GH	: Growth hormone (Hormônio do crescimento)
GLM	: General linear models
GnRH	: Gonadotropin-releasing hormone (Hormônio liberador de gonadotrofinas)
h	: horas
HC	: Histologia clássica
HE	: Hematoxilina-eosina

IAA : Indol 3-acetic acid (Ácido 3-indol acético)

IGF-1 : Insulin like growth factor-1 (Fator de crescimento semelhante à insulina-1)

IGFR1 : Receptor para IGF-1

IgG : Immunoglobulin G (Imunoglobulina G)

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

IU : International units (Unidades internacionais)

K^+ : Íon potássio

kDa : Quilodaltons

KDR : Kinase insert domain receptor

KL : Kit ligand

L : Litro

LABOVIR : Laboratório de Virologia

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

LH : Luteinizing hormone (Hormônio luteinizante)

LIF : Leukemia inhibitory factor (Fator inibidor de leucemia)

LM : Light microscopy (Microscopia de luz)

MEM : Meio essencial mínimo

MEM^+ : Meio essencial mínimo suplementado

MET : Microscopia eletrônica de transmissão

m : mitocôndrias

M : Molar

mg : miligrama

mgc : mural granulosa cells (células da granulosa mural)

min. : minutos

mL : mililitro

mm : milímetros

mM : milimolar

MOIFOPA : Manipulação de oócitos inclusos em folículos ovarianos pré-antrais

mOsm/L : miliosmol/litro

mv : microvilli (microvilo)

n. : número

n	: núcleo
n	: normal
Na+	: Íon sódio
nc	: nucleolus (nucléolo)
ng	: nanograma
NGF	: Nerve growth factor (Fator de crescimento do nervo)
nm	: nanômetros
Nu	: Núcleo
NUBIS	: Núcleo de Biotecnologia de Sobral
O	: Oocyte (Oócito)
P < 0.05	: Probabilidade de erro menor do que 5%
P > 0.05	: Probabilidade de erro maior do que 5%
p.	: página
PACAP	: Pituitary adenylate cyclase-activating polypeptide (Polipeptídeo ativador da adenilato-ciclase pituitária)
PAS	: Periodic acid Schiff (Ácido periódico de Schiff)
PBS	: Phosphate buffer solution (Tampão fosfato salino)
PCR	: Polimerase chain reaction (Reação em cadeia de polimerase)
pH	: potencial hidrogeniônico
PK	: Protein kinase
PPGCV	: Programa de Pós-Graduação em Ciências Veterinárias
RENORBIO	: Rede Nordeste de Biotecnologia
RNA	: Ribonucleic acid (Ácido ribonucléico)
RNAase	: Enzima ribonuclease
RNAm	: Ribonucleic acid messenger (Ácido ribonucléico mensageiro
RT-PCR	:Reverse transcription- polimerase chain reaction (Reação em cadeia polimerase - transcriptase reversa)
s	: sense (senso)
SAS	: Statistical analysis system
SBAC	: Solução à base de água de coco
SEM	: Standard error of means (Erro padrão da média)

StAR	:Steroidogenic acute regulatory protein (Proteína reguladora aguda esteroidogênica)
tc	: theca cells (células da teca)
TEM	: Transmission electron microscopy (Microscopia eletrônica de transmissão)
TGF-β	: Transforming growth factor-β (Fator de crescimento transformante-β)
UBQ	: Ubiquitin (Ubiquitina)
UECE	: Universidade Estadual do Ceará
UFC	: Universidade Federal do Ceará
UnB	: Universidade de Brasília
USA	: Estados Unidos da América
v	: vesicles (vesículas)
VEGF	:Vascular endotelial growth factor (Fator de crescimento do endotélio vascular)
VEGFR-1	: Receptor 1 para VEGF
VEGFR-2	: Receptor 2 para VEGF
VIP	: Vasoactive intestinal peptide (Peptídeo intestinal vasoativo)
VPAC1-R	: Receptor 1 para VIP
VPAC2-R	: Receptor 2 para VIP
VPF	: Vascular permeability factor (Fator de permeabilidade vascular)
X	: Eixo das abscissas
Y	: Eixo das ordenadas
α-MEM	: Alpha minimal essential medium (Meio essencial mínimo alfa)
α-MEM^{+}	: Supplemented alpha minimal essential medium (Meio essencial mínimo alfa suplementado)
μg	: Microgramas
μL	: Microlitro
μm	: Micrômetro
μM	: Micromolar
%	: Porcentagem
~	: Aproximadamente
°C	: Graus Celsius

SUMÁRIO

1. INTRODUÇÃO

Diversas tecnologias de reprodução assistida, tais como a fecundação *in vitro*, a transferência de embriões, a transgenia e a clonagem, vêm sendo desenvolvidas com o intuito de aumentar a utilização do potencial reprodutivo em fêmeas mamíferas. Entretanto, a utilização em larga escala destes procedimentos depende da disponibilidade de oócitos maturos, que constituem apenas uma pequena porção dos oócitos presentes no ovário (MURUVI et al., 2005).

Considerando que os folículos pré-antrais contém a grande maioria dos oócitos presentes nos ovários (cerca de 90%), o cultivo, a maturação e a fecundação *in vitro* dos oócitos inclusos nestes folículos abrem inúmeras possibilidades para a produção de milhares de embriões, bem como para a compreensão das funções ovarianas. No entanto, a maioria desses folículos morre durante a vida pré- ou pós-natal, enquanto estão ainda em quiescência, e nunca entram na complexa via de desenvolvimento, que pode, ou não, culminar com a ovulação (KNIGHT; GLISTER, 2006).

Diante disso, surgiu a biotécnica de Manipulação de Oócitos Inclusos em Folículos Ovarianos Pré-antrais (MOIFOPA), com o objetivo de recuperar um grande número de oócitos inclusos nesses folículos e cultivá-los *in vitro* até sua completa maturação, prevenindo-os assim da morte (atresia) que ocorre *in vivo*. A MOIFOPA, também conhecida como "ovário artificial", visa mimetizar *in vitro* os eventos *in vivo* que culminam com a formação do folículo pré-ovulatório. Essa biotécnica poderá contribuir, decisivamente, no sentido de oferecer condições para a conservação e multiplicação de animais de alto valor genético e/ou em via de extinção (FIGUEIREDO et al., 2008).

Neste contexto, o desenvolvimento de um sistema de cultivo *in vitro* eficiente é um ponto crucial para permitir a obtenção de um grande número de oócitos inclusos em folículos pré-antrais. Entretanto, os mecanismos precisos que controlam o início e a progressão do crescimento folicular ainda estão sendo investigados. Dessa forma, uma melhor compreensão das diferentes substâncias que regulam o desenvolvimento normal dos oócitos e suas células foliculares circundantes é de suma importância para compreender a foliculogênese inicial e para o sucesso dos sistemas de cultivo *in vitro*, contribuindo assim para a posterior produção *in vitro* de embriões.

Dentre os fatores que parecem exercer influência na foliculogênese, podem-se destacar substâncias angiogênicas, tais como angiotensina II (ANG II) e o fator de crescimento do endotélio vascular (VEGF), bem como substâncias presentes em fibras nervosas, como o peptídeo intestinal vasoativo (VIP). Estudos têm demonstrado que esses fatores exercem importantes papéis no controle da foliculogênese (GIOMETTI et al., 2005; ABRAMOVICH; PARBOREL; TESONE, 2006; HULSHOF, 1995). Entretanto, a maioria das pesquisas realizadas com esses fatores é com grandes ruminantes, sendo ainda escassas as pesquisas em caprinos. Visto que os caprinos são considerados animais economicamente atrativos por serem importantes fontes de carne, leite e pele, especialmente na região Nordeste do país, o estudo da expressão e do efeito desses fatores no cultivo *in vitro* de folículos pré-antrais é de fundamental importância para aumentar o potencial reprodutivo desses animais.

Para uma melhor compreensão da importância deste trabalho, a revisão de literatura a seguir aborda aspectos relacionados ao ovário mamífero, foliculogênese e classificação dos folículos ovarianos, regulação da foliculogênese, MOIFOPA, com ênfase no cultivo *in vitro* de folículos pré-antrais caprinos e técnicas para análise folicular.

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 estágios 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).

2.2. Foliculogênese e classificação dos folículos ovarianos

A foliculogênese, evento iniciado na vida pré-natal na maioria das espécies, pode ser definida como o processo de formação, crescimento e maturação folicular, iniciando-se com a formação do folículo primordial e culminando com o estágio de folículo pré-ovulatório (VAN DEN HURK; ZHAO, 2005).

O folículo é considerado a unidade morfológica e funcional do ovário mamífero, cuja função é proporcionar um ambiente ideal para o crescimento e maturação do oócito (CORTVRINDT; SMITZ, 2001a), bem como produzir hormônios e peptídeos (BARNETT et al., 2006). O folículo é composto por um oócito circundado por células somáticas (granulosa e tecais), todavia, durante a foliculogênese, a morfologia folicular é alterada uma vez que o oócito cresce e as células da granulosa circundantes se diferenciam (BRISTOL-GOULD; WOODRUFF, 2006). Os folículos ovarianos podem ser classificados de acordo com o grau de evolução em pré-antrais (primordiais, intermediários, primários e secundários) ou antrais (terciários e pré-ovulatórios) (FIGUEIREDO et al., 2008).

2.3. Regulação da foliculogênese inicial ou fase pré-antral

2.3.1. Formação de folículos primordiais

Ainda na vida pré-natal, na maioria das espécies, os oócitos são circundados por células somáticas (células da pré-granulosa), formando os folículos primordiais (PEPLING; SPRADLING, 1998). Estes por sua vez, são constituídos por um oócito quiescente, esférico ou oval, circundado por células da pré-granulosa de formato pavimentoso. O núcleo do oócito é relativamente grande e ocupa uma posição de central a excêntrica. Nos folículos primordiais, a zona pelúcida ainda não é observada, verificando-se apenas uma justaposição do oócito e células da granulosa, sem nenhuma junção específica (LUCCI et al., 2001).

Um fator que parece ser crucial para o início da formação de folículos primordiais é o Fig-α (BARNETT et al., 2006), um fator transcricional derivado do oócito. Neurotrofinas podem também estar envolvidas na sinalização entre células somáticas e células germinativas no momento da formação dos folículos primordiais. Tal informação é baseada nas mudanças no padrão de expressão da neurotrofina-4 ao longo de toda a formação de folículos primordiais e a presença de seus receptores nos oócitos de ovários humanos e de ratos (ANDERSON et al., 2002). Estudos *in vitro* sugerem que o fator de crescimento do nervo (NGF) pode estar envolvido não apenas na inervação ovariana, mas também na formação folicular em ratas e camundongas (OJEDA et al., 2000). Além disso, o NGF participa na diferenciação de células mesenquimais para formar folículos primordiais em camundongas (DISSEN et al., 2001).

2.3.2. Ativação de folículos primordiais para primário

A ativação dos folículos primordiais é um processo que se dá pela transição dos folículos do “pool” de reserva (folículos quiescentes) para o "pool" de folículos em crescimento (primário, secundário, terciário e/ou pré-ovulatório) (RÜSSE, 1983). Durante a ativação o folículo primordial, o qual se apresenta circundado por uma camada de células da granulosa de morfologia pavimentosa, transforma-se em folículo primário, circundado por somente uma camada de células cubóides (VAN DEN HURK; BEVERS; BECKERS,1997).

Além da mudança da morfologia das células da granulosa, os volumes citoplasmático e nuclear do oócito aumentam consideravelmente (HIRSHFIELD, 1991).

O conhecimento sobre os fatores e mecanismos envolvidos na ativação dos folículos primordiais é escasso, o que pode ser devido às dificuldades de isolamento destes folículos (CAMPBELL, 2009). No entanto, sabe-se que um balanço entre fatores inibitórios e estimulatórios sistêmicos ou de origem local provavelmente regula a ativação dos folículos primordiais.

Em relação aos fatores inibitórios, estudos têm demonstrado que o hormônio anti-mileriano (AMH), membro da superfamília do fator de crescimento transformante-β (TGF-β), promove a inibição, mas não o bloqueio completo, do recrutamento de folículos primordiais para o "pool" de folículos em crescimento (DURLINGER; VISSER; THEMMEN, 2002). Camundongas com nocaute para o gene AMH mostraram um aumento na taxa de recrutamento de folículos primordiais, resultando em uma depleção prematura da reserva ovariana dos animais (DURLINGER et al., 1999). O papel inibitório do AMH sobre os folículos primordiais tem sido reportado também em vacas (GIGLI et al., 2005) e mulheres (CARLSSON et al., 2006). Além disso, a observação de que a expressão de AMH é ausente em folículos primordiais, mas detectada nas células da granulosa de folículos primários até folículos antrais iniciais (DURLINGER et al., 1999), sugere que folículos em crescimento podem exercer uma influência inibitória sobre folículos primordiais quiescentes.

Estudos *in vitro* com bovinos (BRAW-TAL; YOSSEFI, 1997), caprinos (SILVA et al., 2004a, CHAVES et al., 2010a), primatas (FORTUNE et al., 1998) e humanos (HOVATTA et al., 1997) demonstraram que a ativação de folículos primordiais ocorre espontaneamente, isto é, sem a adição de hormônios ou fatores de crescimento, o que pode ser devido à liberação de fatores estimulatórios ou prevenção da produção de fatores inibitórios pelo oócito ou estroma, células da granulosa ou células pré-tecais dentro do tecido (SILVA et al., 2006a).

No que se refere aos fatores estimulatórios, um candidato promissor para promover o início do crescimento de folículos primordiais é o kit ligand (KL). YOSHIDA et al. (1997) demonstraram que o bloqueio do receptor do KL (c-Kit) com anticorpos afeta o desenvolvimento de folículos primordiais. Estudos têm demonstrado que ambos o RNAm e a proteína do KL (SILVA et al., 2006b) e c-Kit (MOTRO; BERNSTEIN, 1993; CLARK et al.,

1996) são encontrados em oócitos e células da granulosa, respectivamente, de todos os estágios de desenvolvimento folicular. Quando adicionado ao meio de cultivo, o KL promoveu a ativação folicular, ou seja, passagem dos folículos do estágio primordial para primário em diferentes espécies (ratas: PARROT; SKINNER, 1999; NILSSON; SKINNER, 2004; DOLE; NILSSON; SKINNER, 2008; camundongas: HUTT; MCLAUGHLIN; HOLLAND, 2006; cabras: CELESTINO et al., 2010).

Da mesma forma, as proteínas morfogenéticas ósseas (BMPs), pertecentes à superfamília TGF-β, têm um papel importante na ativação folicular, uma vez que seu efeito tem sido demonstrado sobre a transição de folículos primordiais para primários em camundongas (LEE et al., 2001; MOORE; SHIMASAKI, 2005) e cabras (CELESTINO et al., dados não publicados). Ovelhas com mutações inativadoras no gene BMP-15 são completamente inférteis, tendo o desenvolvimento bloqueado em estágio de folículo primordial (JUENGEL et al. 2002; HANRAHAN et al., 2004). Além disso, a expressão da BMP-15 (ovelhas: MERY et al., 2007; vacas: BODENSTEINER et al., 1999; mulheres: SHIMASAKI et al., 2004) e dos seus receptores (ratas: ERICKSON; SHIMASAKI, 2003; cabras: SILVA et al., 2004b; vacas: FATEHI et al., 2005) em folículos primordiais sugere que esse fator possua um papel na ativação folicular. Em roedores, a BMP-15 estimulou o desenvolvimento *in vitro* de folículos primordiais e primários (OTSUKA et al., 2000; FORTUNE, 2003). Outros tipos de BMPs, como a BMP-4 e -7, também podem estar envolvidas na ativação folicular. Células tecais/intersticiais produzem BMP-4 e a exposição de ovários a anticorpos neutralizantes para BMP-4 resultou em redução do volume dos ovários, acompanhada da perda progressiva de folículos primordiais, e aumento da apoptose celular (NILSSON; SKINNER, 2003). Além disso, estudos têm mostrado que injeções de BMP-7 na bursa ovariana de ratas reduziram o número de folículos primordiais, e aumentaram de forma concomitante, o número de folículos primários, secundários e antrais (LEE et al., 2001; 2004), suportando uma ação parácrina positiva da BMP-7 derivada da teca sobre as células da granulosa de folículos pré-antrais em crescimento.

O fator de crescimento fibroblástico-2 (FGF-2), também pode influenciar positivamente a transição de folículos primordiais para primários. Os oócitos produzem FGF-2 que agem sobre células da granulosa e células da teca (NILSSON ; PARROTT; SKINNER, 2001). O FGF-2 está expresso em oócitos de folículos primordiais (NILSSON; SKINNER,

2004) e estudos *in vitro* têm mostrado que esse fator é eficiente em estimular a ativação de folículos primordiais de ratas (NILSSON; PARROTT; SKINNER, 2001), mulheres (GAROR et al., 2009) e cabras (MATOS et al., 2007a).

O fator inibitório de leucemia (LIF) também pode promover o desenvolvimento de folículos primordiais. Células da granulosa produzem LIF que age sobre o oócito, bem como sobre outras células da granulosa (NILSSON; KEZELE; SKINNER, 2002), A presença do LIF e de seus receptores foi detectada em folículos primordiais, além de folículos primários e secundários (ratas: NILSSON; KEZELE; SKINNER, 2002; mulheres: ABIR et al., 2004). A exposição de ovários ao anticorpo neutralizante do LIF diminuiu o desenvolvimento folicular espontâneo (NILSSON; KEZELE; SKINNER, 2002). O LIF promoveu um aumento na ativação folicular após cultivo *in vitro* de ovários de ratas (NILSSON; KEZELE; SKINNER, 2002) e de mulheres (ABIR et al., 2004).

Recentemente, estudos realizados com camundongas transgênicas demonstraram que a mutação de genes que controlam a expressão das gonadotrofinas, bem como de seus receptores, afeta diretamente não só a ovulação e formação de corpo lúteo, mas também o processo de formação de folículos primordiais, crescimento folicular e atresia (BARNETT et al., 2006). Em caprinos, o FSH promoveu a ativação folicular após cultivo de folículos primordiais inclusos em pequenos fragmentos de córtex ovariano (MATOS et al., 2007b; MAGALHÃES et al., 2009a,b). Apesar dos receptores para FSH serem expressos a partir de folículos primários (O'SHAUGHNESSY; DUDLEY; RAJAPAKSHA, 1996; SARAIVA et al., 2010a), esse hormônio pode agir indiretamente estimulando a síntese e secreção de fatores parácrinos nos grandes folículos. Além disso, trabalhos reportaram que o FSH estimula a expressão do KL (EPPIG, 2001), o qual é importante na regulação da foliculogênese inicial.

Por fim, algumas substâncias podem ainda estimular a expressão de outros fatores importantes para ativação folicular. Por exemplo, a insulina estimula o efeito do LIF e do KL e pode, portanto, ser um co-regulador na via de sinalização, controlando a transição de folículos primordiais, ou pode fornecer um suporte trófico e permitir que eles respondam ao máximo à sinalização do LIF (KEZELE; NILSSON; SKINNER 2002). Além disso, o LIF e o FGF-2 estimulam a expressão do RNAm do KL em células da granulosa (NILSSON; NILSSON; SKINNER, 2002; NILSSON; SKINNER, 2004).

A Figura 1 ilustra resumida e esquematicamente os diferentes fatores envolvidos na formação de folículos primordias e ativação folicular.

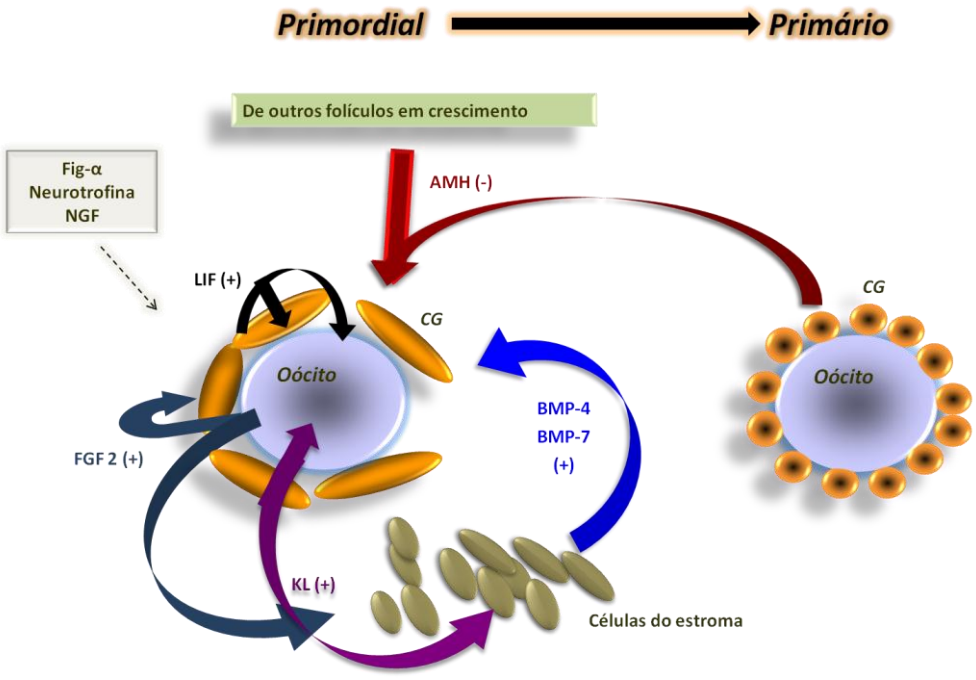

Figura 1. Desenho esquemático ilustrando os fatores envolvidos na formação e ativação folicular. CG: Células da granulosa; CT: Células da teca; (+): efeito estimulatório; (-): efeito inibitório. Adaptado de Knight and Glister (2006).

2.3.3. Progressão de folículo primário para secundário

A passagem de folículo primário para secundário é caracterizada pela proliferação das células da granulosa e um aumento no tamanho e conteúdo protéico do oócito (VAN DEN HURK; ZHAO, 2005). Os folículos secundários são formados por um oócito circundado por duas ou mais camadas de células da granulosa de morfologia cubóide. O núcleo do oócito assume uma posição excêntrica e as organelas começam a mover-se para a periferia. Com o desenvolvimento dos folículos, também aumenta o número de microvilos e inicia-se a formação da zona pelúcida (LUCCI et al., 2001). Neste estágio, o oócito entra em fase de crescimento extensivo, as células da granulosa ao redor se tornam mais proliferativas e uma camada de células da teca se desenvolve em torno das células da granulosa, a partir de células

do estroma intersticial. Após a formação da camada da teca, já se inicia a formação de vasos sanguíneos. Além disso, ocorre a formação de uma extensiva rede de junções do tipo gap, que são canais membranários que permitem a passagem de nutrientes, íons inorgânicos, segundo mensageiros e pequenos metabólitos entre as células (KIDDER; MHAWI, 2002). Uma complexa organização citoplasmática é necessária e depende tanto da produção de produtos de genes novos e organelas, como da modificação e redistribuição daquelas já existentes (PICTON; BRIGGS; GOSDEN, 1998). Dessa forma, como consequência do aumento da síntese de RNAm e de proteínas, o número de ribossomos, mitocôndrias e outras organelas celulares aumentam nos oócitos em crescimento. Além disso, muitas organelas mudam sua aparência e movem-se para a periferia do oócito (VAN DEN HURK; ZHAO, 2005). Essa progressão parece ser controlada, tanto por peptídeos e hormônios esteróides, bem como por fatores de crescimento intraovarianos (FORTUNE, 2003; VAN DEN HURK; ZHAO, 2005).

O crescimento de folículos pré-antrais *in vivo* ocorre independentemente das gonadotrofinas, uma vez que folículos pré-antrais de animais e humanos desenvolvem até o estágio antral com níveis mínimos de gonadotrofinas circulantes (GULYAS et al., 1977; HALPIN et al., 1986; HILLIER, 1994), sendo dessa forma atuante os fatores de crescimento. Entretanto, vários estudos *in vitro* têm sugerido que o FSH desempenha um importante papel no desenvolvimento de folículos pré-antrais (HIRAO et al., 1994; CAIN; CHATTERJEE; COLLINS, 1995; CORTVRINDT; SMITZ; VAN STEIRTEGHEM, 1997; SPEARS et al., 1998; WU; BENJAMIN; CARRELL, 2001). Mais recentemente, alguns autores têm demonstrado que o FSH aumenta o diâmetro de folículos pré-antrais bovinos (ITOH et al., 2002), suínos (WU; TIAN, 2007) e caprinos (MATOS et al., 2007b; MAGALHÃES et al., 2009a) após cultivo *in vitro*.

O fator de crescimento e diferenciação-9 (GDF-9) é um fator essencial para a formação de folículos secundários, uma vez que em ratas com nocaute de gene para o GDF-9, o desenvolvimento folicular além do estágio primário não foi observado (DONG et al., 1996). De forma semelhante, ovelhas com mutações espontâneas inativadoras do gene GDF-9 (HANRAHAN et al., 2004) e ovelhas imunizadas ativamente contra o GDF-9 não apresentaram folículos pré-antrais desenvolvidos no estágio de folículos secundários (JUENGEL et al., 2002). Em cabras, SILVA et al. (2004b) localizaram a proteína GDF-9 em oócitos de todos tipos foliculares e em células da granulosa de folículos primários,

secundários e antrais, mas não em folículos primordiais. A exposição *in vitro* de tecido ovariano ao GDF-9 em roedores (NILSSON; SKINNER, 2002, 2003; WANG; ROY, 2004), cabras (MARTINS et al., 2008) e mulheres (HREINSSON et al., 2002) promoveu a progressão do desenvolvimento de folículos primários. Além disso, outro estudo mostrou que 100 ng/mL de GDF-9 promoveu o crescimento de folículos pré-antrais *in vitro* e suprimiu a apoptose de células da granulosa (ORISAKA et al., 2006).

A BMP-15, outro fator derivado do oócito, parece também ser importante para o crescimento de folículos primários (JUENGEL et al., 2004), sendo as células da granulosa, as células alvo para o ligante BMP-15 (OTSUKA et al., 2000; GALLOWAY et al., 2000). A expressão dos genes que codificam a proteína BMP-15 são essenciais para os estágios iniciais de crescimento folicular e em particular para transição de folículos primários para secundários (MERY et al., 2007). Além disso, alguns autores têm descrito uma alta expressão de RNAm e/ou proteína BMP-15 em oócitos em crescimento ou completamente crescidos (SHIMASAKI et al., 2004; JUENGEL; MCNATTY, 2005; LI et al., 2008). TEIXEIRA et al. (2002) relataram que a expressão desse fator aumenta em uma correlação direta com o crescimento folicular. Em cabras, foi demonstrado um aumento significativo nos níveis de RNAm para BMP-15 durante a transição de folículos primários para secundários, bem como um aumento no número desses folículos após cultivo *in vitro* (CELESTINO et al., dados não publicados).

A expressão da ativina, receptores de ativina, e folistatina (proteína ligada à ativina) tem sido detectada em folículos primários e secundários (RABINOVICI, 1991; MCNATTY, 2000; PANGAS et al., 2002; DRUMMOND et al., 2002; SILVA et al., 2006a). Sob condições *in vitro*, a ativina estimula o crescimento de folículos uni e/ou multilaminares de vacas (HULSHOF et al., 1997), ratas (ZHAO et al., 2001), camundongas (SMITZ et al., 1998) e cabras (SILVA et al., 2006a).

O fator de crescimento epidermal (EGF) é conhecido como um potente fator mitogênico, podendo estimular a proliferação de diferentes tipos celulares (TOYODA et al., 2007). Estudos têm demonstrado a expressão da proteína e RNAm para o EGF e seus receptores em oócitos e células da granulosa de folículos pré-antrais e antrais de ratas, vacas, mulheres, porcas, camundongas e cabras (FENG; KNECHT; CATT, 1987; LONERGAN et al., 1996; QU et al., 2000; SINGH; RUTLEDGE; ARMSTRONG, 1995; HILL et al., 1999;

SILVA et al., 2006c). Outros estudos com cultivo *in vitro* de folículos pré-antrais mostraram que o EGF promove a proliferação de células da granulosa de porcas, roedores e mulheres (MORBECK; FLOWERS; BRITT, 1993; GOSPODAROWICZ; BIALECKI, 1979) e aumenta o diâmetro folicular de vacas, roedores, mulheres e cabras (GUTIERREZ et al., 2000; ROMANO et al., 1994; ROY; KOLE, 1998; SILVA et al., 2004c).

Outro fator mitogênico também importante para a transição de folículos primários para secundários é o FGF-2. Tal fator é importante no controle de uma ampla variedade de funções ovarianas, incluindo mitose de células da granulosa (ROBERTS; ELLIS, 1999), esteroidogênese (VERNON; SPICER,1994), diferenciação (ANDERSON; LEE, 1993) e apoptose (TILLY et al., 1992). Já foi observado que o FGF-2 está expresso em oócitos de folículos primordiais e células da granulosa e da teca de folículos em crescimento de vacas (VAN WEZEL et al., 1995; YAMAMOTO et al., 1997; NILSSON; PARROTT; SKINNER, 2001). Além disso, receptores para a proteína e RNAm para o FGF-2 têm sido demonstrados em folículos em crescimento de vacas (WANDJI; FORTIER; SIRARD, 1992) e ratas (SHIKONE; YAMOTO; NAKANO, 1992; ASAKAI et al., 1993, 1995). Wandji et al. (1996) mostraram que a utilização de 50 ng/mL de FGF-2 aumentou o diâmetro folicular e estimulou a proliferação de células da granulosa em folículos bovinos cultivados por 6 dias. Outros autores também observaram que o FGF-2 promoveu aumento no diâmetro folicular e a proliferação das células da granulosa de vacas (NUTTINCK et al., 1996), gatas (JEWGENOW, 1996) e ratas (NILSSON; PARROTT; SKINNER, 2001).

2.3.4. Progressão de folículo secundário para antral

Após o crescimento dos folículos secundários e organização das células da granulosa em várias camadas, ocorre a formação de uma cavidade repleta de líquido denominada antro. A partir deste estágio, os folículos passam a ser denominados terciários ou antrais. O fluido folicular que preenche esta cavidade contém água, eletrólitos, proteínas séricas e alta concentração de hormônios esteróides secretados pelas células da granulosa (BARNETT et al., 2006). 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. O desenvolvimento dos

folículos antrais é caracterizado por uma fase de crescimento, recrutamento, seleção e dominância (VAN DEN HURK; ZHAO, 2005) sendo a formação de folículos pré-ovulatórios um pré-requisito para a ovulação e formação do corpo lúteo (DRUMMOND, 2006).

Vários experimentos *in vivo* e *in vitro* têm mostrado que o número de folículos secundários, seu tamanho, número de células e taxa de atresia são influenciados pelas gonadotrofinas, das quais o FSH é o fator de sobrevivência predominante (VAN DEN HURK; BEVERS; BECKERS,1997; VAN DEN HURK et al., 2000). O LH também parece ser importante para o desenvolvimento de folículos secundários, uma vez que, através de seus receptores, que podem ser demonstrados na camada da teca de folículos secundários de ratas, ele desencadeia a biossíntese de andrógenos os quais são capazes de estimular a formação de receptores para FSH nas células da granulosa e assim, podem ampliar os efeitos do FSH em folículos secundários (VAN DEN HURK; BEVERS; DIELEMAN, 1999; VAN DEN HURK et al., 2000). A síntese de andrógenos estimulada pelo LH é amplificada por neurotrofinas (VAN DEN HURK; ZHAO, 2005).

O hormônio do crescimento (GH) é outro fator endócrino que pode promover o desenvolvimento de folículos secundários. A presença de RNAm para o receptor de GH tem sido detectada em folículos pré-antrais de ratas (ZHAO et al., 2002). Estudos *in vitro* usando GH têm demonstrado que esse fator desempenha um importante papel controlando as fases iniciais do desenvolvimento folicular. A adição de GH ao meio de cultivo *in vitro* de folículos pré-antrais estimulou a produção de estradiol e a proliferação das células da granulosa e da teca em murinos (KOBAYASHI et al., 2000), além de promover um aumento no diâmetro de folículos pré-antrais de camundongas cultivados *in vitro* (LIU et al., 1998; KIKUCHI et al., 2001).

Além dos hormônios citados, a família FGF, que é composta por um grupo de fatores, é potencialmente importante para o crescimento folicular (BURATINI et al., 2005). McGEE et al. (1999) demonstraram uma supressão dose-dependente da apoptose de células da granulosa e estímulo do crescimento de folículos secundários de ratas cultivadas com o FGF-7. O FGF-2, um potente fator angiogênico (REYNOLDS; REDMER, 1998; PLENDL, 2000), exerce um importante papel na foliculogênese, uma vez que a função ovariana é dependente do estabelecimento e contínuo remodelamento de um complexo sistema vascular, que possibilita os folículos receberem suprimento adequado de nutrientes, oxigênio e suporte

hormonal (ROBINSON et al., 2009). Além do FGF-2, outros fatores angiogênicos também parecem ser importantes para o desenvolvimento folicular nessa fase, dos quais podemos destacar o VEGF e a ANG II. No primeiro Capítulo desta tese, será mostrada uma revisão detalhada acerca da importância de fatores angiogênicos durante o desenvolvimento folicular, incluindo o FGF-2, VEGF e ANG II.

Neurotrofinas e neurotransmissores, como por exemplo, NGFs e VIP respectivamente, também parecem ser bons candidatos para estimular o desenvolvimento folicular precoce, inclusive de folículos secundários. Ovários de camundongas com mutações deletérias ao NGF exibiram acentuada redução do número de folículos primários e secundários, enquanto a proliferação de células da granulosa foi visivelmente reduzida após o cultivo *in vitro* (DISSEN et al., 2001). Já o VIP tem sido apontado como um importante regulador no desenvolvimento de folículos secundários de ratas, bovinos e primatas (VAN DEN HURK et al., 2000; MCGHEE; HSUEH, 2000) e pode estar envolvido na função esteroidogênica das células da granulosa de roedores (VAN DEN HURK; ZHAO, 2005). O segundo Capítulo desta tese refere-se a uma revisão acerca do envolvimento do VIP na fisiologia ovariana.

A Figura 2 ilustra resumida e esquematicamente os diferentes fatores envolvidos no crescimento folicular a partir do estágio primário até antral.

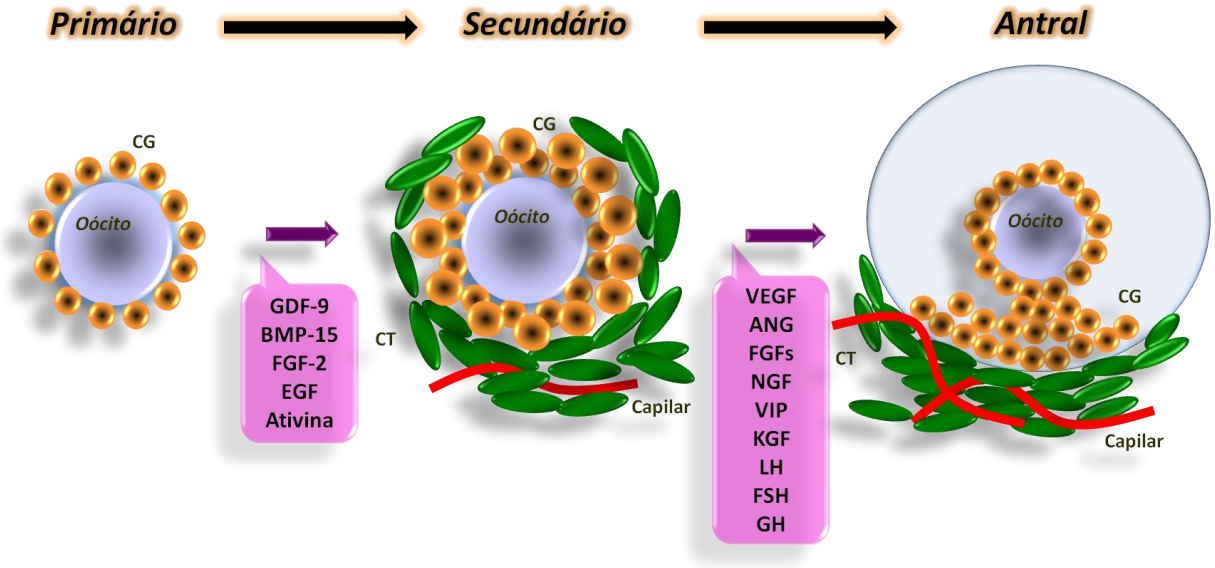

Figura 2. Desenho esquemático ilustrando os fatores envolvidos no crescimento folicular a

partir do estágio primário até antral. CG: Células da granulosa; CT: Células da teca. Adaptado de Knight and Glister (2006).

2.4. Biotécnica de MOIFOPA

Tendo em vista que o ovário mamífero contém milhares de oócitos inclusos em folículos pré-antrais e que poucos destes folículos desenvolvem-se até o estágio de folículo pré-ovulatório, 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 um grande número de oócitos inclusos nos folículos pré-antrais, seguido das etapas de conservação (resfriamento e/ou criopreservação) e/ou cultivo *in vitro* até o estágio de maturação folicular (FIGUEIREDO et al., 2008), representando uma alternativa para o fornecimento de uma população homogênea de oócitos para as biotécnicas de fecundação *in vitro* e clonagem (TELFER, 1996).

Nesse contexto, o cultivo *in vitro* de folículos pré-antrais vem sendo largamente empregado com o intuito de promover a multiplicação e a posterior diferenciação das células da granulosa e assegurar o crescimento e a maturação de seus oócitos, para que os mesmos estejam aptos à produção de embriões (FIGUEIREDO et al., 2008). A secção a seguir abordará os diversos sistemas de cultivo *in vitro* empregados.

2.5. Cultivo *in vitro* de folículos pré-antrais

No que se refere aos sistemas de cultivo *in vitro*, os folículos ovarianos podem ser cultivados inclusos no próprio tecido ovariano (*in situ*) ou na forma isolada. O cultivo *in situ* é considerado um modelo prático uma vez que a manipulação é rápida e nesse cultivo, a manutenção da integridade tridimensional dos folículos e a interação destes com células do estroma são mantidas. Já o cultivo de folículos isolados permite uma melhor perfusão dos nutrientes e o acompanhamento individual dos folículos.

Em roedores, a pequena dimensão dos ovários possibilita o cultivo do órgão inteiro, o que tem sido bastante útil para o estudo da foliculogênese inicial em pequenos mamíferos (FORTUNE, 2003). Por outro lado, em animais domésticos de médio e grande porte, devido às grandes dimensões dos ovários, não é possível utilizar este modelo. Para estes animais, o

cultivo de pequenos fragmentos de córtex ovariano, rico em folículos primordiais, tem sido realizado para o estudo da ativação e crescimento de folículos primários (caprinos: SILVA et al., 2004a; bovinos: BRAW-TAL; YOSSEFI, 1997; babuínos: WANDJI et al., 1997; humanos: HOVATTA et al.,1997). O cultivo de pequenos fragmentos de córtex ovariano tem a vantagem de manter a integridade celular e facilitar a perfusão do meio para o tecido ovariano (TELFER, 1996).

Grande progresso já tem sido observado no cultivo *in vitro* de folículos pré-antrais em diferentes espécies animais. Em felinos (JEWGENOW; STOLTE, 1996) e marsupiais (BUTCHER; ULLMAN, 1996), observou-se o crescimento de folículos pré-antrais isolados, porém, sem a formação de antro. Em humanos, bovinos e caninos, folículos secundários isolados cresceram *in vitro* o estágio antral (ROY; TREACY, 1993; GUTIERREZ et al., 2000; SERAFIM et al., 2010). Resultados mais satisfatórios foram obtidos com suínos (WU; TIAN, 2007), bubalinos (GUPTA et al., 2008), ovinos (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010) e caprinos (SARAIVA et al., 2010b; MAGALHÃES et al., 2011), em que se alcançou a produção de embriões após cultivo *in vitro* de grandes folículos secundários. Entretanto, os melhores resultados do cultivo folicular foram obtidos em roedores, sendo observada a obtenção de crias viáveis a partir do cultivo de oócitos provenientes de folículos pré-antrais de camundongas, nos quais o oócito adquiriu competência para maturação, fertilização e desenvolvimento embrionário (EPPIG; SCHROEDER, 1989; O'BRIEN; PENDOLA; EPPIG, 2003). Tal crescimento foi obtido através do sistema de cultivo em dois passos: (1) cultivo de ovários inteiros para obtenção da transição de folículo primordial para primário e (2) isolamento e posterior cultivo de folículos primários e secundários.

Como pode-se observar, um grande número de sistemas de cultivo *in vitro* têm sido desenvolvidos para promover o crescimento de folículos de diferentes espécies. Além das diferenças dos sistemas de cultivo, existem também muitas diferenças espécies-específicas, como: i) o período para a conclusão da foliculogênese e oogênese; ii) o tamanho dos folículos ovulatórios e oócitos maduros, e iii) as diferenças na natureza, concentrações e efeitos dos fatores de crescimento que influenciam o folículo e produção de oócitos *in vivo*. Essas diferenças são altamente relevantes no desenvolvimento de sistemas que suportam o crescimento *in vitro* completo e maturação do oócito (PICTON et al., 2008). No entanto, em

todos os protocolos, algumas características são vitais para otimizar o crescimento *in vitro*: o fornecimento de nutrientes, eletrólitos, antioxidantes, aminoácidos, substratos energéticos, vitaminas, hormônios e fatores de crescimento (PICTON et al., 2008). Diante disso, nossa equipe vem testando uma série de substâncias no cultivo *in vitro* de folículos pré-antrais caprinos.

2.6. Cultivo *in vitro* de folículos pré-antrais caprinos *in situ*

Os primeiros estudos do LAMOFOPA foram realizados com o intuito de se obter um meio de base para o cultivo *in vitro* de folículos pré-antrais caprinos. SILVA et al. (2004a) ao comparar a solução à base de água de coco (SBAC) com o Meio Essencial Mínimo (MEM), verificaram que o MEM apresentou melhores taxas de sobrevivência e ativação folicular. Esses resultados foram reforçados por MARTINS et al. (2005), em que foi observado uma elevada taxa de degeneração folicular após utilização de SBAC. Além disso, SILVA et al. (2004a) observaram que a adição de suplementos ao meio de base MEM, como hipoxantina e substratos energéticos, tais como piruvato e glutamina, e ITS (Insulina, Transferrina e Selênio) foram considerados importantes para manter a sobrevivência de folículos pré-antrais caprinos cultivados *in vitro*.

Existiu ainda uma clara necessidade de verificar a temperatura e tempo de transporte ideal para ovários caprinos para posterior cultivo *in vitro*. Então, CHAVES et al. (2008) ao testar diferentes tempos e temperaturas de transporte, verificaram que os fragmentos ovarianos resfriados a 4º C por 4 h durante o transporte são melhores para manter a viabilidade e aumentar o crescimento folicular durante o cultivo *in vitro*.

Nossa equipe testou ainda o efeito de alguns hormônios. No que se refere à utilização do FSH (MATOS et al., 2007b; MAGALHÃES et al., 2009a,b) e do estradiol (LIMA-VERDE et al., 2010a), estes apresentaram efeitos positivos na sobrevivência, isto é, percentagens de folículos pré-antrais morfologicamente normais superiores ao controle cultivado (MEM^{+}), bem como na manutenção da integridade ultraestrutural. Tais hormônios tiveram ainda um efeito adicional sobre a ativação e o crescimento folicular. No que se refere ao LH, embora ele tenha mantido a integridade ultraestrutural dos folículos quando adicionado ao meio de cultivo, as percentagens de folículos pré-antrais morfologicamente

normais foram similares ao controle cultivado após sete dias (SARAIVA et al., 2008). Dentre os hormônios testados, o GH na concentração de 10 ng/mL, além de apresentar efeito benéfico para sobrevivência, também foi eficiente para a transição de folículos primordiais para primários (MARTINS et al., 2010). Por outro lado, a androstenediona não apresentou nenhum efeito adicional sobre a sobrevivência de folículos pré-antrais caprinos (LIMA-VERDE et al., 2010b).

Quando os fatores de crescimento foram testados, foi obsevado que dois membros da família FGF, o FGF-10 (CHAVES et al., 2010b) e o FGF-2 (MATOS et al., 2007a), aumentaram a percentagem de folículos normais e promoveram a ativação folicular em relação ao controle cultivado, respectivamente. No entanto, fatores como o KL e a BMP-15, não apenas se destacaram em relação ao controle cultivado, como também apresentaram valores de sobrevivência similares ao controle fresco (CELESTINO et al., 2010; CELESTINO et al., dados não publicados). Todos esses fatores foram também eficientes em manter a integridade ultraestrutural dos folículos após sete dias de cultivo. No que se refere ao crescimento, a ativina A (SILVA et al., 2006a), o FGF-10 (CHAVES et al., 2010b) e a BMP-15 (CELESTINO et al., dados não publicados) aumentaram o diâmetro de folículos pré-antrais caprinos após cultivo *in vitro*. Alguns fatores de crescimento, tais como EGF, KL, BMP-15 e IGF-1 aumentaram a percentagem de folículos primários (CELESTINO et al., 2009; 2010; dados não publicados; MARTINS et al., 2010). No entanto, apenas a BMP-15 e o GDF-9 aumentaram a percentagem de folículos secundários após sete dias de cultivo (CELESTINO et al., dados não publicados; MARTINS et al., 2008), porém com taxas relativamente baixas (~ 20%).

Dentre os antioxidantes testados, pode-se citar o α-tocoferol e a ternatina, no entanto, quando utilizados nas concentrações de 5, 10 ou 15 μM, não foram eficientes para o desenvolvimento de folículos pré-antrais caprinos (LIMA-VERDE et al., 2009). Por outro lado, o ácido ascórbico (50 μg/mL) associado ao FSH (50 ng/mL) obteve elevadas percentagens de folículos pré-antrais normais, os quais ainda mantiveram a integridade ultraestrutural, bem como promoveram a ativação e o crescimento de folículos pré-antrais caprinos (ROSSETTO et al., 2009). Outras interações também foram benéficas, tais como o FSH com FGF-2; e FSH com EGF, as quais aumentaram o diâmetro folicular (MATOS et al.,

2007c; SILVA et al., 2004c) e IGF-1 com GH, os quais promoveram a transição de folículos primordiais para intermediários (MARTINS et al., 2010).

Por outro lado, outros fatores testados (BMP-7: ARAÚJO et al., 2010a; NGF: CHAVES et al., 2010a), não foram eficazes em melhorar a percentagem de folículos pré-antrais morfologicamente normais em relação ao controle cultivado, apesar de terem mantido a ultraestrutura dos folículos após análise por microscopia eletrônica de transmissão (MET). Substâncias, tais como o ácido 3-indol-acético (IAA) (MATOS et al., 2006) e soro fetal bovino (BRUNO et al., 2008), nem mesmo foram capazes de manter a ultraestrutura dos folículos cultivados.

Como se pode observar, apesar de folículos inclusos em tecido ovariano serem mantidos saudáveis, ativarem e crescerem, poucos desses folículos progridem até o estágio de primário e secundário. Na tentativa de melhorar tais resultados, outras substâncias, isoladamente ou em associação, vêm sendo testadas.

2.7. Cultivo *in vitro* de folículos pré-antrais caprinos isolados

Simultaneamente aos experimentos com cultivo *in vitro* de fragmentos ovarianos, experimentos voltados para o cultivo de folículos pré-antrais isolados vêm sendo realizados, com o objetivo de manter a viabilidade, promover a formação da cavidade antral, estimular o crescimento e capacitar os oócitos inclusos nesses folículos a serem maturados e fecundados *in vitro*.

Outros pesquisadores, trabalhando com essa mesma espécie, desenvolveram um sistema de cultivo de folículos secundários isolados, capaz de promover a formação do antro (ZHOU; ZHANG, 2000), bem como manter a sobrevivência e aumentar o diâmetro folicular (ZHOU; ZHANG, 2005). Nosso grupo também foi capaz de obter tais resultados, uma vez que a utilização do meio de base foi capaz de manter a sobrevivência e atingir estágio antral, e a adição de FSH no meio ainda promoveu um aumento na taxa de crescimento folicular (RODRIGUES et al., 2010).

Um grande progresso foi alcançado por nosso grupo, o qual além de estabelecer um sistema de cultivo capaz de estimular o crescimento e formação antral assegurou a retomada da meiose (SILVA et al., 2010). Nesse experimento foram testadas duas concentrações de oxigênio (5 e 20%), em que a concentração de 20% foi mais eficiente em promover o

crescimento folicular e a retomadada da meiose (16,7%) de oócitos oriundos de folículos pré-antrais crescidos *in vitro*.

MAGALHÃES et al. (2010), ao avaliar o efeito de diferentes intervalos de troca de meio (troca a cada 2 ou 6 dias) sobre o desenvolvimento de folículos pré-antrais caprinos isolados, verificaram que sob as mesmas condições, o intervalo de troca de meio a cada 2 dias foi mais eficiente sobre a viabilidade folicular e ainda proporcionou a retomada da meiose (12,5%).

Ainda no que se refere à troca de meio, ARAÚJO et al (2010b) avaliaram a influência de três diferentes protocolos de troca de meio. Nesse trabalho, a adição periódica de meio (5 μl de meio fresco a cada dois dias) foi recomendada por ser mais prática, manter a viabilidade e promover o desenvolvimento *in vitro* de folículos pré-antrais caprinos.

Outras formas de cultivo também foram testadas, como o cultivo de folículos de forma individual ou em grupos. DUARTE et al. (2010) observaram que o co-cultivo de folículos antrais com folículos pré-antrais afetou negativamente o desenvolvimento folicular. Por outro lado, o cultivo de folículos pré-antrais em grupo mostrou taxas superiores de sobrevivência e crescimento em relação aos folículos cultivados individualmente, além de estimular a retomada da meiose em 9,09%. No entanto, essa percentagem também foi muito baixa, o que refletiu a necessidade de melhorar tal sistema de cultivo.

Na tentativa de melhorar tal sistema, e sabendo que os folículos possuem diferentes níveis de exigências nos diferentes estágios de desenvolvimento, foi desenvolvido um meio de cultivo seqüencial utilizando FSH. Esse hormônio foi utilizado em concentrações aumentadas ao longo do cultivo (FSH 100 ng/mL do dia 0 a 6; FSH 500 ng/mL do dia 6 a 12; FSH 1000 ng/mL do dia 12 a 18), promovendo altas percentagens de retomada da meiose (aproximadamente 62%) (SARAIVA et al., 2010a). Diante desse resultado promissor, novos experimentos foram realizados utilizando tal sistema de cultivo.

De fato, a partir da utilização de um meio de base que continha FSH seqüencial suplementado com LH e EGF (SARAIVA et al., 2010b) ou GH (MAGALHÃES et al., 2011), foi possível a obtenção dos primeiros embriões caprinos oriundos de folículos secundários isolados cultivados *in vitro*.

Apesar dos grandes avanços obtidos pela nossa equipe, o rendimento referente à produção de oócitos maturos, bem como a obtenção de embriões a partir de folículos pré-

antrais caprinos ainda é extremamente baixo, o que reforça a necessidade de melhorar tal sistema de cultivo.

2.8 Técnicas para análise folicular

Diferentes técnicas podem ser utilizadas para análise folicular após o cultivo *in vitro* de folículos pré-antrais. Dentre elas, podemos destacar aquelas que permitem a avaliação da qualidade folicular, como por exemplo, a histologia clássica (HC), a microscopia eletrônica de transmissão (MET) e a microscopia de fluorescência. Além disso, existem ainda as técnicas de biologia molecular que permitem o estudo da expressão de genes que codificam ligantes e/ou receptores de diferentes substâncias importantes para a foliculogênese, contribuindo para uma melhor elucidação desse processo. A seguir, será abordada brevemente cada uma dessas técnicas.

2.8.1 Histologia Clássica

A HC é uma técnica importante para avaliação de folículos pré-antrais após cultivo *in vitro*, pois além de permitir uma análise quantitativa, ou seja, de um grande número de folículos cultivados, permite ainda verificar a mudança na morfologia das células da granulosa de pavimentosa para cúbica, por ocasião da ativação folicular, além de analisar a integridade morfológica do oócito e das células da granulosa. Tal técnica permite, portanto a classificação dos folículos quanto ao seu estágio de desenvolvimento (primordial, intermediário, primário ou secundário), e ainda quanto às suas características morfológicas (normais ou atrésicos). Entretanto, a HC possui como desvantagens não permitir a avaliação da integridade de membranas e das organelas citoplasmáticas. Vale salientar que tal técnica pode ser realizada tanto em folículos isolados, como naqueles inclusos em fragmentos de córtex ovariano (MATOS et al., 2007d).

2.8.2 Microscopia Eletrônica de Transmissão

A MET é considerada uma técnica qualitativa e acurada, capaz de permitir a avaliação

da integridade de membranas celulares e organelas citoplasmáticas (SALEHNIA; MOGHADAM; VELOJERDI, 2002). Ela se mostra como uma ferramenta valiosa para detectar modificações morfológicas iniciais devido à atresia, as quais podem ser observadas apenas em nível ultraestrutural, antes de se tornarem mais visíveis e possíveis de serem identificadas por microscopia óptica. Tal técnica é, portanto, capaz de verificar a qualidade do oócito e das células da granulosa (LUCCI et al., 2001; LOPES et al., 2009). Com esta técnica, é possível ainda avaliar a atresia, ou seja, a morte do folículo tanto por apoptose (STALDEMANN; LASSMAN, 2000) como pelo processo degenerativo de necrose (MARTINEZ-MADRID et al., 2007).

2.8.3 Microscopia de Fluorescência

Outra técnica empregada para avaliar a viabilidade folicular após o cultivo *in vitro* é a microscopia de fluorescência, a qual utiliza marcadores fluorescentes, que quando excitados com radiação de baixo comprimento de onda, absorvem energia e emitem luz de comprimento de onda maior (JUNQUEIRA; CARNEIRO, 2005). A microscopia de fluorescência é considerada uma técnica confiável, prática e rápida (CORTVRINDT; SMITZ, 2001b; LOPES et al., 2009). Recentemente, estudos vêm sendo realizados empregando a técnica de fluorescência para avaliar a viabilidade folicular após o cultivo *in vitro* (ROSSETTO et al., 2009; SILVA et al., 2010; MAGALHÃES et al., 2010). Nesses trabalhos, os folículos foram analisados por fluorescência com base na detecção simultânea de células vivas e mortas marcadas por calceína-AM e pelo etídio homodímero-1, respectivamente. Enquanto a primeira sonda detecta atividade da esterase intracelular, enzima característica de células viáveis, a outra cora ácidos nucléicos em células não-viáveis, com ruptura na membrana plasmática (LOPES et al., 2009).

2.8.4 Biologia Molecular

A identificação e quantificação dos níveis de RNAm de diferentes substâncias (ligantes e receptores) que atuam durante a foliculogênese também é considerada uma importante ferramenta para auxiliar na compreensão desse processo, uma vez que permite

detectar alterações nos padrões de expressão gênica que ocorrem em resposta a fenômenos relacionados à sobrevivência, ao crescimento e à diferenciação celular. Desta forma, a capacidade de quantificar os níveis de transcrição de genes específicos é fundamental para garantir a completa investigação das funções foliculares (ZAMORANO; MAHESH; BRANN, 1996). Dentre as técnicas de biologia molecular, podem ser citadas aquelas que detectam, localizam ou identificam os ácidos nucléicos (hibridização *in situ* Southern e Northern Blotting) ou proteínas (Western Blotting e imunohistoquímica), as que podem efetuar a quantificação do DNA (Reação em Cadeia de Polimerase - PCR) ou do RNA (Reação de Transcriptase Reversa de Cadeia da Polimerase - RT-PCR), as que permitem a quantificação da expressão do RNAm mesmo em uma mistura complexa de RNA total (ensaios de proteção de ribonuclease) ou que possibilitem a análise da expressão de milhares de genes simultaneamente (Microarranjos de DNA).

Atualmente, a técnica mais utilizada para quantificar a expressão de RNAm é a RT-PCR em tempo real (qRT-PCR) (KREUZER; MASSEY, 2002). A qPCR, uma variante da RT-PCR convencional, permite uma análise precisa da quantificação da expressão gênica em determinado tecido ou amostra biológica. Esse método utiliza um sistema fluorescente em plataforma, capaz de detectar a luz oriunda da reação de amplificação de um determinado gene no momento real da amplificação (BUSTIN, 2002).

2.8.5 Imunohistoquímica

A imunohistoquímica também vem sendo utilizada com freqüência para avaliar a expressão de fatores de crescimento e hormônios, bem como seus receptores, presentes nos ovários, além de ser utilizada também para verificar a proliferação das células da granulosa. Esta técnica representa um conjunto de procedimentos diagnósticos que utilizam anticorpos (policlonais ou monoclonais) como reagentes de grande especificidade para a detecção de antígenos que marcam estruturas em cortes histológicos ou em células cultivadas (ABIR et al., 2006).

3. JUSTIFICATIVA

Os folículos primordiais representam cerca de 90% de todos os folículos presentes no ovário. Entretanto, os mecanismos responsáveis pelo seu crescimento ainda não são completamente elucidados. 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*, surge a biotécnica de MOIFOPA que permite a recuperação de dezenas a milhares de folículos primordiais a partir de um único ovário. Tais folículos poderiam ser criopreservados para posterior utilização e/ou cultivados *in vitro*.

Dessa forma, sabendo-se da grande relevância social e econômica que a espécie caprina representa para várias regiões do mundo, especialmente o Nordeste Brasileiro, e da escassez de informações sobre as condições necessárias e os fatores envolvidos no crescimento e na maturação oocitária *in vitro,* é de extrema importância o desenvolvimento de um sistema de cultivo *in vitro* capaz de manter a sobrevivência folicular, ativar esses folículos e assegurar o seu posterior crescimento, otimizando o aproveitamento do potencial oocitário desses animais e incrementando a eficiência da reprodução animal.

Alguns estudos têm investigado o efeito de diversos hormônios e fatores de crescimento no cultivo *in vitro* de folículos pré-antrais de animais de laboratórios e animais domésticos. Entretanto, os efeitos de diferentes concentrações de ANG II, VEGF e VIP ainda não foram avaliados no cultivo *in vitro* de folículos pré-antrais caprinos. Para este fim, as técnica de histologia clássica, microscopia de fluorescência e MET foram empregadas para determinar a qualidade de folículos pré-antrais caprinos cultivados *in vitro* e, conseqüentemente, melhor avaliar a eficiência dos meios de cultivo testados. Além disso, foram utilizados ensaios imunohistoquímicos e ferramentas da biologia molecular, com intuito de se conhecer a expressão dessas substâncias no ovário caprino, bem como após cultivo *in vitro*.

4. HIPÓTESE CIENTÍFICA

Diante do exposto, formularam-se as seguintes hipóteses:

1) O receptor para VEGF, bem como o RNAm para os receptores da ANG II (AGTR1 e AGTR2) e para o VIP são expressos de forma diferenciada em folículos ovarianos caprinos;

2) A ANG II, o VEGF e o VIP influenciam a sobrevivência, a ativação e o crescimento *in vitro* de folículos pré-antrais caprinos inclusos em tecido ovariano;

3) O VIP afeta a sobrevivência, a formação de antro, o crescimento e a expressão do RNAm para VIP e receptor de FSH (FSHR) em folículos pré-antrais caprinos isolados.

5. OBJETIVOS

5.1. Objetivo geral

1) Verificar a localização da proteína para o receptorde VEGF- 2 (VEGFR-2) e a expressão do RNAm para os receptores da ANG II (AGTR1 e AGTR2) e para o VIP em folículos ovarianos caprinos.

2) Avaliar o efeito da ANG II, VEGF e VIP sobre o desenvolvimento *in vitro* de folículos pré-antrais caprinos inclusos em tecido ovariano.

3) Avaliar o efeito do VIP sobre o desenvolvimento *in vitro* de folículos pré-antrais caprinos isolados, bem como sobre a expressão do RNAm para o VIP e receptor de FSH (FSHR) em folículos pré-antrais caprinos isolados após cultivo *in vitro*.

5.2. Objetivos específicos

1) Investigar nos compartimentos foliculares (oócitos, células da granulosa, células da teca e células do cumulus) a localização do receptor de VEGF (VEGFR-2), bem como a expressão do RNAm para os receptores da ANG II (AGTR1 e AGTR2) e para o VIP, em folículos caprinos;

2) Avaliar os efeitos da ANG II, VEGF e VIP sobre a sobrevivência, a ativação e o crescimento de folículos pré-antrais caprinos durante o cultivo *in vitro* de fragmentos de córtex ovariano por 1 ou 7 dias;

3) Avaliar o efeito do VIP sobre o a sobrevivência, formação de antro e crescimento de folículos pré-antrais caprinos isolados, bem como sobre a expressão do RNAm para VIP e receptor de FSH (FSHR) em folículos pré-antrais caprinos isolados após cultivo *in vitro*.

Nas páginas seguintes, serão apresentados os resultados desta tese na forma de seis

capítulos referentes a dois artigos de revisão e quatro artigos técnicos. Vale salientar que uma revisão foi publicada em periódico incluso no Qualis CAPES “B3”, dois artigos técnicos já foram publicados em periódicos Qualis “A1” e outros três encontram-se em fase de julgamento.

6. CAPÍTULO 1

Fatores angiogênicos e o desenvolvimento folicular ovariano

(Angiogenic factors and ovarian follicle development)

Periódico: Animal Reproduction v. 6, n. 2, p. 371-379, 2009

Resumo

Os folículos requerem um suprimento sanguíneo adequado de oxigênio, nutrientes e hormônios, além de remover o excesso de CO_2 e outros metabólitos. A aquisição de uma oferta vascular adequada é provavelmente um passo limitante na seleção e maturação do folículo dominante. Desta forma, há um progressivo interesse no estudo dos fatores de crescimento envolvidos no processo de angiogênese. Além disso, uma melhor compreensão sobre os mecanismos que regulam a expressão e a ação desses fatores poderia ser um ponto chave para aumentar o desempenho reprodutivo das fêmeas. Portanto, esta revisão visa resumir os dados atuais sobre a importância dos fatores de crescimento pró e anti-angiogênico que regulam a angiogênese no desenvolvimento folicular ovariano.

Palavras-chave: Angiogênese. Folículo. Fatores de crescimento. Ovário.

Angiogenic factors and ovarian follicle development

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Abstract

Ovarian follicles require an adequate blood supply for oxygen, nutrients and hormones, in addition to eliminating CO_2 and other metabolites. Acquisition of an adequate vascular supply is probably a limiting step in the selection and maturation of the dominant follicle. In this way, there is a progressive interest in the study of the growth factors involved in the angiogenic process. In addition, a better understanding about the mechanisms that regulate the expression and action of these factors could be a key point to increase the reproductive performance in females. Therefore, this review aims to summarize current data on the importance of the pro- and anti-angiogenic growth factors which regulate angiogenesis in ovarian follicle development.

Keywords: angiogenesis, follicle, growth factors, ovary.

Introduction

During embryo development, blood vessels differentiate from endothelial precursors by a process called vasculogenesis. Angiogenesis is the process of new blood vessel development from pre-existing vasculature that occurs in embryos and adults (Stouffer *et al*., 2001). In the last two decades, there was a progressive interest in the study of angiogenesis due to the association of this process with pathological conditions in adult tissues, such as tumoral growth and inflammation (Smith, 2001). In addition, several aspects of human and animal reproduction, such as clinical alterations that occur in the ovary and in the female

reproductive tract depend on angiogenesis (Acosta *et al*., 2003). This process occurs throughout follicular development, allowing adequate nutritional and hormonal supply for ovarian follicle growth and oocyte development, as well as corpus luteum formation (Fraser and Lunn, 2000).

A wide range of growth factors have been identified that promote (pro-angiogenic) or inhibit (anti-angiogenic) angiogenesis (Stouffer *et al*., 2001). However, modulation of the expression and action of these factors can be a key point to increase female reproductive performance. In this review, aspects related to the importance of angiogenesis in ovarian follicle development, as well as the role of the regulatory pro- and anti-angiogenic growth factors, will be discussed.

Blood vessels formation

The vascular system is developed based on two distinct processes: vasculogenesis and angiogenesis. While blood vessels differentiate from endothelial precursors by a process called vasculogenesis during embryo development, in adults further vessel development from pre-existing vasculature occurs by intussusception or sprouting by a process called angiogenesis. Angiogenesis is characterized by a cascade of events that starts with capillary proliferation and culminates with the formation of a new microcirculation composed of arterioles, capillaries and venules (Redmer and Reynolds, 1996). During the development of new blood vessels, some features can be observed such as enzymatic degradation of the basal membrane of the pre-existing vessels, migration of endothelial cells marked by angiogenic stimulus and finally, endothelial cell proliferation (Redmer *et al*., 2001). This neovascularization is completed with the formation of a capillary network and differentiation of new capillaries into arterioles and veins (LeCouter *et al*., 2002).

Studies have demonstrated that circulating endothelial precursor cells, i.e., originated from bone marrow, may contribute to angiogenesis in adults (Carmeliet and Jair, 2000). In addition, recent studies indicated the presence of mitogenic endothelial cells, which are the primary components of capillaries, in specific organs, modulating angiogenic responses in a variety of organs (LeCouter *et al*., 2002).

It is important to note that in several adult tissues, capillary growth rarely occurs and the vascular endothelium represents a stable population of cells with low mitogenic rates (Klagsbrun and D`Amore, 1996). An exception is the rapid growth and regression that occurs in the female genital organs, associated with equivalent changes in its vascular network (Reynolds and Redmer, 1995). The mature ovary shows a highly developed vasculature, reflecting its high metabolic rate, which turns this organ into a unique model for studies of angiogenesis regulation during growth, differentiation and regression of normal tissues in adults (Redmer and Reynolds, 1996).

Follicular angiogenesis

The ovarian follicle is the structural and functional unit of the mammalian ovary, which supplies the necessary environment for oocyte growth and maturation (Telfer, 1996). The follicles are surrounded by somatic cells (granulosa and theca cells) and can be classified in preantral (primordial, primary and secondary) and antral (tertiary and preovulatory) follicles (Hulshof *et al*., 1994). It is known that vasculature is not equally distributed among the population of follicles of the adult ovary, since only theca cell layers, present in later follicular stages, have vessels. Quiescent primordial follicles and slow growing preantral follicles do not have a vascular supply of their own, but instead rely on vessels in the surrounding stroma. Thus, Martelli *et al.* (2009) showed that an autonomous vascular supply starts to be evident in preantral follicles with diameter from 110 μm. However, as a follicular antrum develops, the thecal layer acquires a vascular sheath consisting of two capillary networks located in the theca interna and externa, respectively (Stouffer *et al*., 2001).

Acquisition of an adequate vascular supply is probably a limiting step in the selection and maturation of the dominant follicle destined to ovulate (Stouffer *et al*., 2001). Some studies have shown that angiogenesis is followed by a vasodilatation, a functional adaptation for the occurrence of ovulation, as well as by the development of theca endocrine function (Jiang *et al*., 2003). Thus, there are evidences that theca cells angiogenesis have a primary role in follicular development (Tamanini and De Ambrogi, 2004).

The development and growth of the theca vascular network are probably controlled by paracrine and angiogenic factors produced by granulosa cells. In addition to those factors,

Vascular Endothelial Growth Factor (VEGF), whose levels increase according to follicular growth, can induce the formation of a primitive capillary network during the early phases of antral follicle development. Moreover, the regulation of angiogenesis seems to be dependent on the interaction among other growth factors that can act in different moments, some of them stimulating growth, while others, mediating endothelial cell reorganization in more complexes vascular structures (Grasselli *et al*., 2003).

The degeneration of the capillary network is a relevant phenomenon that causes follicular atresia through the interruption of the metabolic supply for follicular cells. In addition, an increase in vascular density around antral follicles contributes to the inhibition of atresia. However, some studies have suggested that microvascular changes of atretic follicles are a consequence and not the cause of atresia (Macchiarelli *et al*., 1993).

As the corpus luteum begins its formation, thecal capillary sprouts begin to migrate towards and grow into the folds of the stratum granulosum. The growth of new capillaries during luteal angiogenesis follows a cascade of events including changes in the basement membrane, migration and proliferation of endothelial cells and development of capillary lumina (Plendl, 2000).

Pro-angiogenic growth factors

A variety of parameters, including oxygen tension, aging and endocrine or local factors can modulate the expression of angiogenic factors. It is generally believed that a decline in local oxygen concentrations (hypoxia) is a primary initiator of angiogenesis in normal and pathologic tissues (Hazzard and Stouffer, 2000). In the ovary, pro-angiogenic factors promote vascular permeability, supporting antrum formation and the events that induce follicular rupture (Tamanini and De Ambrogi, 2004). Several pro-angiogenic factors are well-known, such as fibroblast growth factor-2 (FGF-2), VEGF, angiotensin II (ANG II), insulin like growth factor-1 (IGF-1), epidermal growth factor (EGF), angiopoietin (ANPT) and endothelin-1 (ET-1). However, those that seem to be most important in angiogenesis are FGF-2, VEGF and ANG II (Redmer *et al*., 2001). Table 1 and Fig. 1 summarize the effects of pro-angiogenic factors on ovarian follicular development.

Table 1. Summary of the effects of angiogenic factors on ovarian follicular development.

Angiogenic factors	Effects on ovarian follicular development
FGF-2	Oocyte and granulosa cells survival
	Primordial follicles activation
	Granulosa and theca cells proliferation
VEGF	Primordial follicles survival
	Mitogenic effect in granulosa cells
	Transition from primary to secondary follicles
ANG II	Regulates oocyte maturation, ovulation and steroidogenesis
IGF-1	Follicular growth and survival
	Increases steroidogenesis

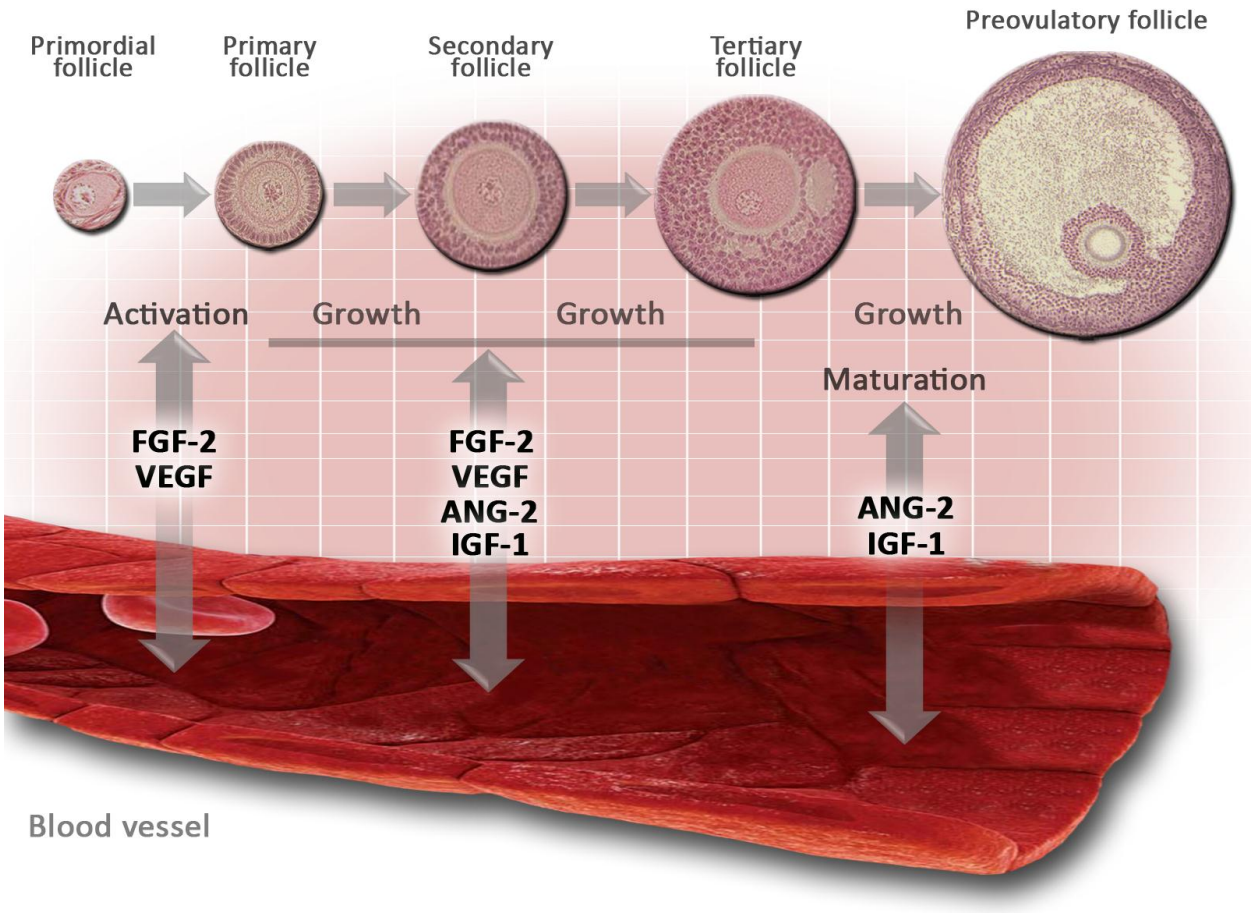

Figure 1. Angiogenic growth factors act in different stages of follicular development.

Fibroblast growth factor-2 (FGF-2)

FGF-2, also known as basic FGF (bFGF), was the first angiogenic factor indentified in the ovary (Gospodarowicz *et al*., 1985). The localization of FGF-2 in endothelial cells suggests that it is an important factor for endothelial growth (Gospodarowicz *et al*., 1985).

FGF-2 is also found in ovarian follicles (rat: Nilsson *et al*., 2001; human: Ben-haroush *et al*., 2005) and corpus luteum (rat: Asakai *et al*., 1993; bovine: Schams *et al*., 1994), while its receptors are expressed in growing follicles (Wandji *et al*., 1995). In medium and large swine follicles, mRNA for FGF-2 and its receptor FGFR-2 was detected in granulosa and theca cells, respectively. In the bovine ovary, the expression of the mRNA for FGF-2 in inner theca significantly increases during final follicular growth; however this expression was weak in granulosa cells (Shimizu *et al*., 2002). This factor exerts an antiapoptotic effect in granulosa cells, favoring the production of other angiogenic factors (Grasselli *et al*., 2002).

Some in vitro studies have shown that addition of FGF-2 to the culture medium promoted primordial and primary follicles growth (Nilsson *et al*., 2001), granulosa and theca cell proliferation (Wandji *et al*., 1996), as well as oocyte survival (Zhou and Zhang, 2005). Recently, Matos *et al*. (2007) demonstrated that FGF-2 at 50 ng/mL, stimulated goat primordial follicle activation after 5 days of in vitro culture.

Vascular Endothelial Growth Factor (VEGF)

VEGF, also known as vascular permeability factor (VPF), is a potent mitogenic factor that stimulates endothelial cell migration. It has also a role in the structural maintenance, increase of capillary permeability (Redmer *et al*., 2001) and a survival factor for endothelial cells of microvessels (Stouffer *et al*., 2001). The VEGF family is composed of at least six members (VEGF A, B, C, D, E and F), and the human VEGF-A gene is organized in eight exons, separated by seven introns. Alternative exon splicing results in the generation of four different isoforms, having 121, 165, 189, and 206 amino acids, respectively, after signal sequence cleavage (VEGF121, VEGF165, VEGF189, VEGF206; Stouffer *et al*., 2001; Ferrara, 2004). VEGF A expression was demonstrated in preantral follicles. The protein VEGF A has been identified in oocytes of human primordial follicles (Otani *et al*., 1999;

Harata *et al*., 2006) and human and rat primary follicles (Celik- Ozenci *et al*., 2003). In swine and bovine follicles, VEGF A is weakly expressed during early development and this expression becomes higher in granulosa and theca cells of dominant follicles (Barboni *et al*., 2000; Greenaway *et al*., 2005). Expression of mRNA for VEGF in the granulosa and theca cells, as well as the protein for VEGF in all follicle compartments and follicular fluid significantly increase according to the stage of follicular development (Yamamoto *et al*., 1997; Greenaway *et al*., 2004; Taylor *et al*., 2004). Recently, VEGF A expression in rats was occasionally observed in early preantral follicles and was always detected in preantral follicles during the late stages of development (Abramovich *et al*., 2009; Martelli *et al*., 2009). Furthermore, there are two known VEGF receptors (VEGFR-1 and VEGFR-2) that bind to VEGF-A. VEGFR-1 is expressed in quiescent and proliferative endothelial cells (Berisha *et al*., 2000) and induced the formation of vessels by VEGF (Boonyaprakob *et al*., 2003). VEGFR-2 is expressed specially in angiogenic endothelial cells and regulates the effects of VEGF on the proliferation and migration of these cells (Celik- Ozenci *et al*., 2003).

High VEGF concentrations cause a destabilization of the blood vessels, resulting in a new vascular network development, while VEGF deficiency results in blood vessel regression (Hanahan, 1997). Furthermore, Hazzard *et al*. (1999) demonstrated that gonadotropins stimulated VEGF secretion in primate preovulatory follicles and can act as regulatory factors of VEGF production. In this way, modulation of the hormones that influence VEGF expression, such as human (hCG) and equine (eCG) chorionic gonadotrophin, luteinizing hormone (LH) and follicle stimulating hormone (FSH), as well as their levels in the follicle, are possibly one of the keys to control ovarian follicular angiogenesis. There are also in vitro (Pepper *et al*., 1992) and in vivo (Asahara *et al*., 1995) indications of synergistic effects between angiogenic growth factors. In bovine, association between VEGF and FGF-2 induced an in vitro angiogenic response, which was stronger and faster than the effect produced by these two factors individually.

Recent in vitro studies suggested that VEGF has a mitogenic effect in granulosa cells and can stimulate follicular growth in rats (Otani *et al*., 1999). In this species, Kezele *et al*. (2005) identified that the gene encoding for VEGF-A is an important regulator of primordial follicle development. In addition, Danforth *et al*. (2003) showed that VEGF increases the number of primary and secondary follicles in rat ovaries. Recently, a study verified that

VEGF promoted the transition from primary to secondary follicles in bovine (Yang and Fortune, 2007). Furthermore, a study associated VEGF production and the increase of blood vessel content to follicular activation, i.e., the transition from the primordial to primary follicle stage (Mattioli *et al*., 2001). Another study showed that endogenous VEGF is essential to rodent primordial follicle survival (Roberts *et al*., 2007). Moreover, the inhibition of VEGF activity produced an increase in ovarian apoptosis through an unbalance in the pro and antiapoptotic protein rate, leading to a great number of atretic follicles (Abramovich *et al*., 2006). Other authors observed that the direct injection of VEGF into the ovary increases vasculature (Shimizu, 2006), the number of antral follicles and inhibits apoptosis (Quintana *et al*., 2004).

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF)

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) was identified as a novel human endothelial cell mitogen, through a bioassay assessing the ability of a library of purified human secreted proteins to promote the growth of primary adrenal cortex capillary endothelial cells (LeCouter *et al*., 2001). EG-VEGF does not belong to the VEGF family or other known families of endothelial mitogens but instead is a member of a structurally related class of peptides including the digestive enzyme colipase, the Xenopus headorganizer, Dickkopf (Glinka *et al*., 1998), venom protein A (VPRA; Joubert and Strydom, 1980) or mamba intestinal toxin-1 ‘‘MIT-1’’ (Schweitz *et al*., 1999), a non-toxic component of Dendroaspis polylepis polylepis venom, and the secreted proteins from Bombina variegata designated Bv8 (Mollay *et al*., 1999). Its receptors were designated EG-VEGFR-1 and EG-VEGFR-2 (Masuda *et al*., 2002). EG-VEGF selectively promoted proliferation, survival and chemotaxis of endothelial cells isolated from steroidogenic tissues (Lin *et al*., 2002). Indeed, exogenous EG-VEGF in the ovary (LeCouter *et al*., 2001) or testis (LeCouter *et al*., 2003) can dramatically affect vascular leakage. Northern blot analysis of a panel of RNAs from a variety of human tissues revealed EG-VEGF expression in ovary, testis, adrenal and placenta (LeCouter *et al*., 2001). In situ hybridization analysis demonstrated that steroidogenic cells within these glands are the source of EG-VEGF (LeCouter *et al*., 2001). Granulosa cells in primordial and primary follicles express EG-VEGF

strongly. As the secondary follicle matures, EG-VEGF expression in granulose cells declines (Ferrara *et al*., 2003). At approximately 5 days post-ovulation, both VEGF-A and EG-VEGF are strongly expressed in a portion of granulosa lutein cells, whereas 8 days post-ovulation EG-VEGF expression is intense in the theca lutein cells, while VEGF expression has diminished to the point where only weak signal remains in the peripheral thecal cells (Corner, 1956).

Angiotensin II (ANG II)

ANG II is a potent vasoactive peptide, which is converted from ANG I by angiotensin conversion enzyme, and induces neovascularization in rabbit retina (Fernandez *et al*., 1985), mouse and human endometrium (Hu *et al*., 1996; Li and Ahmed, 1996; Walsh *et al*., 1997), as well as in the bovine corpus luteum (Walsh *et al*., 1997). ANG II acts by its binding to a group of receptors, classified as ANG receptor type 1 (AT-1) and 2 (AT-2). Both receptors are expressed in the ovary, and their presence and distribution differ significantly among the species and follicular development stages (Pucell *et al*., 1991; Obermüller *et al*., 1998). In rabbit ovaries, AT-2 receptor is expressed predominantly in granulosa cells of preovulatory follicles, while AT-1 is located in theca and stroma cells (Yoshimura *et al*., 1996). In mouse, AT-2 is expressed exclusively in granulosa cells of large atretic follicles, while AT-1 is expressed in all structures of ovarian follicles (Pucell *et al*., 1991). The presence of ANG II receptors in the ovary suggests a possible role of this peptide on this organ. Mitsube *et al*. (2003) reported that only the blockage of AT-2 increases blood flow in the mouse ovary, suggesting that vasoconstriction occurs specifically through this receptor. In addition, another study observed high ANG II levels during proestrous in the mouse, and this fact may be associated with the high vascular proliferation that occurs in this phase of the estrous cycle (Costa *et al*., 2003). Moreover, ANG II improves angiogenic activity of VEGF in bovine microvascular cells (Otani *et al*., 2000).

ANG II regulates oocyte maturation (Kuo *et al*., 1991), ovulation (Yoshimura *et al*., 1996) and steroidogenesis (Yoshimura *et al*., 1993) through the modulation of ovarian blood flow (Mitsube *et al*., 2003). ANG II is well known for its vasoconstrictor action; however this effect was not observed in the ovary after treatment with ANG II (Costa *et al*., 2003). The

vasodilatation was observed in rabbit ovaries perfused in vitro with ANG II and this effect can be due to the release of gonadotropins stimulated by ANG II (Kuo *et al*., 1991). In addition, Shuttleworth *et al*. (2002) suggested a possible role of ANG II in swine early folliculogenesis and steroidogenesis. In bovine, ANG II restored the inhibitory effect of follicular cells on oocyte maturation (Giometti *et al*., 2005; Stefanello *et al*., 2006) and stimulated nuclear and cytoplasmic maturation of swine oocytes (Li *et al*., 2004). In addition, ANG II appears to regulate the induction of several autocrine growth factors, such as platelet derived growth factor, transforming growth factor-β, FGF-2 and IGF-1, and induces the angiogenic activity through the paracrine function of VEGF in microvascular cells (Otani *et al*., 2000).

Insulin like growth factor-1 (IGF-1)

The IGF system appears to have indirect effects on angiogenesis through stimulatory action for VEGF production in luteal cells, as well as through the stimulus of endothelial cell proliferation and differentiation (Schams *et al*., 2001). In bovine, a high expression of IGF-1 in theca interna was observed before the phase of follicular selection, while the expression increased in granulosa cells after this phase. In addition, mRNA for IGF-1 receptor (IGFR-1) was present in theca interna and granulosa cells with increased levels during final follicular development (Schams *et al*., 2002).

In association with FSH, addition of IGF-1 to the in vitro culture medium of preantral follicles stimulated follicular growth in several species (human: Louhio *et al*., 2000; bovine: Gutierrez *et al*., 2000; rats: Zhao *et al*., 2001; mouse: Liu *et al*., 1998). Experiments performed by Zhou and Zhang (2005) showed that IGF- 1 promoted the growth and maintained the viability of oocytes from caprine preantral follicles. In swine, 50 ng/mL of IGF-1 promoted follicular growth, stimulated granulosa cell proliferation and prevented apoptosis of preantral follicles cultured for 4 days in the presence of serum (Mao *et al*., 2004). Furthermore, different concentrations of IGF-1 (10, 50 and 100 ng/mL) increased follicular diameter and steroidogenesis of mouse preantral follicles cultured in vitro for 6, 10 and 12 days (Demeestere *et al*., 2004). In a recent study, Thomas *et al*. (2007) showed that follicular diameter was increased over control levels by addition of 50 ng/ml of IGF-I during 6 days of culture.

Anti-angiogenic growth factors

Angiogenesis is also modulated by inhibitory factors, such as thrombospodin, angiostatin, endostatin, 2-metoxiestradiol, hyaluronic acid, platelet factor-4, tumoral necrose factor α and interferon γ. These substances blocked endothelial cell proliferation and migration, as well as in vitro capillary formation (Espinosa-Cervantes and Rosado-Garcia, 2002).

Regarding thrombospodin 1 and 2, they bind to their receptor CD36 and inhibit angiogenesis, inducing endothelial cell apoptosis. In rat ovaries, the expression of thrombospondin 1 and CD36 mRNA is high in granulosa cells of preantral and antral follicles and is limited in theca cells. Nevertheless, there is no expression of mRNA for thrombospodin 2 in ovarian follicles. Furthermore, thrombospodin 1 is strongly expressed in small follicles where the vascularization is absent, showing that expression of thrombospodins decreases during follicular maturation together with the increase in VEGF levels (Petrik *et al*., 2002).

Platelet factor-4 inhibits angiogenesis both in vivo and in vitro and the inhibitory effects are due to the formation of complexes with FGF-2, inhibiting FGF-2 binding to its receptors (Perollet *et al*., 1998).

Final considerations

Increasing evidence suggests that physiological angiogenesis in ovarian follicles and corpus luteum are fundamental features of mammalian reproduction. Failures in vascular development in these structures may be the reason for several ovarian dysfunctions observed during the estrous cycle and pregnancy. Therefore, it is necessary to evaluate both in vivo and in vitro influences of the angiogenic factors, alone or in association, on the survival (anti-apoptotic effects) of ovarian cells in different species. This information will provide novel opportunities for therapeutic interventions and improvement of the efficiency of assisted reproduction in humans and animals in the future.

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References

Abramovich D, Parborel F, Tesone M. 2006. Effect of a vascular endothelial growth factor (VEGF) inhibitory treatment on the folliculogenesis and ovarian apoptosis in gonadotropin-treated prepubertal rats. *Biol Reprod*, 75:434-441.

Abramovich D, Celin AR, Hernandez F, Tesone M, Parborel F. 2009. Spatiotemporal analysis of the protein expression of angiogenic factors and their related receptors during folliculogenesis in rats with and without hormonal treatment. *Reproduction*, 137:309- 320.

Acosta TJ, Hayashi KG, Ohtani M, Miyamoto A. 2003. Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. *Reproduction*, 125:759-767.

Asahara T, Bauters C, Zheng LP, Takeshita S, Bunting S, Ferrara N. 1995. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation*, 91:365-371.

Asakai R, Tamura K, Eishi Y, Iwamoto M, Kato Y, Okamoto R. 1993. Basic fibroblast growth factor (bFGF) receptors decrease with luteal age in rat ovarian luteal cells: colocalization of bFGF receptors and bFGF in luteal cells. *Endocrinology*, 133:1074-1084.

Barboni B, Turriani M, Galeati G, Spinaci M, Bacci ML, Forni M, Mattioli M. 2000. Vascular endothelial growth factor production in growing pig antral follicles. *Biol Reprod*, 63:858-864.

Ben-haroush A, Abir R, Ao A, Jin S, Kessler-Icekson G, Feldberg D, Fisch B. 2005. Expression of basic fibroblast growth factor and its receptors in human ovarian follicles from adults and fetuses. *Fertil Steril*, 84:1257-1268.

Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R. 2000. Expression and tissue concentration of vascular endothelial growth factor, its receptors and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biol Reprod*, 63:1106-1114.

Boonyaprakob U, Gadsby JE, Hedgpeth V, Routh P, Almond GW. 2003. Expression and localization of vascular endothelial growth factor and its receptors in pig corpora lutea during the oestrous cycle. *Reproduction*, 126:393-405.

Carmeliet P, Jair RK. 2000. Angiogenesis in cancer and other diseases. *Nature*, 407:249-257.

Celik-Ozenci C, Akkoyunhlu G, Kayisli UA, Arici A, Demir R. 2003. Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. *Histochem Cell Biol*, 120:383-390.

Corner GWJ. 1956. The histological dating of the human corpus luteum of menstruation. *Am J Anat*, 9:377-401.

Costa APR, Fagundes-Moura CR, Pereira VM, Silva LFM, Vieira AR, Santos RAS, Reis AM. 2003. Angiotensin-(1–7): A novel peptide in the ovary. *Endocrinology*, 144:1942-1948.

Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. 2003. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. *Biol Reprod*, 68:1736-1741.

Demeestere I, Gervy C, Centner J, Devreker F, Englert Y, Delbaere A. 2004. Effect of insulin-like growth factor-I during preantral follicular culture on steroidogenesis, in vitro oocyte maturation, and embryo development in mice. *Biol Reprod*, 70:1664-1669.

Espinosa-Cervantes MC, Rosado-Garcia A. 2002. Angiogenesis in reproductive physiology: follicular development, formation and maintenance of the corpus luteum. *Ginecol Obstet Mex*, 70:17-27.

Fernandez LA, Twickler J, Mead A. 1985. Neovascularization produced by angiotensin II. *J Lab Clin Med*, 105 141-145.

Ferrara N, Frantz G, LeCouter J, Dillart-Telm L, Pham T, Draksharapu A, Giordano T, Peale F. 2003. Differential expression of the angiogenic factor genes VEGF and EG-VEGF in normal and polycystic human ovaries. *Am J Pathol*, 162:1881-1893.

Ferrara N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*, 25:581- 611.

Fraser HM, Lunn SF. 2000. Angiogenesis and its control in the female reproductive system. *Br Med Bull*, 56: 787-797.

Giometti IC, Bertagnolli AC, Ornes RC, Costa LFS, Carambula SF, Reis AM, Oliveira JF, **Emanuelli IP**, **Gonçalves PB**. 2005. Angiotensin II reverses the inhibitory action produced by theca cells on bovine oocyte nuclear maturation. *Theriogenology*, 63:1014-1025.

Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature*, 391:357-362.

Gospodarowicz D, Cheng J, Lui GM, Baird A, Esch F, Bohlen P. 1985. Corpus luteum angiogenic factor is related to fibroblast growth factor. *Endocrinology*, 117:2383-2391.

Grasselli F, Basini G, Bussolati S, Tamanini C. 2002. Effects of VEGF and bFGF on proliferation and production of steroids and nitric oxide in porcine granulosa cells. *Reprod Domest Anim*, 37:362-368.

Grasselli F, Basini G, Tirelli M, Cavalli V, Bussolati S, Tamanini C. 2003. Angiogenic activity of porcine granulosa cells cocultured with endothelial cells in a microcarrier based three-dimensional fibrin gel. *J Physiol Pharmacol*, 54:361-370.

Greenaway J, Connor K, Pedersen HG, Coomber BL, Lamarre J, Petrik J. 2004. Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. *Endocrinology*, 145:2896-2905.

Greenaway J, Centry PA, Feige J-J, Lamarre J, Petrik JJ. 2005. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. *Biol Reprod*, 72:1071-1078.

Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Webb R. 2000. Growth and antrum formation of bovine preantral follicles in long-term culture in vitro. *Biol Reprod*, 62:1322-1328.

Hanahan D. 1997. Signaling vascular morphogenesis and maintenance. *Science*, 277:55-60.

Harata T, Ando H, Iwase A, Nagasaka T, Mizutani S, Kikkawa F. 2006. Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. *Fertil Steril*, 86:433-439.

Hazzard TM, Molskness TA, Chaffin CL, Stouffer RL. 1999. Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotropin and steroids in macaque granulosa cells during the periovulatory interval. *Mol Hum Reprod*, 5:1115-1121.

Hazzard TM, Stouffer RL. 2000. Angiogenesis in ovarian follicular and luteal development. *Baillieres Best Pract Res Clin Obstet Gynaecol*, 4:883-900.

Hu DE, Hiley CR, Fan TP. 1996. Comparative studies of angiogenic activity of vasoactive intestinal peptide, endothelin-1 and -3 and angiotensin II in a rat sponge model. *Br J Pharmacol*, 117:545-551.

Hulshof SCJ, Figueiredo JR, Bekers JF, Bevers MM, Van Den Hurk R. 1994. Isolation and Characterization of preantral follicles from foetal bovine ovaries. *Vet Q*, 2:78-80.

Jiang JY, Macchiarelli G, Tsang BK, Sato E. 2003. Capillary angiogenesis and degeneration in bovine ovarian antral follicles. *Reproduction*, 125:211-223.

Joubert FJ, Strydom DJ. 1980. Snake venom. The amino acid sequence of protein A from Dendroaspis polylepis polylepis (black mamba) venom. *Hoppe Seylers Z Physiol Chem*, 361:1787-1794.

Kezele PR, Ague JM, Nilsson E, Skinner MK. 2005. Alteration in the ovarian transcriptome during primordial follicle assembly and development. *Biol Reprod*, 72:241-255.

Klagsbrun M, D`Amore PA. 1996. Vascular endothelial growth factor and its receptors. *Cytokine Growth Factor Rev*, 7:259-270.

Kuo TC, Endo K, Dharmarajan AM, Miyazaki T, Atlas SJ, Wallach EE. 1991. Direct effect of angiotensin II on in vitro perfused rabbit ovary. *J Reprod Fertil*, 92:469-474.

LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller G-A, Peale F, Gurney A, Hillan KJ, Ferrara N. 2001. Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature*, 412:877-884.

LeCouter J, Ferrara N. 2002. Endocrine gland-derived VEGF and the emerging hypothesis of organ-specific regulation of angiogenesis. *Nat Med*, 8:913-917.

LeCouter J, Lin R, Frantz G, Tejada M, Peale F, Hillan KJ, Ferrara N. 2003. The EG-VEGF homologue, Bv8, is a potent angiogenic factor. Localization of Bv8 receptors to endothelial cells. *Proc Natl Acad Sci USA*, 100:2685-2690.

Li XF, Ahmed A. 1996. Dual role of angiotensin II in the human endometrium. *Hum Reprod*, 11:95-108.

Li YH, Jiao LH, Liu RH, Chen XL, Wang H, Wang WH. 2004. Localization of angiotensin II in pig ovary and its effects on oocyte maturation in vitro. *Theriogenology*, 61:447-459.

Lin R, LeCouter J, Kowalski J, Ferrara N. 2002. Characterization of EGVEGF signaling in adrenal cortex capillary endothelial cells. *J Biol Chem*, 277:8724-8729.

Liu X, Andoh K, Yokota H, Kobayashi J, Abe Y, Yamada K, Mizunuma H, Ibuki Y. 1998. Effects of growth hormone, activin, and follistatin on the development of preantral follicle from immature female mice. *Endocrinology*, 139:2342-2347.

Louhio H, Hovatta O, Sjöberg J, Tuuri T. 2000. The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. *Mol Hum Reprod*, 6:694-698.

Macchiarelli G, Nottola SA, Vizza E, Familiari G, Kikuta A, Murakami T, Motta PM. 1993. Microvasculature of growing and atretic follicles in the rabbit ovary: a SEM study of corrosion casts. *Arch Histol Cytol*, 56:1-12.

Mao J, Smith MF, Rucker EB, Wu GM, McCauley TC, Cantley JTC, Prather RS, **Didion BA**, **Day BN**. 2004. Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosal cell proliferation and suppression of apoptosis in vitro. *J Anim Sci*, 82:1967-1975.

Masuda Y, Takatsu Y, Terao Y, Kumano S, Ishibashi Y, Suenaga M, Abe M, Fukusumi S, Watanabe T, Shintani Y, Yamada T, Hinuma S, Inatomi N, Ohtaki T, Onda H, Fujino M. 2002. Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochem Biophys Res Commun*, 293:396-402.

Martelli A, Bernabò N, Berardinelli P, Russo V, Rinaldi C, Di Giacinto O, Mauro A, Barboni B. 2009. Vascular supply as a discriminating for pig preantral follicle selection. *Reproduction*, 137:45-58.

Matos MHT, Van den Hurk R, Lima-verde IB, Luque MCA, Santos KDB, Martins FS, Báo SN, Lucci CM, Figueiredo JR. 2007. Effects of fibroblast growth factor-2 on the in vitro culture of caprine preantral follicles. *Cell Tiss Organs*, 186:112-120.

Mattioli M, Barboni B, Turriani M, Galeati G, Zannoni A, Castellani G, Berardinelli P, Scapolo PA. 2001. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. *Biol Reprod*, 65:1014-1019.

Mitsube K, Mikuni M, Matousek M, Zackrisson U, Brannstrom M. 2003. Role of the angiotensin II system in regulation of ovulation and blood flow in the rat ovary. *Reproduction*, 125: 425-435.

Mollay C, Wechselberger C, Mignogna G, Negri L, Melchiorri P, Barra D, Kreil G. 1999. Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. *Eur J Pharmacol*, 374: 189-196.

Nilsson E, Parrot JA, Skinner MK. 2001. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol*, 175:123-130.

Obermüller N, Schlamp D, Hoffmann S, Gentili M, Inagami T, Gretz N, Weigel M. 1998. Localization of the mRNA for the angiotensin II receptor subtype 2 (AT2) in follicular granulosa cells of the rat ovary by nonradioactive in situ hybridization**.** *J Histochem Cytochem*, 46:865-870.

Otani N, Minami S, Yamoto M, Shikone T, Otani H, Nishiyama R, Otani T, **Nakano R**. 1999. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. *J Clin Endocrinol Metab*, 84:3845- 3851.

Otani A, Takagi H, Oh H, Suzuma K, Matsumura M, Ikeda E, Honda Y. 2000. Angiotensin II stimulated vascular endothelial growth factor expression in bovine retinal pericytes. *Invest Ophthalmol Vis Sci*, 41:1192- 1199.

Pepper MS, Ferrara N, Orci L, Montesano R. 1992. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochem Biophys Res Commun*, 189:824-831.

Perollet C, Han ZC, Savona C, Caen JP, Bikfalvi A. 1998. Platelet factor 4 modulates fibroblast growth factor 2 (FGF-2) activity and inhibits FGF-2 dimerization. *Blood*, 91:3289-3299.

Petrik JJ, Gentry PA, Feige JJ, Lamarre J. 2002. Expression and localization of thrombospondin-1 and -2 and their cell surface receptor, CD36, during rat follicular development and formation of the corpus luteum. *Biol Reprod*, 67:1522-1531.

Plendl J. 2000. Angiogenesis and vascular regression in the ovary. *Anat Histol Embryol*, 29:257-266.

Pucell AG, Hodges JC, Sen I, Bumpus FM, Husain A. 1991. Biochemical properties of the ovarian granulosa type 2- angiotensin II receptor. *Endocrinology*, 128:1947-1959.

Quintana R, Kopcow L, Sueldo C, Marconi G, Rueda NG, Barañao RI. 2004. Direct injection of vascular endothelial growth factor into the ovary of mice promotes follicular development. *Fertil Steril*, 82:1101-1105.

Redmer DA, Reynolds LP. 1996. Angiogenesis in the ovary. *Rev Reprod*, 1:182-192.

Redmer DA, Doraiswamy V, Bortnem BJ, Fisher K, Jablonka-Shariff A, Grazul-Bilska AT, Reynolds LP. 2001. Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. *Biol Reprod*, 65:879-889.

Reynolds LP, Redmer DA. 1995. Utero-placental vascular development and placental function. *J Anim Sci*, 73:1839-1851.

Roberts AE, Arbogast LK, Friedman CI, Cohn DE, Kaumaya PT, Danforth D.R. 2007. Neutralization of endogenous vascular endothelial growth factor depletes primordial follicles in the mouse ovary. *Biol Reprod*, 76:218-223.

Schams D, Amselgruber W, Einspanier R, Sinowatz F, Gospodarowicz D. 1994. Localization and tissue concentration of basic fibroblast growth factor in the bovine corpus luteum. *Endocrine*, 2:907-912.

Schams D, Kosmann M, Berisha B, Amselgruber WM, Miyamoto A. 2001. Stimulatory and synergistic effects of luteinising hormone and insulin-like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulose cells. *Exp Clin Endocrinol Diabetes*, 109:155-162.

Schams D, Berisha B, Kosmann M, Amselgruber W. 2002. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. *Domest Anim Endocrinol*, 22:51- 72.

Schweitz H, Pacaud P, Diochot P, Moinier D, Ladzunski M. 1999. MIT(1), a black mamba intestinal toxin with a new and highly potent activity on intestinal contraction. *FEBS Lett*, 461:183-188.

Shimizu T, Jiang J-Y, Sasada H, Sato E. 2002. Changes of messenger RNA expression of angiogenic factors and related receptors during follicular development in gilts. *Biol Reprod*, 67:1846-1852.

Shimizu T. 2006. Promotion of ovarian follicular development by injecting vascular endothelial growth factor (VEGF) and growth differentiation factor 9 (GDF-9) genes. *J Reprod Dev*, 52:23-32.

Shuttleworth G, Pipkin FB, Hunter MG. 2002. In vitro development of pig preantral follicles cultured in a serum-free medium and the effect of angiotensin II. *Reproduction*, 123:807-818.

Smith SK. 2001. Angiogenesis and reproduction. *Br J Obstet Gynaecol*, 108:777-783.

Stefanello JR, Barreta MH, Porciuncula PM, Arruda JN, Oliveira JF, Oliveira MA, Gonçalves PB. 2006. Effect of angiotensin II with follicle cells and insulin-like growth factor-I or insulin on bovine oocyte maturation and embryo development. *Theriogenology*, 66:2068-2076.

Stouffer RL, Martínez-Chequer JC, Molskness TA, Xu F, Hazzard TM. 2001. Regulation and action of angiogenic factors in the primate ovary. *Arch Med Res*, 32:567-575.

Tamanini C, De Ambrogi M. 2004. Angiogenesis in developing follicle and corpus luteum. *Reprod Dom Anim*, 39:206-216.

Taylor PD, Hillier SG, Fraser HM. 2004. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. *J Endocrinol*, 183:1-17.

Telfer EE. 1996. The development of methods for isolation and culture of preantral follicles from bovine and porcine ovaries. *Theriogenology*, 45:101-110.

Thomas FH, Campbell BK, Armstrong DG, Telfer EE. 2007. Effects of IGF-I bioavailability on bovine preantral follicular development in vitro. *Reproduction*, 133:1121-1128.

Walsh DA, Hu DE, Wharton J, Catravas JD, Blake DR, Fan TP. 1997. Sequencial development of angiotensin receptors and angiotensin I converting enzyme during angiogenesis in the rat subcutaneous sponge granuloma. *Br J Pharmacol*, 120:1302-1311.

Wandji SA, Eppig JJ, Fortune JE. 1995. FSH and growth factors affect the growth and endocrine function in vitro of granulosa cells of bovine preantral follicles. *Theriogenology*, 45:817-832.

Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. 1996. Initiation *in vitro* of growth of bovine primordial follicles. *Biol Reprod*, 55:942-948.

Yamamoto S, Konishi I, Tsuruta Y, Nanbu K, Mandai M, Kuroda H, Matsushita K, Hamid AA, Yura Y, Mori T. 1997. Expression of vascular endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary. *Gynecol Endocrinol*, 11:371-381.

Yang MY, Fortune JE. 2007. Vascular endothelial growth factor stimulates the primary to secondary follicle transition in bovine in vitro. *Mol Reprod Dev*, 74:1095-1104.

Yoshimura Y, Karube M, Oda T, Koyama N, Shiokawa S, Akiba M, Yoshinaga A, Nakamura Y. 1993. Locally produced angiotensin II induces ovulation by stimulating prostaglandin production in *in vitro* perfused rabbit ovaries. *Endocrinology*, 133:1609-1616.

Yoshimura Y, Karube M, Aoki H, Oda T, Koyama N, Nagai A, Akimoto Y, Hirano H, Nakamura Y. 1996. Angiotensin II induces ovulation and oocyte maturation in rabbit ovaries via the AT2 receptor subtype. *Endocrinology*, 137:1204-1211.

Zhao J, Taverne MAM, Van Der Weijden GC, Bevers MM, Van Den Hurk R. 2001. Insulin-like growth factor-I (IGF-I) stimulates the development of cultured rat preantral follicle. *Mol Reprod Dev*, 58:287- 296.

Zhou H, Zhang Y. 2005. Regulation of in vitro growth of preantral follicles by growth factors in goats. *Domest Anim Endocrinol*, 28:235-242.

7. CAPÍTULO 2

Envolvimento do peptídeo intestinal vasoativo na fisiologia ovariana

(Involvement of vasoactive intestinal peptide (VIP) on ovarian physiology)

Periódico: Animal Reproduction (Submetido em outubro de 2010)

Resumo

Além do sistema nervoso central, fibras nervosas contendo peptídeo intestinal vasoativo (VIP), foram descritas ao longo do trato genital feminino. Existem crescentes evidências de que o VIP e seus receptores desempenham um papel importante na regulação local da fisiologia ovariana via AMPc. Tem sido relatado que o VIP regula a sobrevivência e crescimento do folículo ovariano, maturação do oócito, ovulação e a esteroidogênese. Portanto, esta revisão visa resumir os dados atuais sobre a importância do VIP na fisiologia ovariana.

Palavras-chave: VIP. Folículos ovarianos. Foliculogênese. Ovulação. Esteroidogênese.

Involvement of vasoactive intestinal peptide (VIP) on ovarian physiology

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Abstract

In addition to the central nervous system, vasoactive intestinal peptide (VIP) containing nerves have been described throughout the female genital tract. There is growing evidence that VIP and their receptors play important roles in the local regulation of ovarian physiology through cAMP pathway. It has been reported that VIP regulates the ovarian follicle survival and growth, oocyte maturation, ovulation and steroidogenesis. Therefore, this review aims to summarize current data on the importance of VIP on ovarian physiology.

Keywords: VIP, ovarian follicles, ovulation, steroidogenesis.

Introduction

Ovarian activity is regulated not only by gonadotropins and steroids, but also by a number of neural inputs and paracrine regulatory mechanisms. The mammalian ovary is innervated by extrinsic nerves, which are both catecholaminergic and peptidergic in nature (Burden, 1985; Ojeda and Lara, 1989; Ojeda et al., 1989). Peptidergic innervation of the ovary was verified, among other ways, by the presence of vasoactive intestinal peptide (VIP) (Ahmed et al., 1986).

In addition to the central nervous system, VIP containing nerves have been described throughout the human female genital tract being most abundant in the vagina, cervix and clitoris and less numerous in the uterine body, oviduct and ovary (Ottesen and Fahrenkrug, 1995), which suggests that such peptides may not play an exclusively neuroendocrine role. There is growing evidence that VIP plays an important role in the female reproductive system by acting as a potential local regulator of ovarian physiology, such as regulation of steroidogenesis, cAMP accumulation, plasminogen activator production and oocyte maturation (Ahmed et al., 1986; Tornell et al., 1988; Johnson and Tilly, 1988). This review will focus on the role of VIP and their receptors in ovarian physiology.

Expression of VIP and their receptors in the ovary

VIP is a member of the structurally related neuropeptide family including pituitary adenylate cyclase-activating polypeptide (PACAP)/secretin/glucagons (Arimura, 1992; Gozes et al., 1999; Miyata et al., 1989). VIP and PACAP act by binding to three types of G protein-coupled VIP/PACAP receptors. PAC1-R binds specifically to both PACAPs and VIP, though with very low affinity; VPAC1-R and VPAC2-R bind to PACAP and VIP with equal affinity (Lutz et al., 1993).

In ovaries, VIP is reported to be produced by nerves fibers innervating follicles at all stages of development of rodent (Ahmed et al., 1986) and avian (Johnson et al., 1994). Hulshof et al. (1994) showed that bovine ovary is innervated by VIP-positive nerve fibers, beginning at the onset of follicular development and increasingly with age. Furthermore, VIP and their receptors immunoreactivity has been found in close association with the ovarian vasculature (Hulshof et al., 1994; Vaccari et al., 2006).

Despite the presence of mRNA for VIP has been detected in ovaries of rats (Gozes and Tsafriri, 1986), its presence was not observed in mice (Barberi et al., 2007). These authors suggested such results due to the fact that this peptide has been originated in extrinsic innervations. In fact, the lack of radioimmunoassayable VIP levels following the transection of the ovarian nerves indicates that ovarian VIP derives mostly from extrinsic innervation of the gland. However, in goats, the presence of mRNA for VIP was detected in all follicular categories (primordial, primary, secondary and antral follicles) and cellular types studied (granulosa and theca cells) (Bruno et al., unpublished data), suggesting a local synthesis of this peptide.

Barberi et al. (2007) showed that the three VIP receptors are expressed in mouse granulosa cells in vivo. Furthermore, other authors have shown that VPAC1-R is the most abundant receptor in ovaries from juvenile mice, and that VPAC2-R levels are lower, while those of PAC1-R are very low (Cecconi et al., 2004). In the rat preovulatory follicles, PAC1-R and VPAC2-R are expressed by granulosa cells, whereas theca/interstitial cells exclusively express VPAC1-R e VPAC2-R (Vaccari et al., 2006). PAC1-R was the main receptor present in the ovary of rat, followed by VPAC2-R and VPAC1-R (Latini et al., 2010).

Signaling pathways and effects of VIP on follicle survival

VIP has been shown to protect several cell types from apoptosis, including thymocytes, prostate cancer and neural cells (Delgado et al., 1996; Said, 1996; Gutierrez-Canas, et al., 2003; Sastry et al., 2006). In the ovary, previous in vitro studies also demonstrated that VIP inhibits apoptosis of rat follicles (Flaws et al., 1995; Lee et al. 1999). Moreover, it has been demonstrated that PACAP and VIP were able to prevent granulosa cell apoptosis of follicles cultured in serum free medium (Vaccari et al., 2006; Barberi et al., 2007). Recently, VIP maintained the ultrastructural integrity of goat preantral follicles after seven days of ovarian tissue culture (Bruno et al., 2010).

It is possible that VIP maintained cell viability through the cAMP pathway. The stimulation of cAMP formation by VIP is probably a transient response that occurs early after culture initiation. In support of this, the ability of VIP to suppress apoptosis was mimicked by treatment of follicles with the adenylyl cyclase activator (forskolin), and this substance increased cAMP production and accumulation within cultured follicles (Flaws et al., 1995). These findings along with previous reports on the role of the cAMP-protein kinase A (PKA) second messenger system in mediating the effects of VIP in target cells (Ojeda et al., 1989; Johnson and Tilly, 1988; Davoren and Hsueh, 1986) provide evidence that VIP, potentially acting at least in part via the cAMP pathway, functions as a novel inhibitor of apoptosis.

Other studies have shown that the neuroprotective effects of VIP are mediated partially via activation of PKA through PKC (Vaudry et al., 2000) and cAMP response element binding protein phosphorylation (Walton and Dragunow, 2000). The effect or mechanisms include inhibition of caspase-3 activity, upregulation of Bcl-2 and suppression of cytoplasmic cytochrome *c* translocation (reviewed by Filippatos et al., 2001). On the other hand, it is plausible that mechanisms independent of PKA activity also partly mediate the actions of VIP on follicle survival. This possibility is supported by previous studies using a mouse pituitary cell line that demonstrated activation of voltage-sensitive calcium channels after VIP stimulation (Schecterson and McKnight, 1991).

In vitro effects of VIP on follicle growth

There are only few papers dealing with VIP effects on the ovarian follicles. In vitro studies revealed that VIP stimulates the development of isolated bovine primary and early secondary follicles (Hulshof, 1995). We have recently demonstrated that VIP is an important factor for the growth of small preantral follicles enclosed in caprine ovarian tissue (Bruno et al., 2010). In addition to the increase of VIP immunoreactivity with the appearance of secondary and antral follicles in bovine ovaries (Hulshof et al., 1994), the contemporary presence of VIP and its receptors around preantral and antral follicles (Vaccari et al., 2006) may explain the role of VIP in follicle growth. Nevertheless, in mice, VIP did not affect follicular development and caused inhibition of follicular growth, antrum formation, granulosa cell proliferation, as well as estradiol production of follicles stimulated by FSH (Cecconi et al., 2004). These conflicting results may be due to study design, differences related to species, culture conditions and different follicular stages analyzed.

Influence of VIP on maturation and ovulation

Studies have shown that VIP stimulates maturation in follicle-enclosed oocytes, but could transiently inhibit or not affect spontaneous maturation of cumulus-enclosed oocytes (Tornell et al., 1988; Apa et al., 1997). Culture of bovine cumulus oocyte complexes in the presence of VIP did not affect nuclear maturation or cumulus expansion, but it retarded cytoplasmic maturation as reflected by delayed cortical granule migration (Beker et al., 2000). Moreover, the addition of VIP to the culture medium did not improve in vitro maturation and fertilization of sheep (Ledda et al., 1996) and buffalo (Nandi et al., 2003) oocytes.

Two possible explanations for the limited response to VIP in events of maturation can be suggested: First, depending on the experimental conditions, cAMP can either stimulate or inhibit meiosis (Hillensjo et al., 1978). It is possible that high doses of VIP sustain high levels of cAMP in the oocyte and prevent meiosis. Such a phenomenon is believed to occur in the presence of forskolin (Ekholm et al., 1984), dbcAMP, or phosphodiesterase inhibitors (Hillensjo et al., 1978). A second explanation for the limited response could be that VIP affects only one subpopulation of follicles or subpopulation of granulosa cells within each

follicle. In fact, Kasson et al. (1985) have shown one predominantly VIP-sensitive and one FSH-sensitive subpopulation of granulosa cells.

Nevertheless, although VIP did not influence maturation, it has been shown to stimulate ovulation in perfused rat ovaries (Schmidt et al., 1990). In mammalian system, VIP or LH, acting via the adenylyl cyclase system, induce an increase in plasminogen activator activity (Beers et al., 1975; Wang and Leung, 1983; Liu et al., 1987), a serine protease that has been implicated in the process of follicular rupture at the time of ovulation (Beers, 1975; Reich et al., 1985) and in the process of follicular cumulus cell expansion and dispersion (Liu et al., 1986).

In addition, the localization of VIP and its receptors in association with blood vessels (Hulshof et al., 1994; Vaccari et al., 2006) suggests that this neuropeptide might be involved in the regulation of ovarian blood flow. In fact, VIP contributes to the increase in blood flow around preovulatory follicles observed after the LH surge (Acosta et al., 2003). This increased ovarian stromal blood flow may, in turn, lead to a greater delivery of gonadotrophins to the granulosa cells of preovulatory follicles (Redmer and Reynolds, 1996), which will be important for the generation of a normal follicle and competent oocyte. The gonadotrophin would consequently stimulate the production of PACAP in the preovulatory follicle. The fact that PACAP induces genes related to ovulation and luteinization, and mediates some of the effects of LH on granulosa cell differentiation at the time of ovulation (Gras et al., 1999; Lee et al., 1999, Park et al., 2000), suggests that PACAP may serve as an ovarian physiological mediator of gonadotrophins in the ovulatory process. This is in agreement with previous demonstration that provides direct evidence of the presence of PAC1-R and the absence of VPAC1-R and VPAC2-R on germinal vesicles oocytes (Vaccari et al., 2006). Further studies are warranted to evaluate the respective roles of PACAP and VIP in ovulatory process.

VIP stimulates ovarian steroidogenesis

Studies have shown that in the periphery, the denervation of ovaries during the early luteal phase of the estrus cycle leads to changes in their morphology and impairs steroidogenic activity in pigs (Jana et al., 2005). Similarly, inhibition of ovarian secretory function and delayed pubertal onset were observed in rats after denervation (Ojeda et al.,

1983; Lara et al., 1990; Forneris and Aguado, 2002). The alterations in gonadal endocrine function are attributed to the loss of the peptidergic supply (for example, VIP) of neuronal fibers (Jana et al., 2005; Ojeda et al., 1983; Lara et al., 1990; Forneris et al., 2002). Therefore, these studies show that VIP is implicated in ovarian steroidogenesis.

In addition, VIP is involved in the regulation of steroidogenic activity, stimulating estradiol and progesterone release from cultured granulosa cells and whole ovaries in vitro (Davoren and Hsueh, 1985; Ahmed et al., 1986; Parra et al., 2007; Kowalewski et al., 2010), progesterone release in vivo (Fredericks et al., 1983), and androgen release from ovarian fragments in vitro (Ahmed et al., 1986). These effects may be related to the ability of VIP to enhance the synthesis of the cholesterol side-chain cleavage enzyme complex (Trzeciak et al., 1986), the rate-limiting step in progesterone biosynthesis and the activity of the aromatase enzyme complex (George and Ojeda, 1987). Studies have provided evidence that VIP is capable of inducing aromatase activity before the ovary became responsive to gonadotropins (George and Ojeda, 1987; Mayerhofer et al., 1997). A possibility to consider is that VIP induction of estradiol release during the estrus cycle may also come from immature follicles. If true, this would suggest that VIP plays a complementary role to that of FSH, the primary mediator of estradiol biosynthesis, in determining the magnitude of estradiol increase under varying physiologic conditions (Parra et al., 2007). These findings strongly suggest a physiological role for this peptide in ovarian steroid biosynthesis.

Little is known about the molecular mechanisms of VIP-mediated steroidogenesis. It is known that VIP increases the levels of cAMP (Tornell et al., 1988, Apa et al., 1997; Vaccari et al., 2006) and subsequently leads to PKA activation, which in turn induces steroidogenesis in granulosa cells. The cAMP/PKA pathway is the major route in the trophic hormone-stimulated regulation of steroidogenic acute regulatory protein (StAR) expression and function. StAR mediates the rate-limiting step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane, and it is the hormonal regulation of StAR expression and activity that allows tissues to accurately control their steroid production (Kowalewski et al., 2010). A reduction of StAR and 3β-hydroxysteroid dehydrogenase expression, enzyme converting pregnenolone to progesterone, accompanied by decreased serum levels of FSH was demonstrated in young VIP knockout mice (Lacombe et al., 2007).

Additional signaling pathways may be involved because the receptors activate multiple intracellular pathways (Spengler et al., 1993; Lutz et al., 1993).

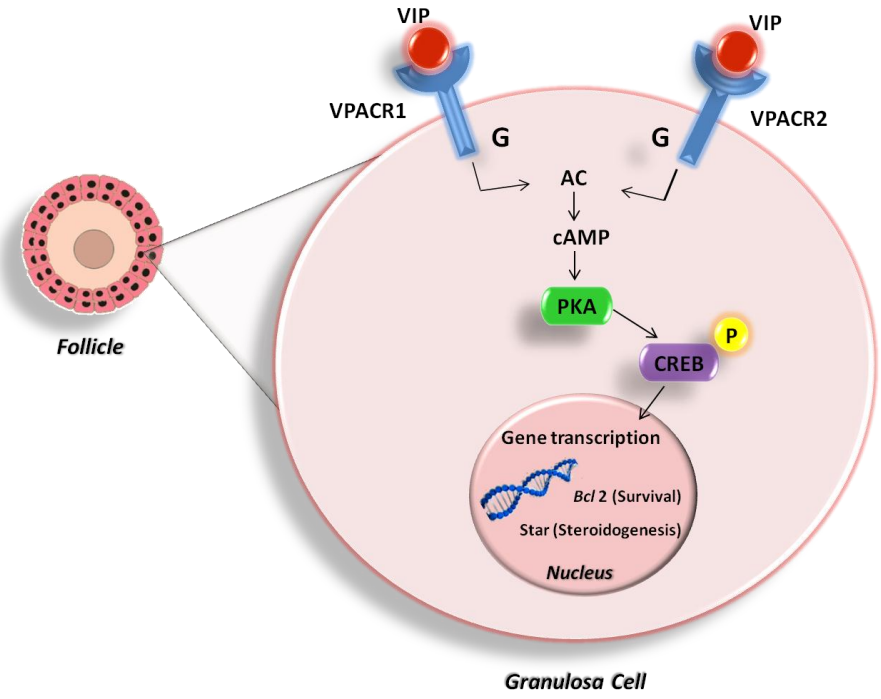

Figure 1. Schematic illustration of the signaling pathways for the effect of VIP in granulosa cells. VIP binds through two G-protein-coupled receptors (VPAC1-R and VPAC2-R). Binding of the peptide to the receptors in the cell membrane results in activation of adenylate cyclase (AC) with increased intracellular formation of cAMP, which in turn activates protein kinase A (PKA). The reactions culminate in activation of cAMP response element binding protein (CREB) via its phosphorylation, followed by upregulation of the StAR and antiapoptotic protooncogene Bcl-2.

Final considerations

Collectively, these results underline the crucial role of VIP and their receptors in the control ovarian folliculogenesis. VIP and its receptors, through cAMP/PKA pathway, regulate the follicle survival and growth, oocyte maturation and ovulation, as well as ovarian steroidogenesis. Further research in this field will greatly advance our understanding of ovarian physiology.

References

Acosta TJ, Hayashi KG, Ohtani M, Miyamoto A. 2003. Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. Reproduction, 125:759-767.

Ahmed CE, Dees WL, Ojeda SR. 1986. The immature rat ovary is innervated by vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. Endocrinology, 118:1682-1689.

Apa R, Lanzone A, Mastrandrea M, Miceli F, Macchione E, Fulghesu AM, Caruso A, Canipari R. 1997. Effect of pituitary adenylate cyclaseactivating peptide on meiotic maturation in follicle-enclosed, cumulus-enclosed, and denuded rat oocytes. Biol Reprod, 57:1074-1079.

Arimura A. 1992. Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research. Regul Pept, 37:287-303.

Barberi M, Muciaccia B, Morelli MB, Stefanini M, Cecconi S, Canipari R. 2007. Expression localisation and functional activity of pituitary adenylate cyclaseactivating polypeptide, vasoactive intestinal polypeptide and their receptors in mouse ovary. Reproduction, 134:281-292.

Beers WH. 1975. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell, 6:379-386.

Beker ARCL, Izadyar F, Colenbrander B, Bevers MM. 2000. Effect of growth hormone releasing hormone (GHRH) and vasoactive intestinal peptide (VIP) on in vitro bovine oocyte maturation. Theriogenology, 53:1771-1782.

Bruno JB, Celestino JJH, Lima-Verde IB, Matos MHT, Lima LF, Name KPO, Araújo VR, Saraiva MVA, Martins FS, Campello CC, Silva JRV, Báo SN, Figueiredo JR. 2010. Vasoactive intestinal peptide improves the survival and development of caprine preantral follicles after in vitro tissue culture. Cells Tissue Organs, 191:414-21.

Burden HW. 1985. The adrenergic innervations of mammalian ovaries. In Ben-Jonathan N, Bahr MM, Weiner RI (Ed.). Catecholamines as Hormone Regulators. New York, Raven Press, pp. 261-278.

Cecconi S, Rossi G, Barberi M, Scaldaferri L, Canipari R. 2004. Effect of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide on mouse preantral follicle development in vitro. Endocrinology, 145:2071-2079.

Davoren JJ, Hsueh AJW. 1985. Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. Biol Reprod, 33:37-52.

Delgado M, Garrido E, Martinez C, Leceta J, Gomariz RP. 1996. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptides (PACAP27) and (PACAP38) protect CD4+CD8+ thymocytes from glucocorticoid-induced apoptosis. Blood, 87:5152-5161.

Ekholm C, Hiilensjö T, Magnusson C, Rosberg S. 1984. Stimulation and inhibition of rat oocyte meiosis by forskolin. Biol Reprod, 30:537-43.

Filippatos GS, Gangopadhyay N, Lalude O, Parameswaran N, Said SI, Spielman W, Uhal BD. 2001. Regulation of apoptosis by vasoactive peptides. Am. J. Physiol. Lung. Cell Mol Physiol, 281:749-761.

Flaws JA, Desanti A, Tilly KI, Javid RO, Kugu K, Johnson AL, Hirshfield AN, Tilly JL. 1995. Vasoactive intestinal peptidemediated suppression of apoptosis in the ovary: potential mechanisms of action and evidence of a conserved antiatretogenic role through evolution. Endocrinology, 136:4351-4359.

Forneris ML, Aguado LI. 2002. Neonatal superior nerve transection disturbs the cyclic activity of the female rats. J Steroids Biochem Mol Biol, 82:75-82.

Fredericks CM, Lundquist LE, Mathur RS, Ashton SH, Landgrebe SC. 1983. Effects of vasoactive intestinal polypeptide upon ovarian steroids, ovum transport and fertility in the rabbit. Biol Reprod, 28:1052-1060.

George FW, Ojeda SR. 1987. Vasoactive intestinal peptide induces aromatase activity before development of primary follicles or responsiveness to follicle-stimulating hormone. Proc Natl Acad Sci USA, 84:5803-5807.

Gozes I, Tsafriri A. 1986. Detection of vasoactive intestinal peptideencoding messenger ribonucleic acid in the rat ovaries. Endocrinology, 119:2606-2610.

Gozes I, Fridkinb M, Hill JM, Brenneman DE. 1999. Pharmaceutical VIP: prospects and problems. Curr Med Chem, 6:1019-1034.

Gras S, Hannibal J, Fahrenkrug J. 1999. Pituitary adenylate cyclaseactivating polypeptide is an auto/paracrine stimulator of acute progesterone accumulation and subsequent luteinization in cultured periovulatory granulosa/lutein cells. Endocrinology, 140:2199-2205.

Gutierrez-Cañas I, Rodriguez-Henche N, Bolanos O, Carmena MJ, Prieto JC, Juarranz MG. 2003. VIP and PACAP are autocrine factors that protect the androgen-independent prostate cancer cell line PC-3 from apoptosis induced by serum withdrawal. Br J Pharmacol, 139:1050-1058.

Hillensjö T, Ekhohn C, Ahrén K. 1978. Role of cyclic AMP in oocyte maturation and glycolysis in the pre-ovulatory rat follicle. Acta Endocrinol, 87:377-388.

Hulshof SCJ, Dijkstra G, Van Der Beek EM, Bevers MM, Figueiredo JR, Beckers JF, Van Den Hurk R. 1994. Immunocytochemical localization of vasoactive intestinal peptide and neuropeptide y in the bovine ovary. Biol Reprod, 50:553-560.

Hulshof SCJ. 1995. Bovine preantral follicles and their development in vitro; Utrecht, Thesis.

Jana B, Dzienis A, Pańczyszyn J, Rogozińska A, Wojtkiewicz J, Skobowiat C, Majewski M. 2005. Denervation of the porcine ovaries performed during the early luteal phase influenced morphology and function of the gonad. Reprod Biol, 5:69-82.

Johnson AL, Tilly JL. 1988. Effects of vasoactive intestinal peptide on steroid secretion and plasminogen activator activity in granulose cells of the hen. Biol Reprod, 38:296-303.

Johnson AL, Li Z, Gibney JA, Malamed S. 1994. Vasoactive intestinal peptide-induced expression of cytochrome p450 cholesterol side-chain cleavage and 17a-hydroxylase enzyme activity in hen granulosa cells. Biol Reprod, 51:327-333.

Kasson BG, Meidan R, Davoren JB, Hsueh AJW. 1985. Identification of subpopulations of rat granulosa cells: sedimentation properties and hormonal responsiveness. Endocrinology, 7:1027-1034.

Kowalewski MP, Dyson MT, Boos A, Stocco DM. 2010. Vasoactive intestinal peptide (VIP)-mediated expression and function of steroidogenic acute regulatory protein (StAR) in granulosa cells. Mol Cell Endocrinol, 328:93-103.

Lacombe A, Lelievre V, Roselli CE, Muller JM, Waschek JA, Vilain E. 2007. Lack of vasoactive intestinal peptide reduces testosterone levels and reproductive aging in mouse testis. J Endocrinol, 194:153-160.

Lara HE, McDonald JK, Ahmed CE, Ojeda SR. 1990. Guanethidine-mediated destruction of ovarian sympathetic nerves disrupts ovarian development and function in rats. Endocrinology, 127:2199-2209.

Latini S, Chiarpotto M, Muciaccia B, Vaccari S, Barberi M, Guglielmo MC, Stefanini M, Cecconi S, Canipari R. 2010. Inhibitory effect of pituitary adenylate cyclase activating polypeptide on the initial stages of rat follicle development. Mol Cell Endocrinol, 320:34-44.

Ledda S, Bogliolo L, Leoni G, Calvia P, Naitana S. 1996. Influence of vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP) and insulin-like growth factor-I (IGF-I) on in vitro maturation of prepubertal and adult sheep oocytes. Zygote, 4:343-348.

Lee J, Park HJ, Choi HS, Kwon HB, Arimura A, Lee BJ, Choi WS, Chun SY. 1999. Gonadotropin stimulation of pituitary adenylate cyclase-activating polypeptide (PACAP) messenger ribonucleic acid in the rat ovary and the role of PACAP as a follicle survival factor. Endocrinology, 140:818-826.

Liu YX, Ny T, Sarkar D, Loskutoff D, Hsueh AJW. 1986. Identification and regulation of tissue plasminogen activator activity in rat cumulus-oocyte complexes. Endocrinology, 119:1578-1587.

Liu YX, Kasson BG, Dahl KD, Hsueh AJ. 1987. Vasoactive intestinal peptide stimulates plasminogen activator activity by cultured rat granulosa cells and cumulus-oocyte complexes. Peptides, 8:29-33.

Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G, Harmar AJ. 1993. The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. FEBS Lett, 334:3-8.

Mayerhofer A, Dissen GA, Costa M, Ojeda SR. 1997. A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. Endocrinology, 138:3320-3329.

Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH. 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun, 164:567-574.

Nandi S, Ravindranatha BM, Gupta PSP, Raghu HM, Sarma PV. 2003. Developmental competence and post-thaw survivability of buffalo embryos produced in vitro: effect of

growth factors in oocyte maturation medium and of embryo culture system Theriogenology, 60:1621-1631.

Ojeda SR, White SS, Aguado LI, Advis JP, Andersen JM. 1983. Abdominal vagotomy delays the onset of puberty and inhibits ovarian function in the female rat. Neuroendocrinology, 36:261-267.

Ojeda SR, Lara HE. 1989. The role of the sympathetic nervous system in the regulation of ovarian function. In Pirke KM, Wuttke W, Schweiger U (Ed.). The Menstrual Cycle and Its Disorders, Berlin, Springer-Verlag, pp. 26-32.

Ojeda SR, Lara HE, Ahmed CE. 1989. The potential relevance of vasoactive intestinal peptide to ovarian physiology. In Adashi E (Ed.). Putative Intraovarian Regulators, New York, Thieme Inc., pp. 52-60.

Ottesen B, Fahrenkrug J. 1995. Vasoactive intestinal polypeptide and other preprovasoactive intestinal polypeptide-derived peptides in the female and male genital tract: localization, biosynthesis, and functional and clinical significance. Am J Obstet Gynecol, 172:1615–1631.

Park HJ, Lee J, Wang L, Park JH, Kwon HB, Arimura A, Chun SY. 2000. Stage-specific expression of pituitary adenylate cyclase-activating polypeptide type I receptor messenger ribonucleic acid during ovarian follicle development in the rat. Endocrinology, 141:702-709.

Parra C, Fiedler JL, Lunal SL, Greiner M, Padmanabhan V, Lara HE. 2007. Participation of vasoactive intestinal polypeptide in ovarian steroids production during the rat estrous cycle and in the development of estradiol valerate-induced polycystic ovary. Reproduction, 133:147-154.

Redmer DA, Reynolds LP. 1996. Angiogenesis in the ovary. Rev Reprod, 1:182-192.

Reich R, Miskin R, Tsafriri A. 1985. Follicular plasminogen activator: involvement in ovulation. Endocrinology, 116:516-521.

Said SI. 1996. Molecules that protect: the defense of neurons and other cells. J Clin Invest, 97:2163-2164.

Sastry KSR, Smith AJ, Karpova Y, Datta SR, Kulik G. 2006. Diverse antiapoptotic signaling pathways activated by vasoactive intestinal polypeptide, epidermal growth factor, and phosphatidylinositol 3-kinase in prostate cancer cells converge on bad. J Biol Chem, 281:20891-20901.

Schecterson LC, McKnight GS. 1991. Role of cyclic adenosine 3',5'- monophosphate-dependent protein kinase in hormone-stimulated /3-endorphin secretion in AtT20 cells. Mol Endocrinol, 5:170-178.

Schmidt G, Jörgensen J, Kannisto P, Liedberg F, Ottesen B, Owman C. 1990. Vasoactive intestinal polypeptide in the PMSG-primed immature rat ovary and its effect on ovulation in the isolated rat ovary perfused in vitro. J Reprod Fertil, 90:465-472.

Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, Journot L. 1993. Differential signal transduction by five splice variants of the PACAP receptor. Nature, 365:170-175.

Tornell J, Carlsson B, Hillensjo T. 1988. Vasoactive intestinal peptide stimulates oocyte maturation, steroidogenesis, and cyclic adenosine 3, 5 -monophosphate production in isolated preovulatory rat follicles. Biol Reprod, 39:213-220.

Trzeciak WH, Schmid CE, Simpson E, Ojeda SR. 1986. Vasoactive intestinal peptide induces the synthesis of the cholesterol side-chain cleavage enzyme complex in cultured rat ovarian granulosa cells. Proc Natl Acad Sci USA, 83:7490-7494.

Vaccari S, Latini S, Barberi M, Teti A, Stefanini M, Canipari R. 2006. Characterization and expression of different pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptors in rat ovarian follicles. J Endocrinol, 191:287-299.

Vaudry D, Gonzalez BJ, Magali B, Pamantung TF, Fontaine M, Fournier A, Vaudry H. 2000. The neuroprotective effect of pituitary adenylate cyclase-activating polypeptide on cerebellar granule cells is mediated through inhibition of the CED3-related cysteine protease caspase-3/CPP32. Proc Natl Acad Sci USA, 97:13390-13395.

Walton MR, Dragunow M. 2000. Is CREB a key to neuronal survival?. Trends Neurosci, 23:48-53.

Wang C, Leung A. 1983. Gonadotropins regulate plasminogen activator production by rat granulosa cells. Endocrinology, 112:1201-1207.

8. CAPÍTULO 3

Expressão dos receptores de angiotensina II em ovários caprinos e melhoria da viabilidade folicular in vitro

(Expression of angiotensin II receptors in the caprine ovary and improvement of follicular viability in vitro)

Periódico: Reproduction, Fertility and Development (Submetido em novembro de 2010).

Resumo

Esse estudo investigou os níveis de RNAm para os receptores de ANG II (AGTR1 e AGTR2) em ovários de cabras e avaliou o efeito da ANG II sobre a sobrevivência e o crescimento de folículos pré-antrais caprinos. Fragmentos ovarianos foram cultivados por um ou sete dias em α-meio essencial mínimo (αMEM^{+}) com ANG II (0, 1, 5, 10, 50 ou 100 ng/mL) e analisados por histologia, microscopia de fluorescência e microscopia eletrônica de transmissão. Os níveis de RNAm para AGTR1 foram similares nos diferentes tipos de folículos pré-antrais; folículos primordiais apresentaram maiores níveis de RNAm para AGTR2 que folículos secundários. Células da granulosa/teca de pequenos ou grandes folículos antrais apresentaram níveis significativamente superiores de RNAm para AGTR1 que seus respectivos COCs. Por outro lado, COCs de grandes folículos antrais tinham níveis de RNAm para AGTR2 que foram significativamente maiores que suas respectivas células da granulosa/teca. ANG II (10 and 50 ng/mL) aumentou a percentagem de folículos pré-antrais normais após sete dias de cultivo comparado com αMEM^{+}. Estudos de fluorescência e ultra-estrutural confirmaram a integridade folicular após sete dias em ANG II (10 ng/mL). Em conclusão, RNAm para AGTR1 e AGTR2 foram detectados em todas categorias foliculares. ANG II (10 ng/mL) manteve a viabilidade de folículos pré-antrais caprinos por sete dias.

Keywords: Angiotensina II. Folículo pré-antral. Cultivo. Caprinos.

Expression of angiotensin II receptors in the caprine ovary and improvement of follicular viability in vitro

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Running head: ANG II and follicular survival

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Abstract

This study investigated the levels of ANG II receptor (AGTR1 and AGTR2) mRNA in goat ovaries and evaluated the effect of ANG II on the *in vitro* survival and growth of caprine preantral follicles. Ovarian fragments were cultured for one or seven days in α-Minimum Essential Medium (αMEM^{+}) with ANG II (0, 1, 5, 10, 50 or 100 ng/mL) and analyzed by histology, fluorescent microscopy and transmission electron microscopy. The levels of AGTR1 mRNA were similar in the different types of preantral follicles; primordial follicles had higher levels of AGTR2 mRNA than secondary follicles. Granulosa/theca cells from either small or large antral follicles had significantly higher levels of AGTR1 mRNA than their respective COCs. Conversely, COCs from large antral follicles had AGTR2 mRNA levels that were significantly higher than their respective granulosa/theca cells. ANG II (10 and 50 ng/mL) increased the percentages of normal preantral follicles after seven days of culture compared with αMEM^{+}. Fluorescence and ultrastructural studies confirmed follicular integrity after seven days in ANG II (10 ng/mL). In conclusion, AGTR1 and AGTR2 mRNAs were detected in all follicular categories. ANG II (10 ng/mL) maintained caprine preantral follicle viability for seven days.

Keywords: angiotensin II; preantral follicles; culture; caprine.

1. Introduction

The formation, growth and maturation process of ovarian follicles is known as folliculogenesis and is influenced by various substances, including hormones and growth factors (Figueiredo *et al*. 2008). Of these substances, Angiotensin II (ANG II) plays an important role in folliculogenesis. ANG II and its receptors are part of a system known as the renin-angiotensin system (RAS) (Yoshimura, 1997). The RAS plays important roles in the synthesis and secretion of prostaglandins and estrogen, the regulation of ovarian follicle development, ovulation and atresia (Pellicer *et al*. 1988; Yoshimura *et al*. 1993).

ANG II acts through two main types of membrane receptors, AGTR1 and AGTR2. ANG II receptors have been detected in the granulosa and inner theca cells of rat antral

follicles (Husain *et al*. 1987). Furthermore, rabbit granulosa cells express high levels of AGTR2 receptors in preovulatory follicles (Yoshimura *et al*. 1996). In bovines, Schauser *et al*. (2001) observed that only theca cells have ANG II receptors, and a predominance of AGTR2 was noted in the outer theca. Recently, Portela *et al*. (2008) detected AGTR1 and AGTR2 mRNA in both the theca and granulosa cells of bovine antral follicles. However, quantification of the steady-state level of AGTR1 and AGTR2 mRNAs during different stages of goat follicular development has not yet been performed.

The activity of ANG II has been related to oocyte maturation, ovulation and steroidogenesis in rabbits (Yoshimura *et al*. 1993; Yoshimura *et al*. 1992; Feral *et al*. 1995) and to meiosis resumption (Giometti *et al*. 2005; Stefanello *et al*. 2006), follicular growth (Nielsen *et al*. 1994) and steroidogenesis (Acosta *et al*. 1999) in cows. Li *et al*. (2004) observed that 100 ng/ml of ANG II stimulated nuclear and cytoplasmic maturation of porcine oocytes. Giometti *et al*. (2005) found that oocytes cultured for 12 h in medium conditioned with follicular cells had inhibited nuclear maturation, and the majority of oocytes remained in the germinal vesicle stage. However, after the addition of ANG II to the medium, this effect was reversed.

The role of ANG II in late folliculogenesis, especially in the processes of ovulation and maturation, is of great interest. However, the effect of this substance on early folliculogenesis is not currently known. Thus, the present study was designed to investigate the influence of different concentrations of ANG II (1, 5, 10, 50 or 100 ng/ml) on the survival, activation (transition from primordial to primary follicles) and growth of preantral follicles enclosed in caprine ovarian tissue cultured for one or seven days.

2. Materials and methods

2.1 Steady-state level of AGTR1 and AGTR2 mRNA in goat ovarian follicles

To evaluate the steady-state level of mRNA, 30 ovaries from 15 goats (*Capra hircus*) were collected at a local slaughterhouse and rinsed in Minimum Essential Medium (MEM) containing antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). After this preparation, 10 ovaries from 5 goats were used to isolate primordial, primary and secondary

follicles. The remaining ovaries were used to collect cumulus–oocyte complexes (COCs), mural granulosa cells, and theca cells from small and large antral follicles. Primordial, primary, and secondary follicles were isolated using a previously described mechanical procedure (Lucci *et al*. 1999). After isolation, these follicles were washed several times to completely remove the stromal cells and were then placed by category into separate Eppendorf tubes in groups of 10. This procedure was completed within 2 h, and all samples were stored at -80°C until the RNA was extracted. From a second group of ovaries (n=15), COCs aspirated from small (1–3 mm) and large (3–6 mm) antral follicles were recovered. Compact COCs were selected from the follicle content as described by van Tol and Bevers (1998). Thereafter, groups of 10 COCs were stored at -80°C until RNA extraction. To collect the mural granulosa and theca cell complex, small (n=10) and large antral follicles (n=10) were isolated from the ovaries (n=5) and dissected free from the stromal tissue with forceps as previously described (van Tol and Bevers, 1998). The follicles were then bisected, and the granulosa and theca cell complexes were collected and stored at -80°C.

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

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

Quantification of the AGTR1 and AGTR2 mRNAs was performed using SYBR Green. PCR reactions were composed of 1 µL cDNA as a template in 7.5 µL of SYBR Green

Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µL of ultra-pure water, and 0.5 µM of each primer. The primers were designed to amplify AGTR1 and AGTR2 mRNA. Glyceraldehyde-2-phosphate dehydrogenase (GAPDH) and β-actin were used as endogenous controls to normalize the steady-state mRNA level of the genes (Table 1). The thermal cycling profile for the first round of PCR was as follows: initial denaturation and activation of the polymerase for 15 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 45 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany). The delta-delta-CT method was used to transform CT values into normalized relative steady-state mRNA levels.

Table 1. Primer pairs used for real-time PCR analyses.

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
GAPDH	TGTTTGTGATGGGCGTGAACCA	s	287-309	GI:27525390
	ATGGCGTGGACAGTGGTCATAA	as	440-462	
β-actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI:28628620
	TCCTTGATGTCACGGACGATTTCC	as	386-410	
AT1	AGCATTGACCGCTACCTGGCTATT	s	367-390	GI: 57619242
	TAGTTGGCAAACTGGCCAAACCTG	as	490-513	
AT2	TACATCTTCAACCTCGCTGTGGCT	s	244-267	GI: 148277605
	TCACAGGTCCAAAGAGCCAGTCAT	as	346-369	

s,sense; as, antisense

2.2 *In vitro* culture of preantral follicles

Ovarian cortical tissues (n=8 ovaries) were collected at a local slaughterhouse from 4 adult (1-3 years old) mixed-breed goats (*Capra hircus*). Immediately postmortem, the ovaries were washed once in 70% alcohol for 10 s and twice in MEM supplemented by HEPES and antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin). Ovary pairs were transported within 1 h to the laboratory in MEM at 4°C (Chaves *et al*. 2008). Unless otherwise stated, the

culture media, synthetic human ANG II, and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

The organ culture system used herein was previously described in detail (Matos *et al*. 2007). In the laboratory, ovarian cortex tissue samples from each ovarian pair were cut into 13 slices (approximately 3 x 3 mm with a 1-mm thickness) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultrastructural analysis (fresh control) or placed in culture for one or seven days. Ovarian pieces were incubated in 1 ml of culture media consisting of MEM supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine and 1.25 mg/mL bovine serum albumin (BSA) in the absence (cultured control) or presence of 1, 5, 10, 50 or 100 ng/mL ANG II in 24-well plates at 39°C in 5% CO_2. Each treatment was repeated four times, and the ovaries of four different animals were consequently used. The culture media were replenished every other day.

2.3 Morphological analysis and assessment of *in vitro* follicular growth

Before culture (fresh control) and after one or seven days in culture, all of the pieces cultured with or without ANG II were fixed in Carnoy's solution for 12 h and then dehydrated in increasing concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the caprine tissue pieces were cut into 7-µm sections, and each section was mounted on a glass slide and stained by periodic acid-Schiff and hematoxylin. Follicle stage and survival were assessed microscopically on serial sections. Coded slides were blindly examined using a microscope (Nikon, Japan) under 400X magnification.

The developmental stages of follicles have been defined previously (Silva *et al*. 2004a) as primordial (one layer of flattened granulosa cells around the oocyte) or growing (intermediate: one layer of flattened to cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells; secondary: two or more layers of cuboidal granulosa cells around the oocyte). Individual follicles were further classified as histologically normal when an intact oocyte surrounded by well-organized granulosa cells in one or more layers without a pyknotic nucleus was observed. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells that were detached from the

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

To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before (fresh control) and after culture in each medium. In addition, follicle and oocyte diameters were measured only in morphologically normal follicles. The follicle diameter was recorded as the length from edge to edge of the basement membrane or from the outside edge of the theca cell layer when present. Oocyte diameter was recorded as the length from edge to edge of the oocyte membrane. Two perpendicular diameters were recorded for each parameter, and the average of these two values was reported. Furthermore, each follicle was examined in every section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting. This ensured that each follicle was only counted once, regardless of its size.

2.4 Viability assessment of follicles cultured *in vitro*

Based on the morphological analysis, the viability of the follicles cultured with the ANG II concentrations that promoted the highest percentages of normal follicle growth was further analyzed using fluorescent probes. Additional pairs of goat ovaries (n=2) were collected from a slaughterhouse and cut into fragments at the laboratory. One of these fragments was immediately processed for follicle isolation, and the remaining fragments were cultured for seven days with ANG II (10 and 50 ng/mL) as described above. After the culture period, fragments were processed for mechanical isolation using the methods described by Lucci *et al*. (1999).

Thereafter, the viability of isolated preantral follicles was assessed through a two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells using calcein-AM and ethidium homodimer-1, respectively. The first probe detected the intracellular esterase activity of viable cells, and the second labeled nucleic acids of non-viable cells that had plasma membrane disruption. The test was performed by adding 4 μM calcein-AM and 2 μM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) to the suspension of isolated follicles and incubating at 37°C for 15 min. After labeling, follicles were washed once by centrifugation at 100 × *g* for 5 min, resuspended in

MEM and mounted on a glass microscope slide in 5 mL anti-fading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching. Finally, follicles were examined using a DMLB fluorescence microscope (Leica, Germany). The emitted fluorescence signals of calcein-AM and ethidium homodimer-1 were collected at 488 and 568 nm, respectively. Oocytes and granulosa cells were considered alive if the cytoplasm stained positively with calcein-AM (green) and the chromatin was not labeled with ethidium homodimer-1 (red).

2.5 Ultrastructural analysis of caprine preantral follicles

For a more detailed evaluation of follicular morphology after histological analysis, ultrastructural studies were performed on the fragments of fresh control and treated with the lowest concentration that maintained their viability (10 ng/mL). A section with a maximum dimension of 1 mm^3 was cut from each fragment of the ovarian tissue and fixed in Karnovsky solution (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2) for 3 h at room temperature (approximately 25°C). After three washes in sodium cacodylate buffer, specimens were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The samples were then dehydrated using a gradient of acetone solutions and then embedded in SPIN-PON resin (Sigma Company, St Louis, MO). Subsequently, semi-thin sections (3 µm) were cut, stained with toluidine blue and analyzed using light microscopy at 400 × magnification. Ultra-thin sections (60–70 nm) were obtained from preantral follicles, which were classified as morphologically normal in semi-thin sections according to the criteria adopted in histology. Subsequently, the ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined under a Morgani-FEI transmission electron microscope operating at 80 kV. The following parameters were evaluated: the density and the integrity of ooplasmic and granulosa cell organelles, vacuolization and the integrity of the basement and nuclear membranes.

2.6 Statistical analysis

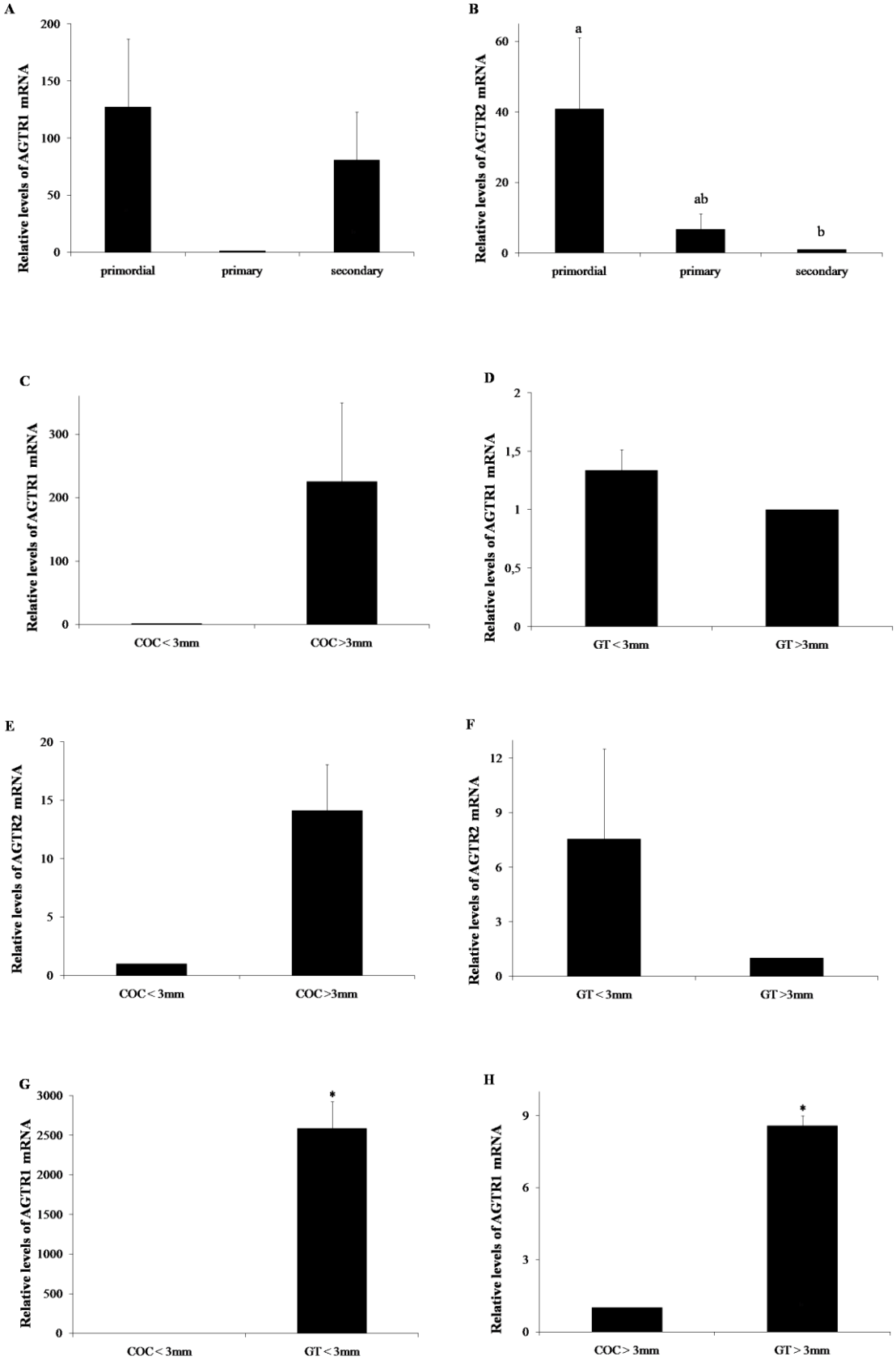
mRNA expression data from primordial, primary and secondary follicles were analyzed using the Kruskal-Wallis test, and the t test was used for paired comparisons of mRNA expression in small and large antral follicles ($P<0.05$). Percentages of the surviving follicles at both stages (i.e., primordial and developing) and follicle and oocyte diameters were calculated for one or seven days of the various treatments. These data were initially analyzed using Kolmogorov–Smirnov and Bartlett's tests to confirm a normal distribution and homogeneity of variance, respectively. Analysis of variance was then performed using the GLM procedure of SAS (1999), and Dunnett's test was applied to compare ANG II-treated groups with the control and αMEM^{+} groups (Steel *et al*. 1997). To avoid type II errors that may result from the high coefficient of variation, Duncan's test was applied to compare results for cultures with different ANG II concentrations, and Student's t-test was used to compare the means between one and seven days of culture. Data obtained from fluorescence microscopy, which was used to validate the histological findings, were also pooled and analyzed by a Chi-square test. Differences were considered to be significant when $P<0.05$, and results are expressed as means ± standard error of the mean (SEM).

3. Results

3.1. Steady-state level of AGTR1 and AGTR2 mRNA in goat ovarian follicles

The quantification of AGTR1 receptor mRNA expression did not demonstrate differences among the preantral follicles, i.e., primordial, primary and secondary follicles ($P>0.05$ – Figure 1A). Primordial follicles had significantly higher levels of AGTR2 receptor mRNA than the secondary follicle stages ($P<0.05$), but the levels did not differ significantly from those of primary follicles ($P>0.05$ – Fig. 1B).

No differences were observed in the AGTR1 and AGTR2 receptor mRNA levels for COCs or granulosa/theca cells collected from small and large antral follicles ($P>0.05$ – Figure 1C, D, E, F). Granulosa/theca cells from either small or large antral follicles had significantly higher levels of AGTR1 mRNA than their respective COCs ($P<0.05$ – Figure 1G, H). Conversely, COCs from large antral follicles had AGTR2 mRNA levels that were significantly higher than their respective granulosa/theca cells ($P<0.05$ – Figure 1I, J).


A
Relative levels of AGTR1 mRNA
200
150
100
50
0
primordial
primary
secondary
B
Relative levels of AGTR2 mRNA
60
40
20
0
a
ab
b
primordial
primary
secondary
C
Relative levels of AGTR1 mRNA
300
200
100
0
COC < 3mm
COC >3mm
D
Relative levels of AGTR1 mRNA
2
1,5
1
0,5
0
GT < 3mm
GT >3mm
E
Relative levels of AGTR2 mRNA
20
15
10
5
0
COC < 3mm
COC >3mm
F
Relative levels of AGTR2 mRNA
12
9
6
3
0
GT < 3mm
GT >3mm
G
Relative levels of AGTR1 mRNA
3000
2500
2000
1500
1000
500
0
*
COC < 3mm
GT < 3mm
H
Relative levels of AGTR1 mRNA
9
6
3
0
*
COC > 3mm
GT > 3mm

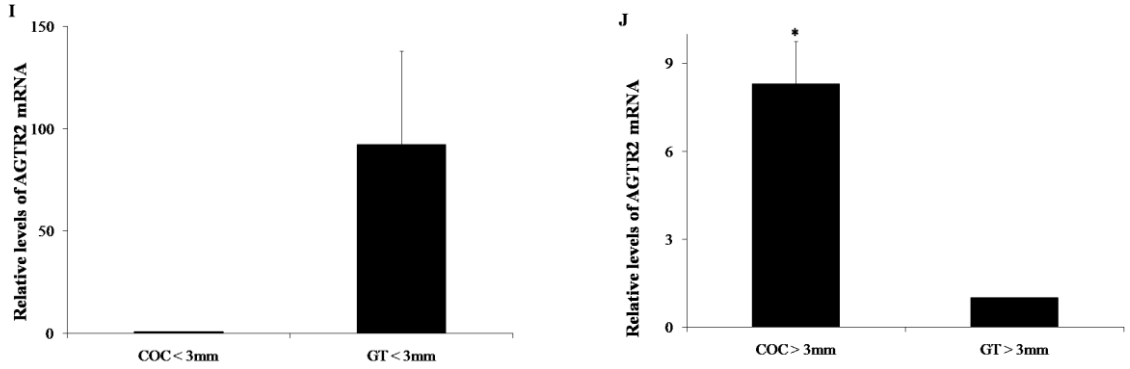


Figure 1. Steady-state levels of AGTR1 and AGTR2 mRNAs in goat ovarian follicles (means ± SEM). A, B) Primordial, primary and secondary follicles. C, E) COCs from small and large antral follicles. D, F) Granulosa/theca cells from small and large antral follicles. G, I) COCs and granulosa/theca cells from small antral follicles. H, J) COCs and granulosa/theca cells from large antral follicles. [a,b] ($P<0.05$).

3.2 Effect of ANG II on follicular survival after *in vitro* culture

The present study analyzed a total of 1,560 caprine preantral follicles. Figure 2 shows histological sections of primordial (A), intermediate (B) and primary (C) morphologically normal follicles after seven days of culture in 10 ng/mL ANG II. As shown in Figure 3, a significant reduction ($P<0.05$) in the percentage of morphologically normal follicles was observed in all treatments when compared to the fresh control (90.82%), except when the fragments were cultured for one day in 10 ng/mL ANG II (81.67%; $P>0.05$). Furthermore, after seven days, a significantly higher percentage of normal follicles ($P<0.05$) was observed in tissues that were cultured with 10 or 50 ng/mL ANG II than those cultured with 100 ng/mL ANG II or the control (αMEM^{+}). The percentage of follicles with normal morphology was similar on days 1 and 7 of culture for all treatments, except for the αMEM^{+} and 100 ng/ml ANG II treatments ($P<0.05$).

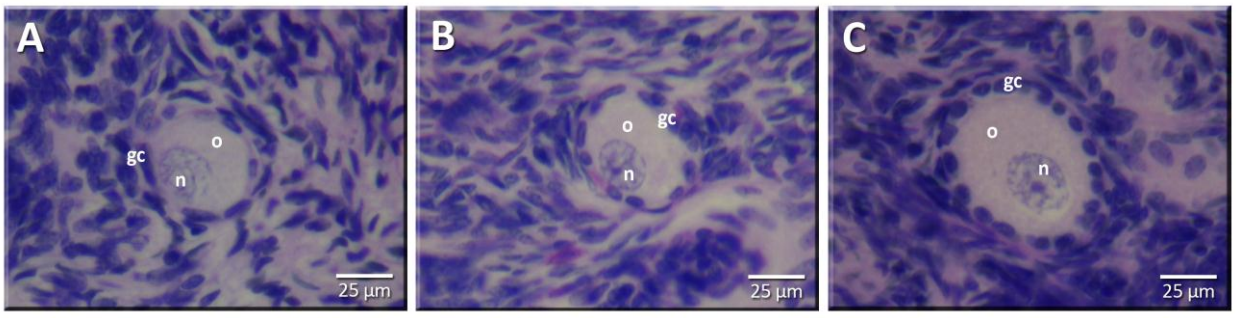


Figure 2. Histological sections, stained with periodic-acid-Schiff hematoxylin, showing primordial (A), intermediate (B) and primary (C) normal follicles after culture in 10-ng/mL ANG II for 7 days. o: oocyte; n: oocyte nucleus; gc: granulosa cells. Magnification was at 400x.

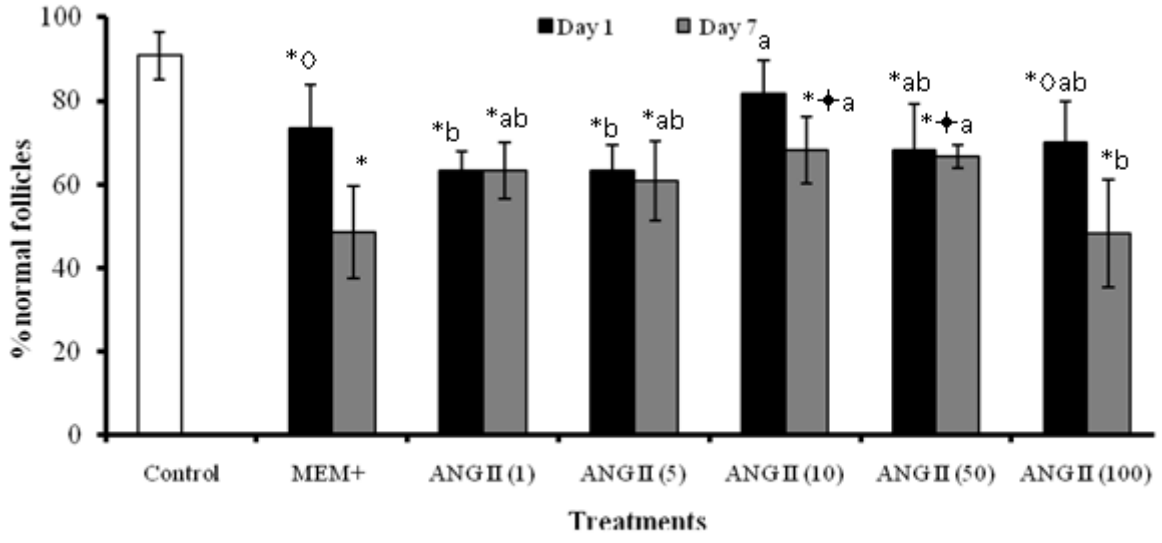


Figure 3. Percentage (mean ± S.E.M.) of morphologically normal caprine preantral follicles in the fresh control and after *in vitro* culture for one or seven days in the absence or presence of ANG II. *: differs significantly from control follicles ($P<0.05$); ✦: differs significantly from αMEM^{+} alone for each day of culture ($P<0.05$); a, b, c: differs significantly between ANG II at different concentrations ($P<0.05$); ◊: differs significantly between days of culture within a single treatment ($P<0.05$).

3.3 Assessment of preantral follicle viability by fluorescence

Based on the results of the morphological analysis, a viability trial was performed using the treatments that promoted a higher percentage of normal follicles. Caprine preantral follicles (n=30) were analyzed after seven day culture in αMEM^{+} supplemented with 10 (Figure 4A, B) or 50 ng/mL ANG II (Figure 4C, D). The results of this quantitative analysis showed that the percentage of viable follicles was similar in the fresh control group (85%) and those cultured in 10 and 50 ng/mL ANG II (93.33% and 90%, respectively) (P>0.05).

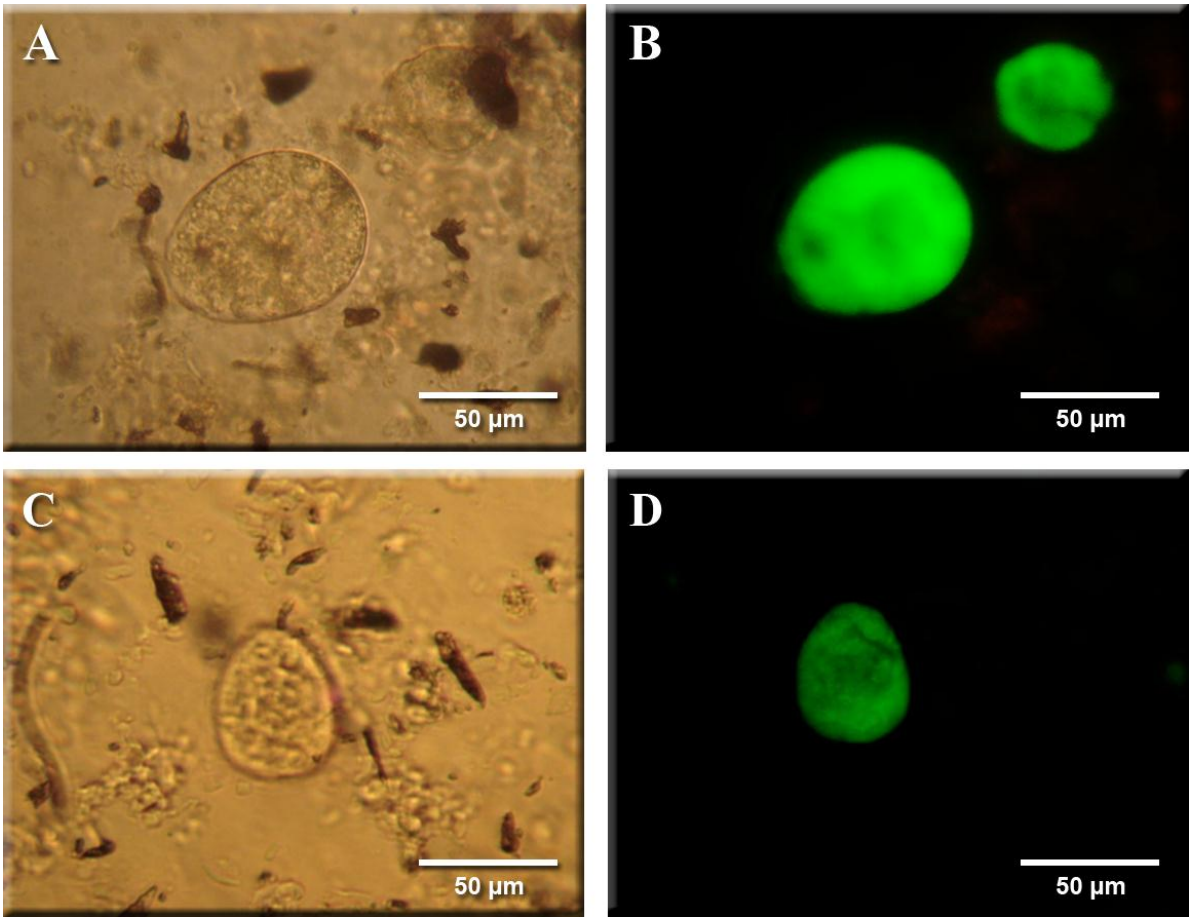


Figure 4. Viability of caprine preantral follicles as determined using fluorescent probes. Isolated preantral follicles after culture in 10 (A) and 50 (C) ng/mL ANG II were classified as viable if cells were labeled by calcein-AM (green fluorescence) (B, D) (B). Scale bars=50 µm.

3.4 Ultrastructural analysis of caprine preantral follicles

TEM studies were performed in non-cultured follicles (fresh control) and in follicles that were cultured for seven days in the lowest concentration of ANG II (10 ng/mL), which showed the best results for follicular survival and viability. The ultrastructural characteristics of follicles from the fresh control (Figure 5A) and those cultured with ANG II were similar (Figure 5B). The follicles showed intact basement and nuclear membranes and a large oocyte nucleus. In addition, vesicles and organelles were uniformly distributed in the ooplasm, especially the mitochondria. Granulosa cells were ultrastructurally normal and showed an elongated and large nucleus with an irregular membrane.

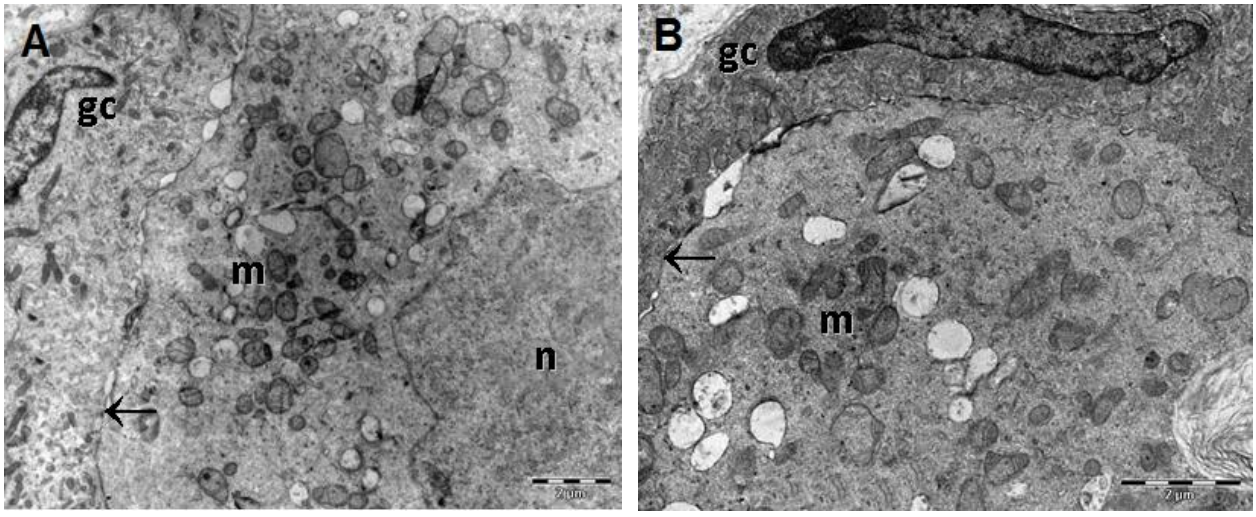


Figure 5. Ultrastructural analysis of non-cultured (fresh control) (A) and cultured caprine preantral follicles (B), which were cultured for seven days in medium containing 10 ng/mL ANG II. o, oocyte; n, oocyte nucleus; gc, granulosa cells; m, mitochondria; arrow, oocyte membrane. (A, B: bar=2 µm; C: bar=1 µm). Three to five follicles per group were examined, and the photomicrographs are representative examples.

3.5 Follicular activation and growth after culturing with ANG II

Figure 6 (A and B) shows the percentage of primordial and growing follicles, respectively, in ovarian cortical tissue before and after *in vitro* culture. After one or seven days of culture, a significant reduction ($P<0.05$) in the percentage of primordial follicles was

observed in all treatments and was associated with an increase ($P<0.05$) in the percentage of growing follicles compared to the fresh control.

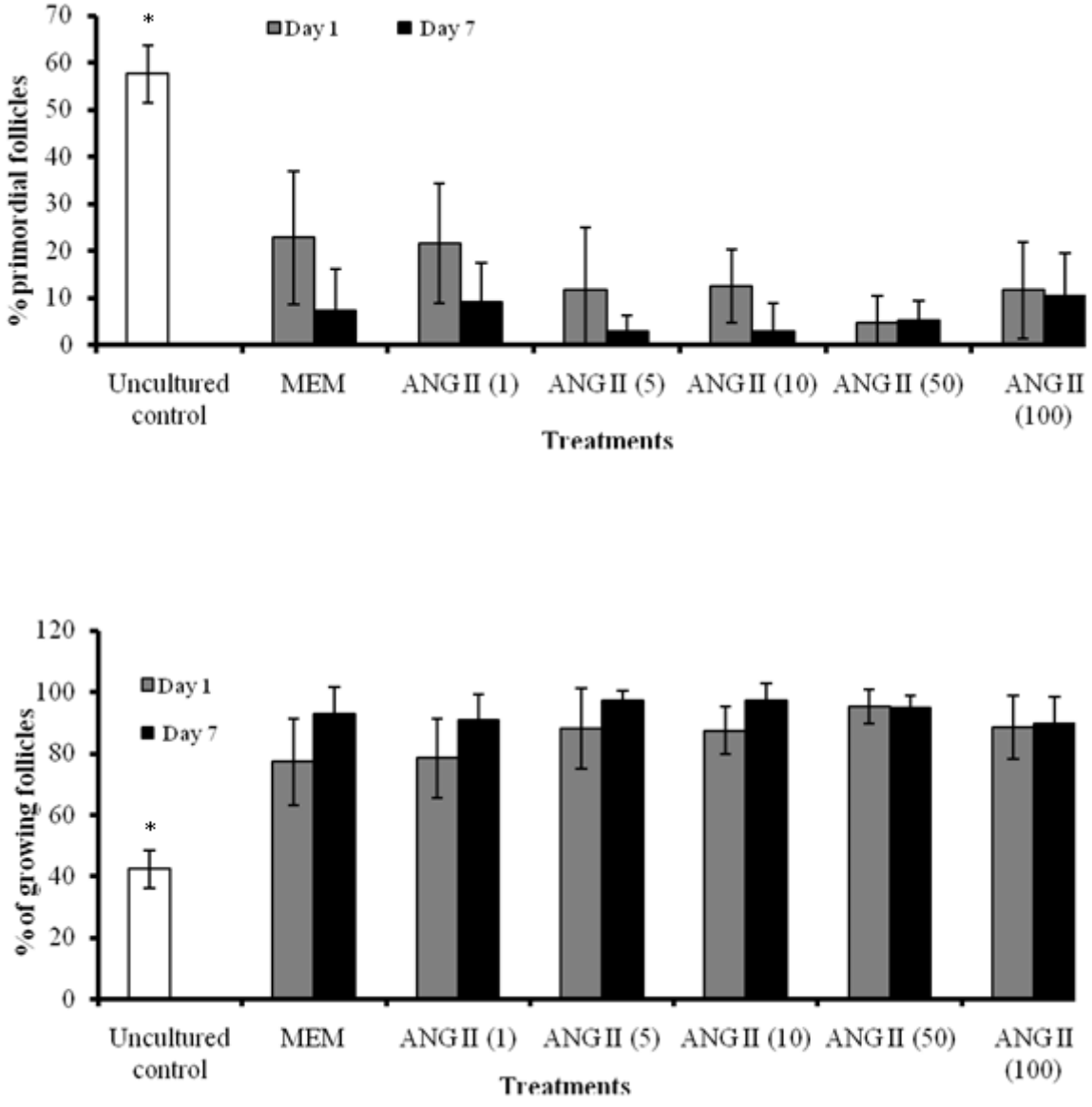


Figure 6. Percentage (mean ± S.E.M.) of primordial (A) and growing (B) follicles in the fresh control and after *in vitro* culture for one or seven days in the absence or presence of ANG II. *: differs significantly from control follicles ($P<0.05$).

Table 2 shows the diameter of follicles and oocytes after one or seven days culture in the presence or absence of ANG II. Regarding the follicular diameter, tissues cultured with

ANG II for seven days showed values similar to the fresh control and the cultured control (P>0.05). Furthermore, with progression of the culture period, only the treatments that contained ANG II maintained their follicular diameter (P>0.05). This result was not found with αMEM^{+}, in which there was a significant decrease in diameter (P<0.05). Oocyte diameter was similar between 10 and 50 ng/mL ANG II and the fresh control. This was not observed in the other treatments, which demonstrated a significant decrease in diameter (P<0.05). Similar to follicular diameter, αMEM^{+} alone decreased the oocyte diameter with the progression of culture (P<0.05).

Table 2. Caprine oocyte and follicle diameters (mean ± SEM) in the fresh control (non-cultured) and after culture for one or seven days at different concentrations of ANG II.

	Follicular diameter (μm)		Oocyte diameter (μm)	
Control	54.04 ± 4.09		43.47 ± 6.12	
Treatments	Day 1	Day 7	Day 1	Day 7
αMEM^{+}	57.84 ± 6.38 ◊	49.03 ± 8.08	43.10 ± 6.21 ◊	35.41 ± 5.27 *
ANG II 1	51.04 ± 7.84✦	50.89 ± 7.20	40.23 ± 5.14	38.00 ± 6.43 *
ANG II 5	49.23 ± 7.42✦	48.09 ± 7.71	38.37 ± 5.89*✦	36.43 ± 5.65*
ANG II 10	52.09 ± 6.24✦	55.10 ± 7.75	39.95 ± 4.14	39.76 ± 5.65
ANG II 50	54.51 ± 7.62	52.01 ± 8.30	39.67 ± 5.08	38.74 ± 6.30
ANG II 100	52.02 ± 6.76	51.71 ± 7.66	40.58 ± 6.45	37.35 ± 4.93*

*: differs significantly from control follicles (P<0.05); ✦: differs significantly from αMEM^{+} alone for each day culture (P<0.05); ◊: differs significantly between days of culture within a single treatment (P<0.05).

4. Discussion

This study showed, for the first time, the AGTR1 and AGTR2 mRNA levels in goat follicles and examined the effects of ANG II on the survival and development of preantral follicles after *in vitro* culture of caprine ovarian tissue for seven days.

Analysis of the steady-state level of mRNA encoding AGTR1 demonstrated similar levels in preantral follicles. Primordial follicles had higher AGTR2 mRNA levels than secondary follicles. Contrary to our results, in mice, Yoon *et al.* (2006) observed an increase in the AGTR2 mRNA expression using microarray analysis during the transition from primary to secondary follicle. The discrepancy may reflect a physiological difference between species.

In the present study, the AGTR1 mRNA levels were higher in granulosa/theca cells from small and large antral follicles when compared to their respective COCs. However, COCs from large antral follicles showed significantly higher AGTR2 mRNA levels than their respective granulosa/theca cells. The effects of ANG II on blood pressure regulation are mediated by AGTR1, whereas the physiological roles of the AGTR2 receptor are still uncertain (Paul *et al*. 2006). Increasing evidence supports a role of AGTR2 in the regulation of growth and differentiation of neuronal tissue (Steckelings *et al*. 2005). Therefore, in late folliculogenesis, we suggest that AGTR1 regulates blood flow while AGTR2 controls oocyte growth. Some studies using RT-PCR have demonstrated the presence of AGTR1 and AGTR2 mRNA in both the granulosa and theca cells of bovine antral follicles (Berisha *et al*. 2002; Portela *et al*. 2008). It is difficult to compare the results of these studies with our results because the mRNA was measured and compared in different cell types (granulosa and theca cells).

We observed high rates of follicular survival after seven days in tissues that were cultured in medium supplemented with 10 and 50 ng/mL ANG II when compared to the cultured control. These preantral follicles were further analyzed using a viability assessment, which suggested that these treatments were efficient in maintaining caprine preantral follicle viability. The fluorescent probes calcein-AM and ethidium homodimer-1 have been used to successfully assess the viability of bovine and caprine early-staged follicles (Schotanus *et al*. 1997; van den Hurk *et al*. 1998; Rossetto *et al*. 2009). This method can be used to analyze the

viability of follicles, thus offering a new approach for investigating the metabolic and developmental aspects of folliculogenesis *in vitro*. Furthermore, after the ultrastructural analysis, important structures in addition to the basement and nuclear membranes, such as the mitochondria and granulosa cells, were preserved even after seven days of culture in the presence of 10 ng/ml ANG II. The beneficial effect of ANG II on follicular viability may have occurred directly through receptor binding or indirectly through an interaction with other substances. As previously mentioned, we did not verify differences in AGTR1 receptor expression in preantral follicles in this work. However, AGTR2 mRNA levels were significantly higher in primordial follicles than in secondary follicles, but they did not differ from the primary stage. The ovarian fragments consisted mostly of early preantral follicles (i.e., primordial and intermediate). Thus, it is possible that ANG II mediates its effect on viability through AGTR2. A recent study showed that AGTR2 expression in the granulosa cells of bovine antral follicles is also related to the maintenance of follicular viability because ANG II concentration and AGTR2 expression were higher in healthy follicles compared to atretic follicles (Portela *et al*. 2008)**.** Moreover, Stefanello *et al*. (2006) found that when ANG II was associated with insulin-like growth factor-1 (IGF-1), it reversed the inhibitory effect of follicular cells on the nuclear maturation of bovine oocytes, resulting in a higher rate of embryo development. In addition to IGF-I, ANG II appears to regulate the induction of other autocrine growth factors, such as fibroblast growth factor-2 (FGF-2) (Itoh *et al*. 1993). Thus, IGF-1 and/or FGF-2 may have functioned as a survival factor for the follicles in the current study. The importance of IGF-1 (unpubl. data) and FGF-2 (Matos *et al*. 2007) in maintaining the morphology of caprine preantral follicles after *in vitro* culture for seven days has also been demonstrated.

After seven days of culture in the current study, a high percentage of growing follicles was observed in all treatments when compared to the fresh control. However, no difference between ANG II-treated and αMEM^{+} follicles was found, which indicates that ANG II did not affect caprine follicular activation. These results agree with those obtained by Harata *et al*. (2006) in humans. This study found ANG II immunolocalization to be weak in oocytes of primordial, primary and secondary follicles. Nevertheless, medium composition is known to be an important factor for success during the *in vitro* culture of preantral follicles. The αMEM that was used in our study is one of the richest formulations of medium for cellular culture

and is composed of 21 essential amino acids, B-vitamin complexes, vitamins C and D, inorganic salts and pyruvate. This medium was also supplemented with glutamine, hypoxanthine, BSA and ITS, which are substances essential for follicular development (Silva *et al*. 2004b). The supplements made the medium more complex and nutrient-rich, which may be responsible for the efficiency of follicular activation that was observed in all treatments. In other studies, spontaneous activation was also described after culturing preantral follicles in αMEM^{+} without the addition of hormones and growth factors (Lima-Verde *et al*. in press; Chaves *et al*. 2010).

Although ANG II maintained follicle and oocyte diameters throughout the culture period, it did not affect follicle or oocyte diameter. Shuttleworth *et al*. (2002) also found no significant effect of ANG II (10^{-10} mol/l) on the diameter of pig preantral follicles or antrum formation after 16 or 30 days of culture. Furthermore, Portela *et al*. (2008) observed that the addition of ANG II to bovine granulosa cells in culture did not affect cell proliferation or estradiol secretion, but it inhibited mRNA that encoded serine protease inhibitor E2, a protein that is involved in tissue remodeling. We believe that the lack of effect on diameter was associated with a low mitogenic stimulation caused by ANG II during the initial categories of follicular development.

In conclusion, this study demonstrated that only AGTR2 was expressed differently in the preantral follicle categories and that AGTR1 and AGTR2 were differently expressed in the different compartments of the antral follicles. In addition, 10 ng/mL ANG II improved the survival of caprine preantral follicles after seven days of *in vitro* culture. These findings suggest that ANG II plays an important role in early follicular viability and that it could have a significant application in improving the quality of oocytes that are used for *in vitro* maturation and fertilization.

Acknowledgments

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Disclosures

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

References

Acosta, T. J., Berisha, B., Ozawa, T., Sato, K., Schams, D., and Miyamoto, A. (1999). Evidence for a local endothelin-angiotensin-atrial natriuretic peptide systemin bovine mature follicles *in vitro*: effects on steroid hormones and prostaglandin secretion. *Biol. Reprod.* **61**, 1419-1425.

Berisha, B., Schams, D., Miyamoto, A. (2002). The mRNA Expression of Angiotensin and Endothelin System Members in Bovine Ovarian Follicles During Final Follicular Growth. *J. Reprod. Developm.***48**, 573-582.

Chaves, R. N., Alves, A. M. C. V., Duarte, A. B. G., Araújo, V. R., Celestino, J. J. H., Matos, M. H. T., Lopes, C. A. P., Campello, C. C., Name, K. P. O., Báo, S. N., and Figueiredo, J. R. (2010). Nerve growth factor promotes the survival of goat preantral follicles cultured *in vitro*. *Cells. Tissues. Organs.* **192**, 272-82.

Chaves, R. N., Martins, F. S., Saraiva, M. V. A., Celestino, J. J. H., Lopes, C. A. P., Correia, J. C., Lima-Verde, I. B., Matos, M. H. T., Báo, S. N., Name, K. P. O., Campello, C. C., Silva, J. R. V., and Figueiredo, J. R. (2008). Chilling ovarian fragments during transportation improves viability and growth of goat preantral follicles cultured *in vitro*. *Reprod. Fertil. Dev.* **20**, 640-647.

Feral, C., LeGall, S., and Leymarie, P. (1995). Angiotensin II modulates steroidogenesis in granulosa and theca in the rabbit ovary: Its possible involvement in atresia. *Eur. J. Endocrinol*. **133**, 747-753.

Figueiredo, J. R., Rodrigues, A. P. R., Amorim, C. A., Silva, J. R. V. (2008). Manipulação de Oócitos Inclusos em Folículos Ovarianos Préantrais- MOIFOPA. In 'Biotécnicas Aplicadas à Reprodução Animal'. (Eds P. B. D. Gonçalves, J. R. Figueiredo and V. J. F. Freitas) pp. 303-327. (Editora Roca: São Paulo, SP.)

Giometti, I. C., Bertagnolli, A. C., Ornes, R. C., Da Costa, L. F. S., Carambula, S. F., Reis, A. M., De Oliveira, J. F. C., Emanuelli, I. P., and Gonçalves, P. B. D. (2005). Angiotensin II reverses the inhibitory action produced by theca cells on bovine oocyte nuclear maturation. *Theriogenology* **63**, 1014-1025.

Harata, T., Ando, H., Iwase, A., Nagasaka, T., Mizutani, S., and Kikkawa, F. (2006). Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. *Fertil. Steril*. **86,** 433-439.

Husain, A., Bumpus, F. M., Silva, P., and Speth, R. C. (1987). Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2489-2493.

Itoh, H., Mukoyama, M., Pratt, R. E., Gibbons, G. H., and Dzau, V. J. (1993). Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. *J. Clin. Invest*. **91**, 2268-2274.

Li, Y. H., Jiao, L. H., Liu, R. H., Chen, X. L., Wang, H., and Wang, W. H. (2004). Localization of angiotensin II in pig ovary and its effects on oocyte maturation *in vitro*. *Theriogenology* **61**, 447-459.

Lima-Verde, I. B., Rossetto, R., Matos, M. H. T., Celestino, J. J. H., Bruno, J. B., Silva, C. M. G., Faustino, L. R., Mororó, M. B. S., Araújo, V. R., Campello, C. C., and Figueiredo, J. R. (in press). Androstenedione and follicle stimulating hormone involvement in the viability and development of goat preantral follicles *in vitro*. *Anim. Reprod*.

Lucci, C. M., Amorim, C. A., Báo, S. N., Figueiredo, J. R., Rodrigues, A. P. R., Silva, J. R., and Gonçalves, P. B. D. (1999). Effect of the interval of serial sections of ovarian in the tissue chopper on the number of isolated caprine preantral follicles. *Anim. Reprod. Sci*. **56**, 39-49.

Matos, M. H. T., van den Hurk, R., Lima-Verde, I. B., Luque, M. C. A., Santos, K. D. B., Martins, F. S., Báo, S. N., Lucci, C. M., Figueiredo, J. R. (2007). Effects of fibroblast growth factor-2 on the *in vitro* culture of caprine preantral follicles. *Cells Tissues Organs*. **186**, 112-120.

Nielsen, A. H., Hagemann, A., Svenstrup, B., Nielsen, J., and Poulsen, K. (1994) Angiotensin-II receptor density in bovine ovarian follicles relates to tissue renin and follicular size. *Clin. Exp. Pharmacol. Physiol*. **21**, 463-469.

Paul, M., Mehr, A. P., Kreutz, R. (2006). Physiology of Local Renin-Angiotensin Systems. *Physiol. Rev.* **86**, 747-803.

Pellicer, A., Palumbo, A., DeCherney, A. H., and Naftolin, F. (1988). Blockage of ovulation by an angiotensin antagonist. *Science* **240**, 1660-1661.

Portela, V. M., Gonçalves, P. B. D., Veiga, A. M., Nicola, E., Buratini, J. J., and Price, C. A. (2008). Regulation of angiotensin type 2 receptor in bovine granulosa cells. *Endocrinology* **149**, 5004-5011.

Rossetto, R., Lima-Verde, I. B., Matos, M. H. T., Saraiva, M. V. A., Martins, F. S., Faustino, L. S., Araújo, V. R., Silva, C. M., Name, K. P., Báo, S. N., Campello, C. C., Figueiredo, and J. R., Blume, H. (2009). Interaction between ascorbic acid and follicle-stimulating hormone maintains follicular viability after long-term *in vitro* culture of caprine preantral follicles. *Domest. Anim. Endocrinol*. **37,** 112-123.

Schauser, K. H., Nielsen, A. H., Winther, H., Dantzer, V., and Poulsen, K. (2001). Localization of the renin-Angiotensin system in the bovine ovary: cyclic variation of the angiotensin II receptor expression. *Biol. Reprod*. **65**, 1672-1680.

Schotanus, K., Hage, W. J., Vanderstichele, H., and van den Hurk, R. (1997). Effects of conditioned media from murine granulosa cell lines on the growth of isolated bovine preantral follicles. *Theriogenology* **48**, 471-83.

Shuttleworth, G., Broughton Pipkin, F. B., and Hunter, M. G. (2002). *In vitro* development of pig preantral follicles cultured in a serum-free medium and the effect of angiotensin II**.** *Reproduction* **123**, 807-818.

Silva, J. R. V., van den Hurk, R., Costa, S. H. F., Andrade, E. R., Nunes, A. P. A., Ferreira, F. V. A., Lôbo, R. N. B., and Figueiredo, J. R. (2004b). Survival and growth of goat primordial follicles after *in vitro* culture of ovarian cortical slices in media containing coconut water. *Anim. Reprod. Sci*. **81**, 273-286.

Silva, J. R. V., van den Hurk, R., Matos, M. H. T., Santos, R. R., Pessoa, C., Moraes, M. O., and Figueiredo, J. R. (2004a). Influences of FSH and EGF on primordial follicles during *in vitro* culture of caprine ovarian cortical tissue. *Theriogenology* **61**, 1691-1704.

Steckelings, U. M., Kaschina, E., Unger, Th. (2005). The AT2 receptor- A matter of love and hate. *Peptides* **26**, 1401-1409.

Steel, R. G. D., Torrie, J. H., and Dickey, D. A. (1997). 'Principles and procedures of statistics: a biometrical approach.' (McGraw-Hill: New York, NY).

Stefanello, J. R., Barreta, M. H., Porciuncula, P. M., Arruda, J. N., Oliveira, J. F., Oliveira, M. A., and Gonçalves, P. B. (2006). Effect of angiotensin II with follicle cells and insulin-like growth factor-I or insulin on bovine oocyte maturation and embryo development. *Theriogenology* **66**, 2068-2076.

van den Hurk, R., Spek, E. R., Hage, W. J., Fair, T., Ralph, J. H., and Schotanus, K. (1998). Ultrastructure and viability of isolated bovine preantral follicles. *Hum. Reprod. Update*. **4**, 833-841.

van Tol, H.T., and Bevers, M. M. (1998). Theca cells and theca-cell conditioned medium inhibit the progression of FSH-induced meiosis of bovine oocytes surrounded by cumulus cells connected to membrane granulosa. *Mol. Reprod. Dev.* **51**, 315-321.

Yoon, S-J., Kim, K-H., Chung, H-M., Choi, D-H., Lee, W-S., Cha, K-Y., and Lee, K-A. (2006). Gene expression profiling of early follicular development in primordial, primary, and secondary follicles. *Fert. Sterility.* **85**, 193-203.

Yoshimura, Y. (1997). The ovarian renin-angiotensin system in reproductive physiology. *Front. Neuroendocrinol*. **18**, 247-291.

Yoshimura, Y., Karubc, M., Koyama, N., Shiokawa, S., Nanno, T., and Nakamura, Y. (1992). Angiotensin II directly induces follicle rupture and oocyte maturation in the rabbit. *FEBS. Lett*. **307**, 305-308.

Yoshimura, Y., Karube, M., Aoki, H., Oda, T., Koyama, N., Nagai, A., Akimoto, Y., Hirano. H., and Nakamura, Y. (1996). Angiotensin II induces ovulation and oocyte maturation in rabbit ovaries via the AT2 receptor subtype. *Endocrinology* **137**, 1204-1211.

Yoshimura, Y., Karube, M., Oda, T., Koyama, N., Shiokawa, S., Akiba, M., Yoshinaga, A., and Nakamura, Y. (1993). Locally produced angiotensin II induces ovulation by stimulating prostaglandin production *in vitro* perfused rabbit ovaries. *Endocrinology* **133**, 1609-1616.

9. CAPÍTULO 4

Expressão do receptor do fator de crescimento do endotélio vascular (VEGF) em ovários de cabras e melhoria da sobrevivência e crescimento de folículos pré-antrais caprinos com VEGF

(Expression of vascular endothelial growth factor (VEGF) receptor in goat ovaries and improvement of in vitro caprine preantral follicle survival and growth with VEGF)

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Resumo

Os objetivos do presente estudo foram avaliar o efeito do fator de crescimento endotelial vascular (VEGF) sobre a sobrevivência e crescimento de folículos pré-antrais caprinos após cultivo in vitro, bem como verificar a expressão de receptor do VEGF (VEGFR)-2 em ovários caprinos. Fragmentos ovarianos foram cultivadas por um dia ou 7 dias em meio essencial mínimo (MEM) isoladamente ou suplementado com diferentes concentrações de VEGF (1, 10, 50, 100 e 200 ng mL-1). Tecidos não-cultivados (controle fresco) e cultivados foram processados para estudos histológico e ultraestrutural. Os resultados mostraram que 200 ng mL-1 de VEGF resultou em percentagem similar de folículos pré-antrais normais após 1 e 7 dias de cultivo comparado com o controle. Comparado ao meio base de cultivo sozinho, um aumento nos diâmetros folicular e oocitário foi observado na presença de 10 ng mL^{-1} VEGF após 7 dias de cultivo. A análise ultraestrutural confirmou a integridade folicular após 7 dias de cultivo na presença de 200 ng mL^{-1} de VEGF. Estudo imunohistoquímico demonstrou a expressão de VEGFR-2 em oócitos e células da granulosa de todos estágios foliculares, exceto em células da granulosa de folículos primordiais. Em conclusão, o presente estudo mostrou que o VEGF manteve a integridade ultraestrutural folicular e promoveu o crescimento folicular. Além disso, VEGFR-2 foi detectado em oócitos de folículos ovarianos caprinos em todos estágios de desenvolvimento e em células da granulosa de folículos em desenvolvimento.

Palavras-chave: VEGF. Cultivo in vitro. Folículos pré-antrais. Caprinos.

Expression of vascular endothelial growth factor (VEGF) receptor in goat ovaries and improvement of *in vitro* caprine preantral follicle survival and growth with VEGF

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Abstract. The aim of the present study was to evaluate the effect of vascular endothelial growth factor (VEGF) on the survival and growth of goat preantral follicles after *in vitro* culture and to verify the expression of VEGF receptor (VEGFR)-2 in goat ovaries. Ovarian fragments were cultured for 1 or 7 days in minimal essential medium (MEM) with different concentrations of VEGF (1, 10, 50, 100 or 200 ng mL^{-1}). Non-cultured (fresh control) and cultured tissues were processed for histological and ultrastructural studies. The results showed that 200 ng mL^{-1} VEGF resulted in a similar percentage of normal preantral follicles after 1 and 7 days of culture compared with control. Compared with basic culture medium alone, an increase in follicular and oocyte diameters was observed in the presence of 10 ng mL^{-1} VEGF after 7 days culture. Ultrastructural analysis confirmed follicular integrity after 7 days culture in the presence of 200 ng mL^{-1} VEGF. Immunohistochemical studies demonstrated the expression of VEGFR-2 in oocytes and granulosa cells of all follicular stages, except in granulosa cells of primordial follicles. In conclusion, the present study has shown that VEGF maintains follicular ultrastructural integrity and promotes follicular growth. In addition, VEGFR-2 is expressed in oocytes of caprine ovarian follicles at all developmental stages and in granulosa cells of developing follicles.

Additional keyword: culture.

Introduction

Preantral follicles, which consist of primordial, primary and secondary follicles, have no vascular supply of their own and depend on vessels in the surrounding stroma (Stouffer *et al*. 2001). However, during antrum development, the thecal layer acquires a vascular sheath consisting of two capillary networks located in the theca interna and externa. These newly formed ovarian blood vessels provide an increased supply of gonadotropins, growth factors, oxygen and steroid precursors, as well as other substances, to the growing follicle. The acquisition of an adequate vascular supply is possibly a rate-limiting step in the selection and maturation of the dominant follicle destined to ovulate (Stouffer *et al*. 2001). Both the ovarian follicle and corpus luteum have been shown to produce several angiogenic factors. Of these factors, vascular endothelial growth factor (VEGF) is thought to play a pivotal role in the regulation of angiogenesis in the ovary (Tamanini and De Ambrogi 2004).

In addition to being a potent mitogenic factor, VEGF (also known as 'vascular permeability factor') has an important role in regulating vascular structure and increasing capillary permeability (Redmer *et al*. 2001). Moreover, there is evidence that VEGF is a survival factor for endothelial cells in certain types of microvessels (Stouffer *et al*. 2001). In the ovary, VEGF appears to be involved in several processes, including follicular survival and growth (Danforth *et al*. 2003; Quintana *et al*. 2004; Roberts *et al*. 2007). Some studies have demonstrated that in the follicle and follicular fluid, the expression of VEGF protein increases significantly according to the stage of follicular development (Barboni *et al*. 2000; Greenaway *et al*. 2005). Others have demonstrated the expression of VEGF in oocytes of primordial follicles (in humans; Otani *et al*. 1999; Harata *et al*. 2006), in primary follicles of rats (Celik-Ozenci *et al*. 2003) and humans (Harata *et al*. 2006) and in granulosa and theca cells of secondary follicles (in the bovine; Yang and Fortune 2007). It has been shown that VEGF acts via two tyrosine kinase family receptors, namely flt-1 (VEGF receptor (VEGFR)-1) and flk-1/KDR (VEGFR-2; Shibuya 1995; Ferrara and Davis-Smyth 1997), which are expressed in theca cells of swine ovaries (Shimizu *et al*. 2003). The intraovarian VEGF/VEGFR-2 pathway seems to be critical for follicular development because blockade of VEGFR-2 function alters the secretion of follicular hormones, indicating that the intraovarian effects of VEGF are mediated by this receptor (Zimmermann *et al*. 2002). The mRNAs encoding VEGF and its

receptors have been shown to be expressed in granulosa and theca cells, respectively, of swine antral follicles (Shimizu *et al*. 2002); more recently, expression of VEGF and its receptors has been localised to the bovine fetal ovarian cortex (Yang and Fortune 2007).

In vivo studies have shown that direct administration of VEGF into the rat ovary increases vascularisation (Shimizu 2006) and the number of primary, secondary (Danforth *et al*. 2003) and antral follicles, and reduces apoptosis (Quintana *et al*. 2004). Moreover, inhibition of VEGF activity reduces the survival of primordial follicles from rodents (Roberts *et al*. 2007) and increases follicular apoptosis (Abramovich *et al*. 2006). Iijima *et al*. (2005) treated rats with different isoforms of VEGF and observed angiogenesis, follicular development and an increase in the number of ovulated oocytes, which were fertilised and showed competent normal development. Recently, an *in vitro* study showed that 10 ng mL^{-1} VEGF promoted the transition from primary to secondary follicles in bovine ovaries (Yang and Fortune 2007). It is important to note that most of the studies with VEGF have been performed in rodents and have concentrated on the final stages of follicular development. Moreover, *in vivo* studies have been performed using a single concentration of VEGF, with the results based only on histological evaluation.

Thus, the aims of the present study were to: (*1*) verify the expression of VEGFR-2 at different follicular stages in goat ovaries; (*2*) investigate the effects of different concentrations of VEGF on survival, initiation of primordial follicle growth (activation) and further follicular growth after *in vitro* culture of caprine ovarian tissue; and (*3*) evaluate the ultrastructure of caprine preantral follicles cultured *in vitro* in the absence or presence of VEGF.

Materials and methods

Chemicals

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

Source of ovaries

Ovarian cortical tissues (*n*=18 ovaries) were collected at a local slaughterhouse from nine adult (1–3 years old) mixed-breed goats (*Capra hircus*). Immediately postmortem, ovaries

were washed once in 70% alcohol for 10 s and twice in minimum essential medium (MEM) supplemented with 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Pairs of ovaries were transported within 1 h to the laboratory in MEM at 4◦C.

Experimental protocol

The organ culture system used in the present study has been described in detail elsewhere (Matos *et al*. 2007). Briefly, ovarian tissue samples from each ovarian pair were cut into 13 slices (3×3×1 mm) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultrastructural analysis (fresh control) or placed in culture for 1 or 7 days. Caprine tissues were transferred to 24-well culture dishes containing 1 mL culture medium. Culture was performed at 39◦C in 5% CO_2 in a humidified incubator and all media were incubated for 1 h before use. The basic culture medium (cultured control) was called MEM^+ and consisted of MEM (pH 7.2–7.4) supplemented with ITS (insulin 6.25 ng mL^{-1}, transferrin 6.25 ng mL^{-1} and selenium 6.25 ng mL^{-1}), 0.23 mm pyruvate, 2 mm glutamine, 2 mm hypoxanthine and 1.25 mg mL^{-1} bovine serum albumin (BSA). For the different experiments, this medium was supplemented with different concentrations of recombinant human VEGF (1, 10, 50, 100 or 200 ng mL^{-1}). Each treatment was repeated four times and the culture medium was replenished every other day.

Morphological analysis and assessment of in vitro *follicular growth*

Before culture (fresh control) and after 1 or 7 days in culture, all tissue pieces were fixed in Carnoy's solution for 12 h and then dehydrated in increasing concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the tissues pieces were cut into 7 μm sections and each section was mounted on glass slides and stained with periodic acid-Schiff (PAS)–haematoxylin. Follicle stage and survival were assessed microscopically on serial sections. Coded slides were examined under a microscope (Nikon, Japan) at ×400 magnification with the observer blinded to treatment group.

The developmental stages of the follicles have been defined previously (Silva *et al*. 2004) as primordial (one layer of flattened granulosa cells around the oocyte) or growing (intermediate, one layer of flattened to cuboidal granulosa cells; primary, one layer of cuboidal granulosa cells; and secondary, two or more layers of cuboidal granulosa cells

around the oocyte). These follicles were classified individually as histologically normal when an intact oocyte was present and was surrounded by granulosa cells that were well organised in one or more layers and had no pyknotic nuclei. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus and/or disorganised granulosa cells detached from the basement membrane. Overall, 120 follicles were evaluated for each treatment (30 follicles per treatment in one repetition × 4 repetitions = 120 follicles).

To evaluate follicular activation, the percentage of healthy primordial and growing follicles was calculated before (fresh control) and after culture in each medium. In addition, follicle and oocyte diameters were measured in healthy follicles only. Follicle diameter was recorded as the length from edge to edge of the basement membrane or from the outside edge of the theca cell layer when present. Oocyte diameter was recorded as the length from edge to edge of the oocyte membrane. Two perpendicular diameters were recorded for each parameter and the average of these two values was reported. Care was taken to count each follicle only once, as we have done in our earlier studies (Matos *et al*. 2007). Each follicle was examined in every section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once, regardless of its size.

Ultrastructural analysis

For better evaluation of follicular morphology, ultrastructural studies were performed on fragments of fresh control tissues (non-cultured) and those with the best results during the histological analysis after 7 days culture. Briefly, small pieces (1 mm^3) of caprine ovarian tissues were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 m sodium cacodylate buffer (pH 7.2) for 4 h at room temperature. After fixation, fragments were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mm calcium chloride in 0.1 m sodium cacodylate buffer for 1 h. Samples were dehydrated through an acetone gradient and tissues were embedded in Spurr's resin. Semithin sections (3 μm) were cut on an ultramicrotome (Reichert Supernova, Heidelberg, Germany) for light microscopy studies and stained with Toluidine blue. The ultrathin sections (60–70 nm) were contrasted with uranyl acetate and lead citrate and examined under a Jeol 1011 (Jeol, Tokyo, Japan) transmission

electron microscope (TEM). Parameters such as the density and integrity of ooplasmic and granulosa cell organelles, vacuolisation and basement membrane integrity were evaluated.

Viability assessment of follicles cultured in vitro

Based on the results of histological analysis, the viability of follicles cultured with the concentration of VEGF that provided the best outcome was further analysed using a more accurate method of assessment based on fluorescent probes.

Caprine preantral follicles from ovarian fragments cultured for 7 days with VEGF (200 ng mL^{-1}) were isolated by the mechanical method of Lucci *et al*. (1999). Briefly, using a tissue chopper (Mickle Laboratory Engineering, Gomshal, Surrey, UK) adjusted to a sectioning interval of 75 μm, samples were cut into small pieces, which were placed in MEM and suspended 40 times using a large Pasteur pipette (diameter approximately 1600 μm) and then subsequently 40 times with a smaller Pasteur pipette (diameter approximately 600 μm) to dissociate preantral follicles from the stroma. The material obtained was passed through 500- and 100-μm nylon mesh filters, resulting in a suspension containing preantral follicles smaller than 100 μm in diameter. This procedure was performed within 10 min at room temperature.

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

Immunohistochemical localisation of VEGFR 2

Localisation of VEGFR-2 was performed on serial 5-μm sections cut from the ovaries of five different goats. These sections were mounted on poly-L-lysine-coated slides, dried overnight at 37◦C, deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase was blocked by incubating the deparaffinized sections in 3% (v/v) hydrogen peroxide in methanol for 10 min. The sections were then washed with 1× phosphate-buffered saline (PBS) and the epitopes were activated by microwaving the sections for 7 min at 900 W in 0.01 m citrate buffer (pH 6.0). After microwave treatment, the sections were washed in PBS–0.05% (v/v) Tween (PBS-T) before being incubated for 30 min with 5% normal goat serum in PBS to minimize non-specific binding. Then, sections were incubated with mouse monoclonal anti-VEGFR-2 antibodies (clone KDR-1) diluted 1 : 20. After incubation with the antibody, sections were washed three times with PBS and incubated for 45 min with biotinylated secondary antibody (anti-mouse IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 200 in PBS containing 5% normal goat serum. Next, sections were washed three times in PBS-T before being incubated for 45 min with an avidin–biotin complex (1 : 600; Vectastain Elite ABC kits; Vector Laboratories, Burlingame, CA, USA). The sections were then washed three times in PBS and stained with diaminobenzidine (DAB; 0.05% (w/v) DAB in 10 mm TRIS-HCl, pH 7.6, 0.03% (v/v) H_2O_2) for a maximum of 10 min. The stained sections were rinsed in PBS and water and counterstained for 10 s in Mayer's haematoxylin. Finally, the sections were washed for 10 min under running tap water and subsequently dehydrated in a graded ethanol series. This was followed by xylene treatment and mounting in Pertex (Cellpath, Hemel, Hempstead, UK). Staining intensity was scored as absent (−), weak (+), moderate (++) or strong (+++). Controls for non-specific staining were performed by: (*1*) replacing the primary antibody with IgGs from the same species in which the specific antibody was raised, at the same concentration; and (*2*) incubation with DAB reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. For immunohistochemical analysis, follicles were classified as described earlier for culture.

Statistical analysis

The percentage of surviving follicles at both stages (i.e. primordial and developing) obtained after 1 or 7 days of various treatments, as well as data regarding follicle and oocyte diameters,

were initially submitted to Kolmogorov–Smirnov and Bartlett's tests to confirm normal distribution and homogeneity of variance, respectively. Analysis of variance was then performed using the GLM procedure of SAS (1999) and Dunnett's test was applied to compare VEGF-treated groups with the control and MEM^+ groups (Steel *et al*. 1997). In order to avoid Type II error because of the high coefficient of variation, Duncan's test was applied to compare different VEGF concentrations and Student's *t*-test was used to compare means between 1 and 7 days of culture. Differences were considered significant when $P < 0.05$. Results are expressed as the mean ± s.e.m.

Results

Effect of VEGF on follicular survival after in vitro *culture*

In the present study, 1800 caprine preantral follicles were analysed. As shown in Fig. 1, after 7 days culture, the percentage of normal follicles was similar to that of the fresh control only for cells cultured in the presence of 200 ng mL^{-1} VEGF (83.3% *v.* 65.5%, respectively; $P > 0.05$). In addition, compared with tissues cultured in the presence of MEM^+, a significantly higher percentage of normal follicles ($P < 0.05$) was observed after culture in the presence of 50, 100 and 200 ng mL^{-1} VEGF for 7 days. These same concentrations of VEGF resulted in a significantly greater ($P < 0.05$) percentage of normal follicles than did other concentrations (1 and 10 ng mL^{-1} VEGF) after 1 or 7 days culture.

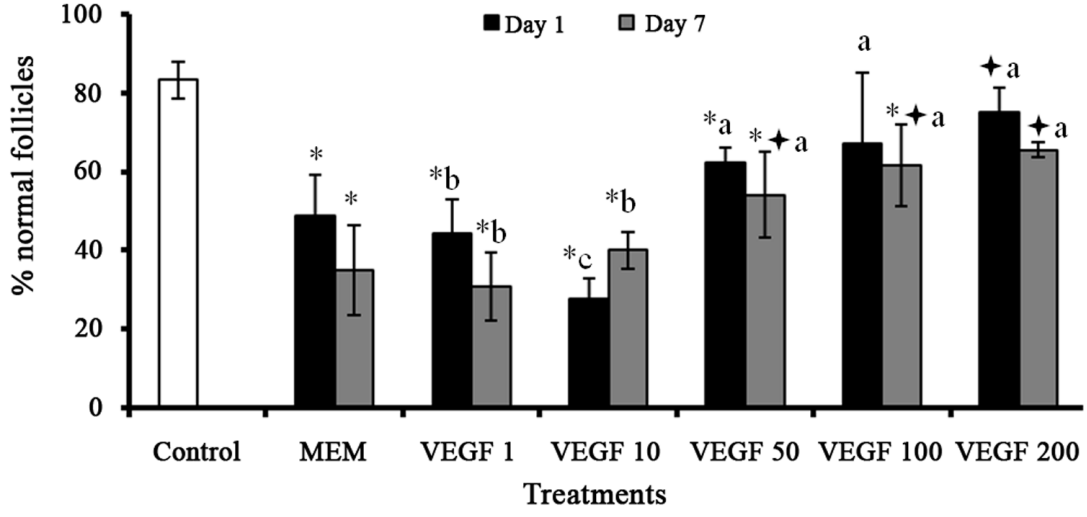

Fig. 1. Percentage (mean ± s.e.m.) of healthy caprine preantral follicles in the fresh control and after *in vitro* culture for 1 or 7 days in the absence or presence of vascular endothelial

growth factor (VEGF). *P <0.05 compared with control follicles; ✦P <0.05 compared with minimum essential medium (MEM) alone on each day of culture. Different superscript letters indicate significant differences between different concentrations of VEGF (P <0.05).

Follicular activation and growth after culture with VEGF

The percentage of primordial and growing follicles in fresh tissues was 35.1% and 64.9%, respectively. In all treatments, after 1 or 7 days culture, there was no significant effect of VEGF on follicular activation (i.e. we did not observe any significant reduction in the percentage of primordial follicles or any increase in the percentage of growing follicles; P >0.05).

With regard to follicular growth, tissues cultured in the presence of 200 ng mL^{-1} VEGF had significantly larger (after 1 day) and smaller (after 7 days) follicular and oocyte diameters than the fresh control (non-cultured). When compared with MEM^{+}, a significant increase (P <0.05) in follicular and oocyte diameters was observed following treatment with 10 ng mL^{-1} VEGF for 7 days. In addition, over the same period, 10 ng mL^{-1} VEGF was more efficient than 50, 100 and 200 ng mL^{-1}VEGF in increasing oocyte diameter (P <0.05; Table 1).

Ultrastructural analysis of caprine preantral follicles cultured with VEGF

The ultrastructural features of follicles from fresh control and 200 ng mL^{-1} VEGF-treated groups were similar. These follicles showed intact basement and nuclear membranes and a large oocyte nucleus. In addition, the organelles were uniformly distributed in the ooplasm, especially the mitochondria. Both the smooth and rough endoplasmic reticula were present, either as isolated aggregations or as complex associations with mitochondria and vesicles. Granulosa cells were ultrastructurally normal and well organised around the oocyte, showing an elongated and large nucleus with an irregular membrane (Fig. 2). Microvillar extensions of the plasma membranes of oocyte and granulose cells were visible in the space between these compartments.

Table 1. Caprine oocyte and follicle diameters (mean ± s.e.m.) in fresh control (non-cultured) and after culture for 1 or 7 days in the absence or presence of vascular endothelial growth factor.

	Follicular diameter (μm)		Oocyte diameter (μm)	
Control	66.90 ± 9.26		53.92 ± 8.88	
Treatments	Day 1	Day 7	Day 1	Day 7
MEM^+	69.22 ± 8.99	57.32 ± 11.45 *	55.77 ± 5.97	45.58 ± 9.71 *
VEGF 1	65.66 ± 13.85 ^{b}	64.43 ± 10.90 ^{a}	52.36 ± 9.86 ^{b}	50.52 ± 9.26 ^{ab}
VEGF 10	66.90 ± 12.04 ^{b}	68.96 ± 11.52 $^{✦a}$	52.68 ± 8.53 ^{b}	55.29 ± 9.93 $^{✦a}$
VEGF 50	73.70 ± 8.87 ^{b}	63.19 ± 11.58 ^{a}	57.44 ± 8.54 ^{ab}	48.05 ± 7.40 ^{bc}
VEGF 100	67.36 ± 15.52 ^{b}	63.52 ± 12.59 ^{a}	52.37 ± 10.44 ^{b}	45.62 ± 7.37 $^{* bc}$
VEGF 200	82.35 ± 12.73 $^{*✦a}$	55.93 ± 5.75 ^{*b}	62.26 ± 6.89 ^{*a}	42.49 ± 6.86 ^{*c}

VEGF1, 10, 50, 100 or 200 represent VEGF at 1, 10, 50, 100 or 200 ng mL^{-1}, respectively. MEM^+, minimum essential medium (pH 7.2–7.4) supplemented with ITS (insulin 6.25 ng mL^{-1}, transferrin 6.25 ng mL^{-1} and selenium 6.25 ng mL^{-1}), 0.23 mm pyruvate, 2 mm glutamine, 2 mm hypoxanthine and 1.25 mg mL^{-1} bovine serum albumin. *P <0.05 compared with control follicles; ✦P <0.05 compared with MEM^+ alone on each day of culture. Different superscript letters indicate significant differences between different concentrations of VEGF (P <0.05)

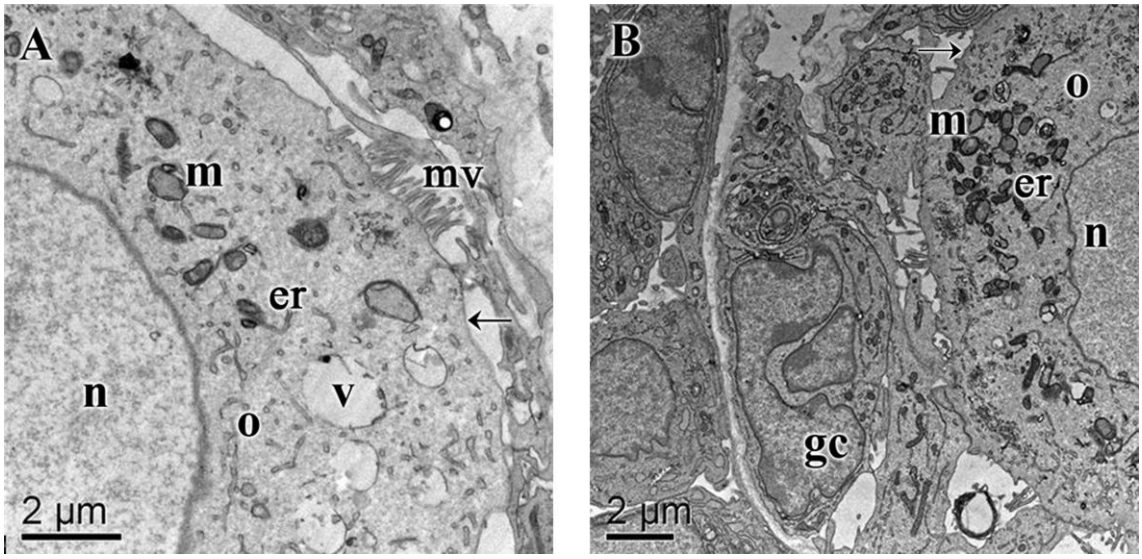

Fig. 2. Ultrastructural analysis of (*a*) non-cultured (fresh control) and (*b*) cultured caprine preantral follicles, cultured for 7 days in medium containing 200 ng mL^{-1} vascular endothelial growth factor (VEGF). o, oocyte; n, oocyte nucleus; gc, granulosa cells; m, mitochondria; v, vesicles; er, endoplasmic reticulum; mv, microvilli; arrow, oocyte membrane. Scale bars = 2µm.

Viability of follicles cultured with VEGF

In the present study, five caprine preantral follicles were analysed after 7 days culture with 200 ng mL^{-1} VEGF. After this qualitative analysis, all follicles (100%) remained viable, as assessed by calcein-AM–ethidium homodimer assays, the percentage of normal follicles was similar to that seen in the fresh control group (Fig. 3).

Immunohistochemical localisation of VEGFR-2 in caprine preantral follicles

Immunohistochemical analysis was performed to localize VEGFR-2 in caprine preantral and antral follicles. The results demonstrated a moderate reaction for VEGFR-2 in oocytes of follicles from all developmental stages. Granulosa cells of primordial follicles showed a negative response for VEGFR-2, but a weak and moderate immunoreaction was observed in granulose cells of primary and secondary follicles, respectively. In addition, cumulus and mural granulose cells showed a weak reaction for VEGFR-2. Nevertheless, theca cells did not

show a positive reaction for VEGFR-2. No staining was observed in negative controls incubated with VEGFR-2 (Table 2; Fig. 4).

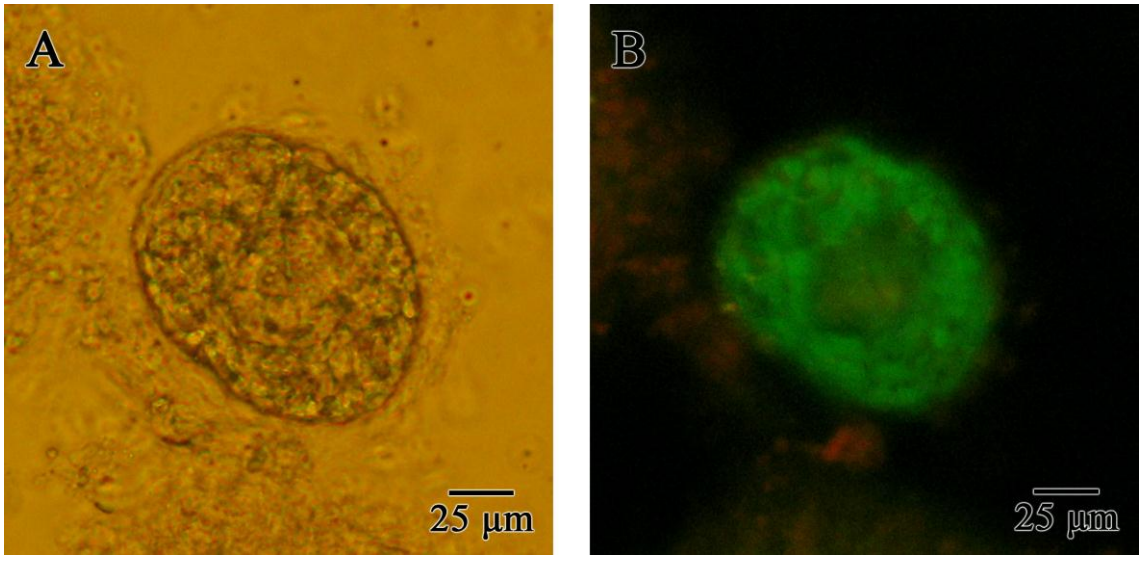

Fig. 3. Viability of caprine preantral follicles, as determined using fluorescent probes. An isolated preantral follicle after culture in 200 ng mL^{-1} vascular endothelial growth factor (VEGF) that was classified as viable (*a*) because cells were labelled by calcein-AM (green fluorescence; *b*). Scale bars = 25µm.

Table 2. Relative intensity of immunohistochemical staining for vascular endothelial growth factor receptor-2 in goat ovarian follicles (−) absent; (+) weak; (++) moderate; NA, not applicable

Follicular compartments	Follicular categories			
	Primordial	Primary	Secondary	Antral
Oocyte	++	++	++	++
Granulosa	-	+	++	+
Theca	NA	NA	-	-
Cumulus	NA	NA	NA	+

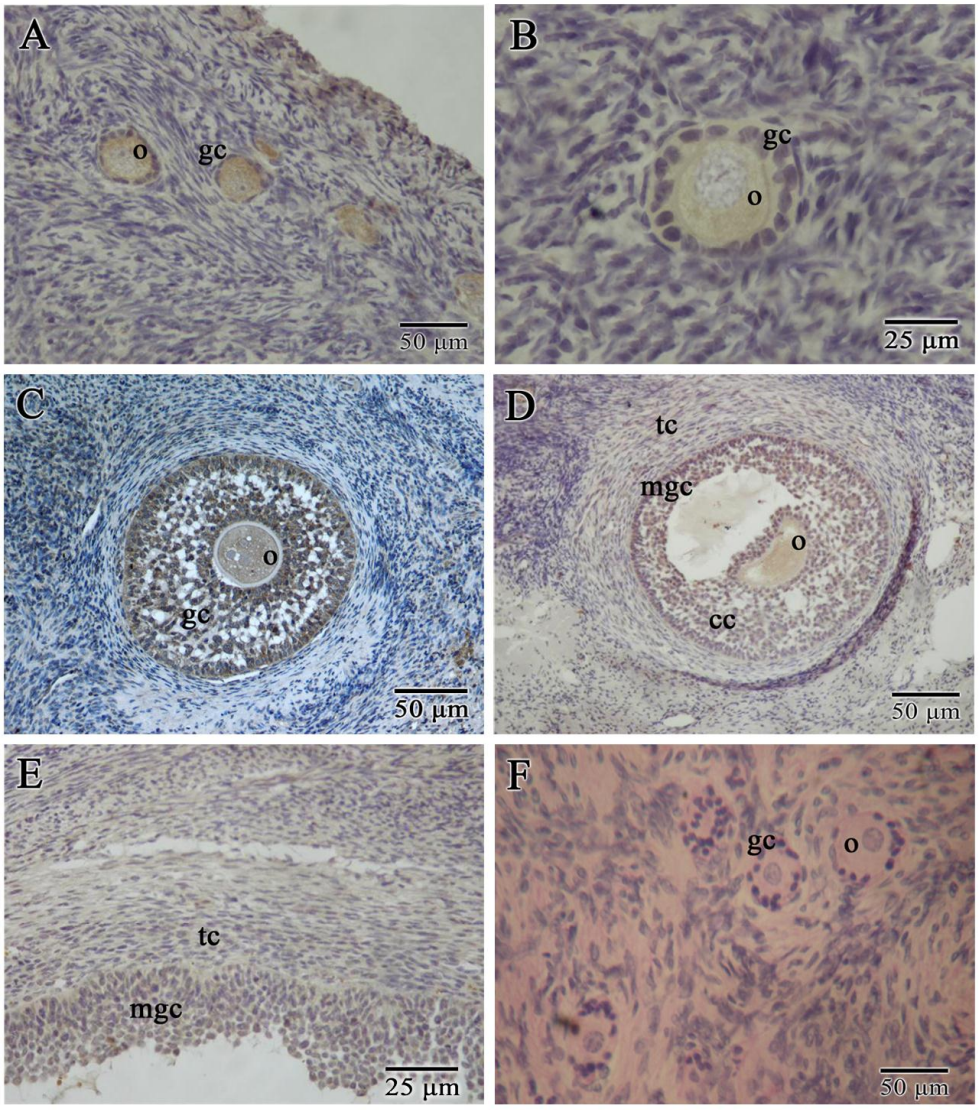

Fig. 4. Vascular endothelial growth factor receptor-2 immunoreactivity in different structures found within goat ovaries: (*a*) primordial follicle, (*b*) primary follicle, (*c*) secondary follicle, (*d*) antral follicle, (*e*) mural granulosa and theca cells from an antral follicle and (*f*) negative control. o, oocyte; gc, granulose cells; mgc, mural granulosa cells; cc, cumulus cells; tc, theca cells. Scale bars=50µm (*a*, *c*, *d*, *f*); 25µm (*b*, *e*).

Discussion

The present study demonstrated the importance of VEGF in the survival and growth of preantral follicles after *in vitro* culture of caprine ovarian tissue. The concentrations of VEGF

used in the present study (i.e. 1 and 10 ng mL^{-1}) were chosen based on the physiological concentrations of VEGF observed in swine follicular fluid (Barboni *et al*. 2000; Mattioli *et al*. 2001; Galeati *et al*. 2003). In addition, we also examined supraphysiological concentrations of VEGF (50, 100 and 200 ng mL^{-1}) that had not been tested previously, except for 100 ng mL^{-1} VEGF, which has been tested in the *in vitro* culture of bovine preantral follicles (Yang and Fortune 2007).

After 7 days culture with 200 ng mL^{-1} VEGF, follicular survival was similar to that of the fresh control, suggesting that this concentration of VEGF is the most adequate for the maintenance of preantral follicle survival after culture. In the ovary, previous studies in rats and cows have demonstrated a strong association between VEGF and follicular survival and inhibition of apoptosis after culture of endothelial and granulosa cells (Greenaway *et al*. 2004; Abramovich *et al*. 2006; Roberts *et al*. 2007). In the present study, culture with 1 and 10 ng mL^{-1} VEGF resulted in a lower percentage of normal follicles compared with culture in the presence of higher concentrations of VEGF (i.e. 50, 100 and 200 ng mL^{-1}), demonstrating a dose-dependent effect of VEGF on caprine preantral follicle survival *in vitro*.

According to Nilsson and Skinner (2004), follicular activation occurs when primordial follicles leave the resting pool. They undergo a primordial to primary follicle transition and the surrounding squamous pregranulosa cells become cuboidal granulosa cells and begin to proliferate. In the present study, no effect of VEGF was observed on follicular activation after 1 or 7 days of culture, as determined by the absence of a reduction in the percentage of primordial follicles and a further increase in the percentage of growing follicles. These results are similar to those obtained in cow ovaries, in which VEGF at 1, 10 and 100 ng mL^{-1} had no effect on the number of primordial or primary follicles after 10 days of *in vitro* culture compared with the fresh control (Yang and Fortune 2007). In addition, an *in vivo* study showed that injection of low doses of VEGF (5 or 10 ng mL^{-1}) into rat ovaries did not increase the number of primary and secondary follicles after 48 h and that such an increase was observed only after the use of higher doses of VEGF (i.e. 500 ng mL^{-1}; Danforth *et al*. 2003).

In the present study, the addition of 10 ng mL^{-1} VEGF to the culture medium was more efficient than MEM^{+} alone in increasing oocyte and follicular diameters after 7 days culture. It seems that VEGF is important in the regulation of follicular growth, because

inhibition of VEGF activity with neutralizing antibodies or a soluble form of its receptor has been reported to interrupt follicular growth and the proliferation of monkey granulosa cells (Danforth *et al*. 2003). Wulff *et al*. (2001) demonstrated that blockade of VEGF results in a reduction of theca cell proliferation in the secondary and tertiary follicles of primates. However, in the present study, there was a reduction in oocyte and follicular diameter after 7 days in the presence of 200 ng mL^{-1} VEGF compared with the fresh control. It is known that granulosa cells are the main cell type responsible for VEGF production (Mattioli *et al*. 2001; Galeati *et al*. 2003). Thus, the VEGF produced by these cells in addition to the VEGF added to the culture medium at high concentration (200 ng mL^{-1}) may have promoted accelerated follicular growth. Late preantral follicles, which have an elevated metabolism and increased uptake of nutrients, were not able to maintain their viability. Then, after 7 days, only the morphology of small preantral follicles, which have a low metabolism and are less sensitive to degeneration (Hirshfield 1991; Wandji *et al*. 1996; Silva *et al*. 2002), was preserved.

Using TEM, it was observed that in addition to the basal and nuclear membranes, important structures, such as the mitochondria, endoplasmic reticulum and granulosa cells were preserved even after 7 days culture in the presence of 200 ng mL^{-1} VEGF. In the present study, we observed microvilli, which are important in the communication between oocytes and granulosa cells, allowing the exchange of substances and, consequently, enabling adequate follicular development. In addition to ultrastructural analysis, the viability of preantral follicles cultured for 7 days in the presence of 200 ng mL^{-1} VEGF was further assessed, with the results indicating that this treatment is efficient in maintaining caprine preantral follicle viability. The fluorescent probes calcein-AM and ethidium homodimer-1 have been used successfully to assess the viability of bovine early stage follicles (Schotanus *et al*. 1997; van den Hurk *et al*. 1998). This method can be used to analyse the viability of follicles, thereby offering a new approach for investigating metabolic and developmental aspects of folliculogenesis *in vitro*.

The present study demonstrated the expression of VEGFR-2 in oocytes and granulosa cells of all follicular stages, except in granulosa cells from primordial follicles. This latter finding may be explained by the fact that VEGF did not promote changes in granulosa cell morphology and further granulosa cell proliferation, which characterises follicular activation. Although absent in granulosa cells of primordial follicles, VEGFR-2 was expressed in the

oocytes of these follicles. Some authors have demonstrated the expression of VEGFR-1 and VEGFR-2 mRNA, particularly in the thecal cell layer (Shimizu *et al*. 2002, 2003), from the late follicular stages onward. In addition, on the basis of experiments in which rats were treated with antibodies that bind toVEGFR-2, it has been reported that VEGFR-2 is important for primordial follicle survival (Roberts *et al*. 2007). Based on the results of the present study, in other types of follicles (i.e. primary and secondary), stimulation with VEGF may increase oocyte diameter, which may or may not be associated with granulosa cell proliferation, thus promoting follicular growth. Zimmermann *et al*. (2003) verified that in hypophysectomised primates VEGFR- 2 activity is necessary to increase the gonadotropin-dependent proliferation of granulosa cells.

Conclusion

The present study showed that different concentrations of VEGF have different effects on caprine preantral follicle survival and development. Specifically, to obtain more viable follicles and to promote preantral follicle growth, 200 and 10 ng mL^{-1} VEGF, respectively, should be used. Moreover, the expression of VEGFR-2 in oocytes of caprine ovarian follicles in all developmental stages and in granulosa cells of developing follicles suggests that VEGF may have a physiological function in mammalian reproduction.

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References

Abramovich, D., Parborel, F., and Tesone, M. (2006). Effect of a vascular endothelial growth factor (VEGF) inhibitory treatment on the folliculoggenesis and ovarian apoptosis in gonadotropin-treated prepubertal rats. *Biol. Reprod.* **75**, 434–441. doi:10.1095/BIOLREPROD.106. 051052

Barboni, B., Turriani, M., Galeati, G., Spinaci, M., Bacci, M. L., Forni, M., and Mattiolo, M. (2000). Vascular endothelial growth factor production in growing pig antral follicles. *Biol. Reprod.* **63**, 858–864. doi:10.1095/ BIOLREPROD63.3.858

Celik-Ozenci, C., Akkoyunhlu, G., Kayisli, U. A., Arici, A., and Demir, R. (2003). Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. *Histochem. Cell Biol.* **120**, 383–390. doi:10.1007/ S00418-003-0586-4

Danforth, D. R., Arbogast, L. K., Ghosh, S., Dickerman, A., Rofagha, R., and Friedman, C. I. (2003).Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. *Biol. Reprod.* **68**, 1736–1741. doi:10.1095/BIOLREPROD.101.000679

Ferrara, N., and Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. *Endocr. Rev.* **18**, 4–25. doi:10.1210/ER.18.1.4

Galeati, G., Spinaci, M.,Govoni,N., Zannoni, A., Fantinati, P., Seren, E., and Tamanini, C. (2003). Stimulatory effects of fasting on vascular endothelial growth factor (VEGF) production by growing pig ovarian follicles. *Reproduction* **126**, 647–652. doi:10.1530/REP.0.1260647

Greenaway, J., Connor, K., Pedersen, H. G., Coomber, B. L., Lamarre, J., and Petrik, J. (2004).Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. *Endocrinology* **145**, 2896–2905. doi:10.1210/EN.2003- 1620

Greenaway, J., Centry, P. A., Feige, J.-J., Lamarre, J., and Petrik, J. J. (2005). Thormbospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. *Biol. Reprod.* **72**, 1071–1078. doi:10.1095/ BIOLREPROD.104.031120

Harata, T., Ando, H., Iwase, A., Nagasaka, T., Mizutani, S., and Kikkawa, F. (2006). Localization of angiotensin II, the AT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucine aminopeptidase, and vascular endothelial growth factor in the human ovary throughout the menstrual cycle. *Fertil. Steril.* **86**, 433–439. doi:10.1016/J.FERTNSTERT.2006.01.041

Hirshfield,A. N. (1991). Development of follicles in mammalian ovary. *Int. Rev. Cytol.* **124**, 43–101. doi:10.1016/S0074-7696(08)61524-7

Iijima, K., Jiang, J.-Y., Shimizu,T., Sasada, H., and Sato, E. (2005). Acceleration of follicular development by administration of vascular endothelial growth factor in cycling female rats. *J. Reprod. Dev.* **51**, 161–168. doi:10.1262/JRD.51.161

Lucci, C. M.,Amorim, C.A., Báo, S.N., Figueiredo, J. R., Rodrigues,A. P. R., Silva, J. R., and Gonçalves, P. B. D. (1999). Effect of the interval of serial sections of ovarian in the tissue chopper on the number of isolated caprine preantral follicles. *Anim. Reprod. Sci.* **56**, 39–49. doi:10.1016/S0378- 4320(99)00031-7

Matos, M. H. T., Van Den Hurk, R., Lima-Verde, I. B., Luque, M. C. A., Santos, K. D. B., Martins, F. S., Báo, S. N., Lucci, C. M., and Figueiredo, J. R. (2007). Effects of fibroblast growth factor-2 on the *in vitro* culture of caprine preantral follicles. *Cells Tissues Organs* **186**, 112–120. doi:10.1159/000103016

Mattioli, M., Barboni, B., Turriani, M., Galeati, G., Zannoni, A., Castellani, G., Berardinelli, P., and Scapolo, P. A. (2001). Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. *Biol. Reprod.* **65**, 1014–1019. doi:10.1095/ BIOLREPROD65.4.1014

Nilsson, E. E., and Skinner, M. K. (2004). Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Mol. Cell. Endocrinol.* **214**, 19–25. doi:10.1016/ J.MCE.2003.12.001

Otani, N., Minami, S., Yamoto, M., Shikone, T., Otani, H., Nishiyama, R., Otani,T., and Nakano, R. (1999).The vascular endothelial growth factor/ *fms*-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. *J. Clin. Endocrinol. Metab.* **84**, 3845–3851. doi:10.1210/JC.84.10.3845

Quintana, R., Kopcow, L., Sueldo, C., Marconi, G., Rueda, N. G., and Barañao, R. I. (2004). Direct injection of vascular endothelial growth factor into the ovary of mice promotes follicular development. *Fertil. Steril.* **82**, 1101–1105. doi:10.1016/J.FERTNSTERT.2004.03.036

Redmer, D. A., Doraiswamy, V., Bortnem, B. J., Fisher, K., Jablonka- Shariff, A., Grazul-Bilska, A. T., and Reynolds, L. P. (2001). Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. *Biol. Reprod.* **65**, 879–889. doi:10.1095/ BIOLREPROD65.3.879

Roberts, A. E., Arbogast, L. K., Friedman, C. I., Cohn, D. E., Kaumaya, P. T., and Danforth, D. R. (2007). Neutralization of endogenous vascular endothelial growth factor depletes primordial follicles in the mouse ovary. *Biol. Reprod.* **76**, 218–223. doi:10.1095/BIOLREPROD. 106.050880

Schotanus, K., Hage, W. J., Vanderstichele, H., and van den Hurk, R. (1997). Effects of conditioned media from murine granulosa cell lines on the geowth of isolated bovine preantral follicles. *Theriogenology* **48**, 471–483. doi:10.1016/S0093-691X(97)00256-2

Shibuya, M. (1995). Role of VEGF–Flt receptor system in normal and tumor angiogenesis. *Adv. Cancer Res.* **67**, 281–316. doi:10.1016/S0065- 230X(08)60716-2

Shimizu,T. (2006). Promotion of ovarian follicular development by injecting vascular endothelial growth factor (VEGF) and growth differentiation factor 9 (GDF-9) genes. *J. Reprod. Dev.* **52**, 23–32. doi:10.1262/ JRD.17072

Shimizu, T., Jiang, J.-Y., Sasada, H., and Sato, E. (2002). Changes of messenger RNA expression of angiogenic factors and related receptors during follicular development in gilts. *Biol. Reprod.* **67**, 1846–1852. doi:10.1095/BIOLREPROD.102.006734

Shimizu, T., Jiang, J.-Y., Iijima, K., Miyabayashi, K., Ogawa,Y., Sasada, H., and Sato, E. (2003). Induction of follicular development by direct single injection of vascular endothelial growth factor gene fragments into the ovary of miniature gilts. *Biol. Reprod.* **69**, 1388–1393. doi:10.1095/ BIOLREPROD.103.016311

Silva, J. R., Ferreira, M.A. L., Costa, S. H. F., Santos, R. R., Carvalho, F. C.A., Rodrigues,A. P. R., Lucci, C. M., Báo, S.N., and Figueiredo, J. R. (2002). Degeneration rate of preantral follicles in the ovaries of goats. *Small Ruminant Res.* **43**, 203–209. doi:10.1016/S0921-4488(02)00017-2

Silva, J. R., Van Den Hurk, R., Matos, M. H. T., Santos, R. R., Pessoa, C., Moraes, M. O., and Figueiredo, J. R. (2004). Influences of FSH and EGF on primordial follicles during *in vitro* culture of caprine ovarian cortical tissue. *Theriogenology* **61**, 1691 1704. doi:10.1016/ J.THERIOGENOLOGY.2003.09.014

Statistical Analysis System (SAS) (1999). 'SAS/STAT User's Guide.' (SAS Institute: Cary, NC.)

Steel, R. G. D., Torrie, J. H., and Dickey, D. A. (1997). 'Principles and Procedures of Statistics: A Biometrical Approach, 3rd edn.' (McGraw- Hill: NewYork.)

Stouffer, R. L., Martínez-Chequer, J. C., Molskness, T. A., Xu, F., and Hazzard, T. M. (2001). Regulation and action of angiogenic factors in the primete ovary. *Arch. Med. Res.* **32**, 567–575. doi:10.1016/S0188-4409(01)00323-X

Tamanini, C., and De Ambrogi, M. (2004). Angiogenesis in developing follicle and corpus luteum. *Reprod. Domest. Anim.* **39**, 206–216. doi:10.1111/J.1439-0531.2004.00505.X

van den Hurk, R., Spek, E. R., Hage, W. J., Fair, T., Ralph, J. H., and Schotanus, K. (1998). Ultrastructure and viability of isolated bovine preantral follicles. *Hum. Reprod. Update* **4**, 833–841. doi:10.1093/ HUMUPD/4.6.833

Wandji, S. A., Srsen, V., Voss, A. K., Eppig, J. J., and Fortune, J. E. (1996). Initiation *in vitro* of growth of bovine primordial follicles. *Biol. Reprod.* **55**, 942–948. doi:10.1095/BIOLREPROD55.5.942

Wulff, C., Wiegand, S. J., Saunders, P. T., Scobie, G. A., and Fraser, H. M. (2001). Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). *Endocrinology* **142**, 3244–3254. doi:10.1210/EN.142.7.3244

Yang, M. Y., and Fortune, J. E. (2007). Vascular endothelial growth factor stimulates the primary to secundary follicle transition in bovine *in vitro. Mol. Reprod. Dev.* **74**, 1095–1104. doi:10.1002/MRD.20633

Zimmermann, R. C., Xiao, E., Bohlen, P., and Ferin, M. (2002). Administration of anti vascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey. *Endocrinology* **143**, 2496–2502. doi:10.1210/EN.143.7.2496

Zimmermann, R. C., Hartman, T., Kavic, S., Pauli, S. A., Bohlen, P., Sauer, M.V., and Kitajewski, J. (2003).Vascular endothelial growth factor receptor 2-mediated angiogenesis is essential for gonadotropin-dependent follicle development. *J. Clin. Invest.* **112**, 659–669.

10. CAPÍTULO 5

Peptídeo intestinal vasoativo melhora a sobrevivência e desenvolvimento de folículos pré-antrais caprinos após cultivo de tecido in vitro

(Vasoactive intestinal peptide improves the survival and development of caprine preantral follicles after in vitro tissue culture)

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Resumo

O objetivo deste estudo foi avaliar o efeito do peptídeo intestinal vasoativo (VIP) na sobrevivência, ativação e crescimento de folículos pré-antrais após o cultivo in vitro. O córtex ovariano foi dividido em pequenas peças e um fragmento foi imediatamente fixado (controle). Os fragmentos restantes foram cultivados in vitro por um ou sete dias a 39^{o}C e 5% de CO_2, em Meio Essencial Mínimo suplementado (MEM^{+}) com ou sem diferentes concentrações de VIP (1, 10, 50, 100 e 200 ng / ml). Fragmentos ovarianos não-cultivados (controle fresco) e cultivados foram processados para análise histológica e Microscopia Eletrônica de Transmissão (MET). Os folículos foram classificados como primordiais ou em desenvolvimento, bem como normais ou degenerados. Nossos resultados indicam que quando comparado ao controle, todas as concentrações de VIP, exceto 200 ng / ml, resultaram em percentagens similares de folículos pré-antrais normais após um e sete dias de cultivo. O cultivo de tecido do córtex ovariano por um ou sete dias aumentou a percentagem de ativação folicular em todos os tratamentos, quando comparado ao controle, exceto com 1 ng / ml de VIP após um dia. No entanto, nenhuma diferença foi observada entre os folículos tratados-VIP e tratados-MEM^{+}. Além disso, após sete dias de cultivo, maiores diâmetros foliculares e oócitários foram observados em folículos cultivados com 10 ng / ml VIP em relação ao MEM^{+} sozinho. A MET mostrou integridade ultraestrutural de folículos após sete dias de cultivo em 10 ng / ml VIP. Em conclusão, este estudo demonstrou que o VIP manteve a integridade folicular e estimulou o crescimento de folículos pré-antrais caprinos.

Palavras-chave: VIP. Folículo pré-antral. Cultivo. Caprino.

Vasoactive Intestinal Peptide Improves the Survival and Development of Caprine Preantral Follicles after in vitro Tissue Culture

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Abstract

The aim of this study was to evaluate the effect of vasoactive intestinal peptide (VIP) on the survival, activation and growth of goat preantral follicles after in vitro culture. The ovarian cortex was divided into small pieces and one fragment was immediately fixed (control). The remaining fragments were cultured in vitro for 1 or 7 days at 39 ° C and 5% CO^2, in supplemented minimum essential medium (MEM^+) with or without different concentrations of VIP (1, 10, 50, 100 or 200 ng/ml). Noncultured (fresh control) and cultured ovarian fragments were processed for histological analysis and transmission electron microscopy. Follicles were classified as primordial or developing, and as normal or degenerated. Our findings indicate that when compared with control, addition of all concentrations of VIP except 200 ng/ml resulted in similar percentages of normal preantral follicles after 1 and 7 days of culture. Culture of ovarian cortex tissue for 1 and 7 days increased the percentage of follicular activation in all treatments when compared with control, except with 1 ng/ml of VIP after 1 day. However, no difference was observed between VIP-treated and MEM^+-treated follicles. In addition, after 7 days of culture, the highest follicular and oocyte diameters were observed in follicles cultured with 10 ng/ml VIP relative to MEM^+ alone. Transmission electron microscopy showed ultrastructural integrity of follicles after 7 days of culture in 10 ng/ml VIP. In conclusion, this study demonstrates that VIP maintains follicular integrity and stimulates caprine preantral follicle growth.

Key Words: Vasoactive intestinal peptide, Preantral follicles, Culture, Caprine.

Introduction

Ovarian activity is regulated not only by gonadotropins and steroids, but also by a number of neural inputs and paracrine regulatory mechanisms. The mammalian ovary is innervated by extrinsic nerves, which are both catecholaminergic and peptidergic in nature [Burden, 1985; Ojeda and Lara, 1989; Ojeda et al., 1989]. Peptidergic innervation of the ovary was verified, among other ways, by the presence of vasoactive intestinal peptide (VIP) [Ahmed et al., 1986]. This neuropeptide was originally isolated from porcine duodenum and is involved in smooth muscle relaxation and vasodilation [Said and Mutt, 1970]. VIP has been found in the central nervous system as well as in a wide variety of organs and peripheral tissues, including lung, testis, adrenal and ovary [Cecconi et al., 2004].

Additional findings, summarized below, suggest VIP may play a vital role in the acquisition of cyclic ovarian function. VIP has been shown to stimulate in vitro androgen and estradiol release [Davoren and Hsueh, 1985; Parra et al., 2007]. In addition, VIP stimulation induced aromatase and follicle-stimulating hormone (FSH) receptor expression at both the transcript and protein level in the rat neonatal ovary several days before the ovary became responsive to gonadotropins [Mayerhofer et al., 1997]. In bovine tissue, primary follicle formation is accompanied by the outgrowth of VIP-containing nerves [Hulshof et al., 1994]. Furthermore, this neuropeptide is reported to be produced by nerve fibers innervating follicles at all stages of development [Ahmed et al., 1986; Hulshof et al., 1994; Johnson et al., 1994].

Other in vitro studies revealed that VIP stimulates the development of isolated bovine primary and early secondary follicles [Hulshof, 1995] and promotes avian granulose cell survival by inhibiting apoptosis in preovulatory follicles [Flaws et al., 1995]. Moreover, many of the effects of VIP on ovarian granulosa cells are similar to the action of gonadotropins [Ahmed et al., 1986; Johnson and Tilly, 1988; Ojeda et al., 1989; Johnson et al., 1994], suggesting that VIP may be important for regulating gonadotropin independent development and ovarian follicle survival. These data confirm previous findings concerning the participation of sympathetic innervation in the control of follicular development. However, there are no reports on the possible role of VIP in caprine preantral follicle viability and development in vitro. The aim of this study, therefore, was to evaluate whether different

concentrations of VIP influence the survival, activation and growth of caprine preantral follicles in vitro cultured for 1 or 7 days.

Materials and Methods

Chemicals

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

Source of Ovaries

Ovarian cortical tissues were obtained from 4 mixed-breed goats (n = 8 ovaries) collected at a local slaughterhouse. Immediately postmortem, the ovaries were washed first in 70% alcohol and then twice in minimum essential medium (MEM) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin (Vetec, Rio de Janeiro, Brasil). Ovary pairs were transported within 1 h to the laboratory in MEM at 33 ° C.

Experimental Protocol

Our organ culture system was described in detail previously [Silva et al., 2004; Matos et al., 2007a]. Ovarian tissue samples from the same ovarian pair were cut in slices (3 x 3 x 1 mm) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultrastructural analysis (control – day 0) or placed in culture for 1 or 7 days. Caprine tissues were transferred to 24-well culture dishes containing 1 ml of culture media. Culture was performed at 39°C in 5% CO_2 in a humidified incubator and all media were incubated for 1 h prior to use. The basic culture medium (cultured control) was called MEM^+ and consisted of MEM (pH 7.2 7.4) supplemented with ITS (insulin 6.25 ng/ml, transferrin 6.25 ng/ml and selenium 6.25 ng/ml), 0.23 m M pyruvate, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml of bovine serum albumin. To test the effects of VIP, different concentrations of this peptide (0, 1, 10, 50, 100 or 200 ng/ml) were added to the culture medium. The experiment was carried out in 4 replicates and the culture media was replenished every other day.

Morphological Analysis and Assessment of in vitro Follicular Growth

Fresh (noncultured) control and cultured tissues were fixed in 10% formalin for 12 h and then dehydrated in increasing concentrations of ethanol. After paraffin (Synth, São Paulo, Brasil) embedding, the caprine tissues pieces were cut into 7 - µm sections and stained by periodic acid Schiff and hematoxylin. Sections were examined by microscopy (Nikon, Tokyo, Japan) under 400 x magnifications.

Preantral follicles were classified as described previously [Gougeon and Chainy, 1987], as primordial (1 layer of flattened granulosa cells around the oocyte), intermediate (1 layer of flattened to cuboidal granulosa cells), primary (1 layer of cuboidal granulosa cells) and secondary (2 or more layers of cuboidal granulose cells around the oocyte). Individual follicles were further classified as histologically normal when an intact oocyte, surrounded by well organized granulosa cells in 1 or more layers, with no pyknotic nucleus, was present. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus and/or disorganized granulosa cells detached from the basement membrane. Overall, 120 follicles were evaluated for each treatment (30 follicles per animal).

To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before (fresh control) and after culture in each medium. In addition, follicle and oocyte diameters were measured only in healthy follicles with the aid of an ocular micrometer. Both diameters, from the basement membrane, at right angles to each other in the largest crosssection of each growing oocyte and follicle, were measured and averaged. To minimize the possibility of counting more than once, only follicles with a visible oocyte nucleus were recorded. Further, each follicle was examined in every section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, regardless of its size.

Ultrastructural Analysis of Caprine Preantral Follicles

For more in-depth evaluation of follicular morphology, ultrastructural studies were performed on fragments of fresh control and treatment groups that maintained follicular morphology during histological analysis. Small pieces (1 mm^3) of caprine ovarian tissues were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 h at room temperature. After fixation, fragments were postfixed in 1%

osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h. Subsequently, the samples were dehydrated in a gradient of acetone solutions and the tissues were embedded in Spurr's resin. Semithin sections (3 µm) were cut on an ultramicrotome (Reichert Supernova, Heidelberg, Germany) for light microscopy studies and stained with toluidine blue. Ultrathin sections (60–70 µm) were contrasted with uranyl acetate and lead citrate, and examined under a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope. Parameters such as density and integrity of ooplasmic and granulosa cell organelles, vacuolization and basement membrane integrity were evaluated.

Statistical Analysis

Means of surviving follicles at all stages (primordial and developing – intermediate, primary or secondary) determined after 1 or 7 days of culture in the various treatments were subjected to analysis of variance using the GLM procedure of the SAS software and Dunnett's test to compare VIP-treated groups against control and MEM^+; Duncan test was used to compare differences among VIP concentrations [Steel et al., 1997]. Student's t test was used to compare means between 1 and 7 days of culture. Differences were considered to be significant when $p < 0.05$ and data were expressed as mean ± standard error of means (SEM).

Results

Effect of VIP on the Follicular Survival after Culture

In the present study, a total of 1,800 caprine preantral follicles were analyzed. Figure 1 shows a histological section of caprine ovarian tissue cultured for 7 days in MEM^+ supplemented with 10 ng/ml of VIP. Present in this figure are morphologically normal follicles with a round oocyte, a nucleus without pyknosis and well-organized granulose cells around the oocyte. In addition, degenerated follicles show oocytes with pycnotic nucleus and disorganized granulosa cells around the oocyte. After 1 or 7 days of in vitro culture, it was verified that percentages of normal preantral follicles were similar ($p > 0.05$) to fresh control in all treatment groups, except for MEM^+ alone or with the addition of 200 ng/ml of VIP ($p < 0.05$) (fig. 2).

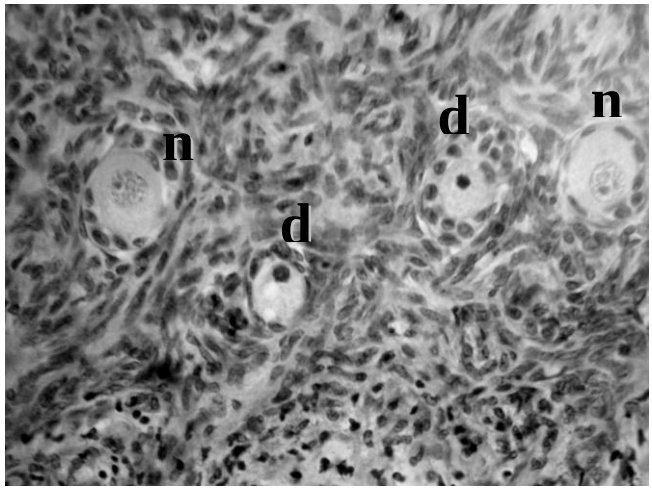

Fig. 1. Histological section of caprine tissue cultured for 7 days in 10 ng/ml of VIP showing normal (n) and degenerated (d) follicles after staining with periodic acid Schiff–hematoxylin.

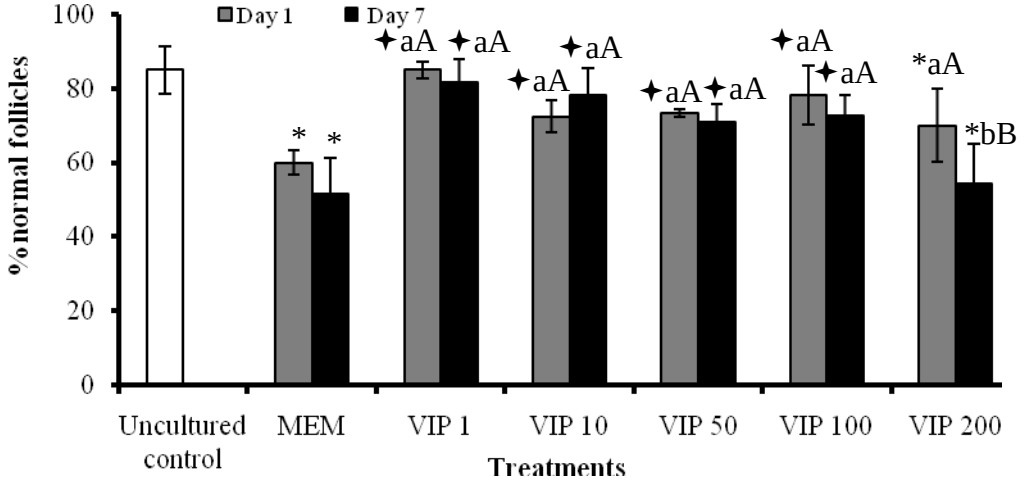

Fig. 2. Percentage (mean ± SEM) of morphologically normal preantral follicles in control (noncultured) ovaries and ovaries cultured in vitro for 1 or 7 days, in the absence or presence of VIP.

Furthermore, after 1 or 7 days, treatment with all concentrations of VIP significantly increased ($p < 0.05$) the percentage of normal follicles when compared to MEM^{+} alone, except 200 ng/ml of VIP ($p < 0.05$). In fact, treatment with 200 ng/ml VIP resulted in a

significant reduction ($p < 0.05$) in the percentage of normal follicles with the progression of the culture period from 1 to 7 days.

Follicular Activation and Growth after in vitro Culture with VIP

Figure 3a and b shows the percentage of primordial and growing follicles, respectively, in ovarian cortical tissue before and after in vitro culture. In all treatments tested, including MEM^+, after 1 or 7 days of culture, a significant reduction ($p < 0.05$) in the percentage of primordial follicles was observed, as well as an increase ($p < 0.05$) in growing follicles compared with fresh control, except when 1 ng/ml of VIP ($p > 0.05$) was used after 1 day of culture. However, after comparing VIP treatments with MEM^+, no significant differences were observed (except for 1 ng/ml after 1 day of culture). That is, the addition of VIP has no effect on follicular activation. However, VIP addition was most effective in promoting an increase in follicular diameter, since after 1 or 7 days of culture, ovaries treated with 10 ng/ml of VIP had a significantly larger follicular diameter ($p < 0.05$) than control, MEM^+ alone and MEM^+ supplemented with different concentrations of VIP, except when compared to ovaries treated with 1 ng/ml of VIP ($p > 0.05$) (table 1). With regard to oocyte growth, after 1 or 7 days of culture, this same concentration of 10 ng/ml of VIP resulted in oocytes with diameters greater than MEM + oocytes ($p < 0.05$). In addition, after 1 day of culture, oocyte diameter was significantly greater ($p < 0.05$) with 10 ng/ml of VIP when compared to other VIP concentrations.

Ultrastructural Analysis of Caprine Preantral Follicles Cultured with VIP

Based on histological results, transmission electron microscopy studies were performed in noncultured follicles (fresh control) and in follicles cultured for 7 days in MEM^+ plus 10 ng/ml of VIP. The ultrastructural characteristics of follicles from fresh tissue and those cultured in medium with 10 ng/ml of VIP for 7 days appeared similar (fig. 4). These follicles showed intact basal and nuclear membranes, nuclei with decondensed chromatin, as well as some vesicles and organelles uniformly distributed in the cytoplasm. Granulosa cells were normal, with elongated nuclei and a high nucleus cytoplasm ratio. In addition, mitochondria were the most predominant organelles observed in both oocyte and granulose cells.

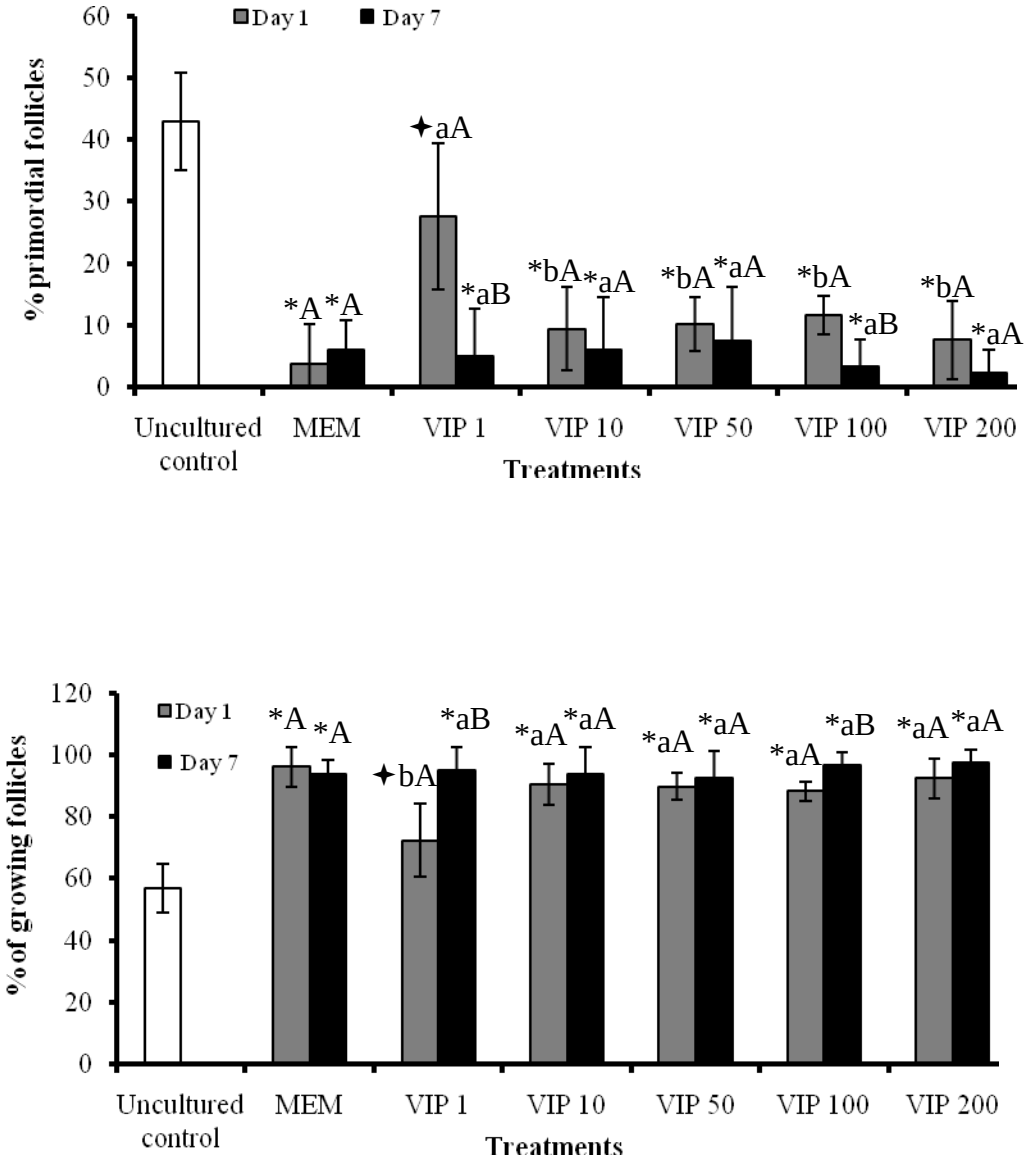

Fig. 3. Percentage (mean ± SEM) of primordial (**a**) and growing (**b**) follicles in control (non-cultured) ovaries and ovaries cultured in vitro for 1 or 7 days in the absence or presence of VIP.

Table 1. Oocyte and follicle diameters (mean ± SEM) in control (noncultured) and treatments after in vitro culture for 1 or 7 days in the absence or presence of VIP.

	Follicular diameter (µm)		Oocyte diameter (µm)	
Control	51.2 ± 6.0		38.6 ± 6.1	
Treatments	Day 1	Day 7	Day 1	Day 7
MEM	50.8 ± 8.1	50.5 ± 8.4	37.0 ± 6.7	37.2 ± 4.0
1 ng/ml VIP	54.3 ± 11.0 [c,d]	54.5 ± 7.1 [c,d]	39.2 ± 7.3 [d]	40.0 ± 5.6 [c,d]
10 ng/ml VIP	58.7 ± 9.6 [a,b,c]	57.7 ± 7.7 [a,b,c]	44.1 ± 7.4 [a,b,c]	42.9 ± 5.8 [b,c]
50 ng/ml VIP	48.9 ± 7.5 [d]	50.0 ± 6.6 [d]	36.4 ± 5.3 [d]	36.6 ± 5.9 [d]
100 ng/ml VIP	50.6 ± 7.3 [d]	51.9 ± 6.6 [d]	38.3 ± 5.7 [d]	39.0 ± 6.2 [d]
200 ng/ml VIP	50.1 ± 7.5 [d]	51.2 ± 4.9 [d]	39.5 ± 7.4 [d]	37.0 ± 3.4 [d]

[a] $p < 0.05$: differs significantly from control follicles; [b] $p < 0.05$: differs significantly from MEM alone in each day culture. Superscript letters c and d indicate significant differences ($p < 0.05$) between VIP at different concentrations. There were no significant differences between days 1 and 7 of culture ($p > 0.05$).

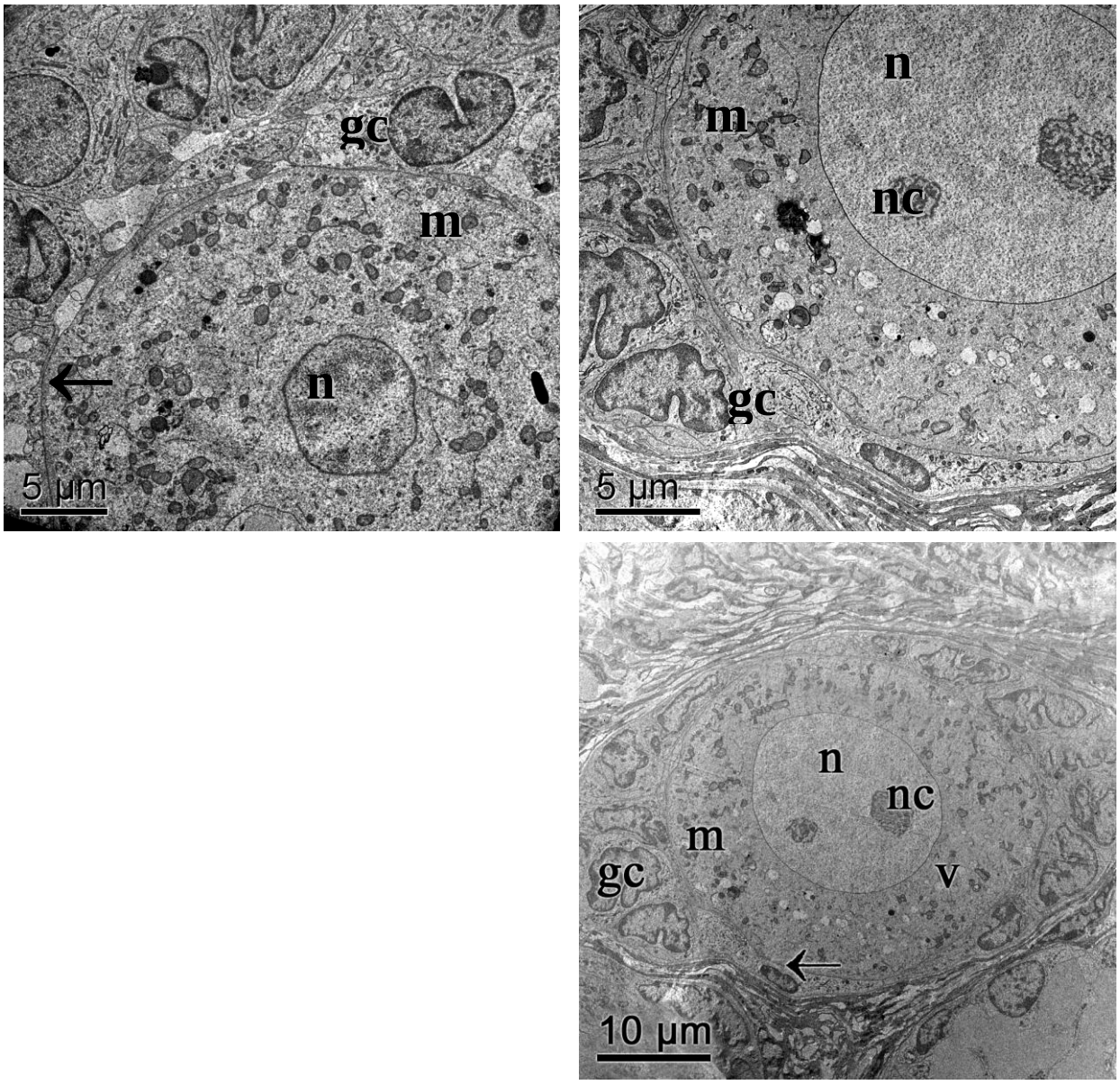

Fig. 4. Ultrastructural analysis of non-cultured preantral follicle (**a**) and follicle cultured for seven days in medium containing 10 ng/ml VIP (**b** , **c**). n = Nucleus; gc = granulosa cell; nc = nucleulus; m = mitochondria; v = vesicles; arrow = oocyte membrane.

Discussion

The present study demonstrated, for the first time, the effects of different concentrations of VIP on caprine follicular viability and early development in vitro. Several studies have demonstrated that VIP influences important ovarian functions, such as regulation of steroidogenesis [Ahmed et al., 1986], cAMP accumulation [Tornell et al., 1988], plasminogen activator production [Johnson and Tilly, 1988] and oocyte maturation [Tornell et al., 1988].

In our study, ovarian fragments from fresh control ovaries contained 85% histologically normal preantral follicles. Silva et al. [2002] reported that approximately 12% of goat preantral follicles in fresh ovaries were degenerated. In previous studies characterizing ovarian follicular populations, 4.8 and 0.20% of caprine and ovine preantral follicles were degenerated, respectively [Lucci et al., 1999; Amorim et al., 2000]. Additionally, follicular survival rates (approximately 85%) in fresh control ovaries similar to our results have been previously described by other authors [Matos et al., 2007a; Chaves et al., 2008].

The addition of different concentrations of VIP (1, 10, 50 or 100 ng/ml) to the culture medium was important to maintain preantral follicle viability after 1 or 7 days of culture. VIP has been shown to protect several cell types from apoptosis, including thymocytes, prostate cancer and neural cells [Flaws et al., 1995; Delgado et al., 1996; Said, 1996; Gutierrez-Canas et al., 2003; Sastry et al., 2006]. Reduction in lung injury by VIP is associated with inhibition of caspase activation and upregulation of bcl- 2, both of which suppress cell death and promote cell survival [Fraser and Evan, 1996; Said and Dickman, 2000]. In the ovary, previous in vitro studies also demonstrate that VIP inhibits apoptosis of rat follicles [Flaws et al., 1995; Vaccari et al., 2006] as well as avian granulosa cells [Flaws et al., 1995]. In our study, however, the highest concentration of VIP (that is, 200 ng/ml) promoted a decrease in follicular survival, indicating this VIP concentration may be toxic to the early stages of caprine follicular development. More studies are necessary to determine the molecular mechanism of VIP action on caprine follicular survival.

According to Nilsson and Skinner [2004], follicular activation occurs when primordial follicles leave the resting pool, undergo a primordial to primary follicle transition, and surrounding squamous pregranulosa cells become cuboidal and begin to proliferate. It is

known that follicular activation also occurs due to the increase in oocyte diameter. In the present study, all treatments tested, including MEM^{+}, increased the percentage of growing follicles after 7 days of culture in relation to fresh control. However, there was no difference between VIP-treated and MEM^{+} follicles, which indicates that VIP did not have an effect on follicular activation. Nevertheless, VIP addition was effective in promoting an increase in oocyte and follicular diameter after both 1 and 7 days of culture. The presence of VIP receptors in granulosa, theca and interstitial cells [Vaccari et al., 2006] may explain the effect of VIP on the increase in follicular diameter. Another possibility is that VIP probably acts by increasing the oocyte diameter and/or number of granulosa cells but not interfering in the changing of granulosa cell morphology (flat to cuboidal). It is likely that other growth factors, such as fibroblast growth factor-2 or epidermal growth factor [Matos et al., 2007b; Celestino et al., 2009], may be necessary in the culture medium to promote caprine follicular activation. In addition, VIP immunoreactivity is reported to increase with the appearance of secondary and antral follicles in bovine ovaries, which suggests a role for this peptide in follicular growth [Hulshof et al., 1994]. Furthermore, VIP stimulated in vitro development of isolated primary and secondary bovine follicles [Hulshof, 1995]. In accordance with our results, a recent study in buffalo showed that 50 ng/ml VIP increased preantral follicle diameter [Gupta et al., 2002]. Nevertheless, in mice, VIP did not affect follicular development and caused inhibition of follicular growth, antrum formation, granulosa cell proliferation as well as estradiol production of follicles stimulated by FSH [Cecconi et al., 2004]. These conflicting results may be due to study design, differences related to species, culture conditions and different follicular stages analyzed.

In the present work, treatment with 10 ng/ml VIP and the noncultured control maintained the ultrastructural integrity of preantral follicles after 7 days of culture. Recently, Matos et al. [2007a] showed similar results in the culture of caprine ovarian tissue in medium containing FSH. Normal follicles had an ultrastructure similar to that previously described in the goat [Lucci et al., 2001] and other species: swine [Greenwald and Moor, 1989]; bovine [Van Wezel and Rodgers, 1996]; and human [Oktay et al., 1997].

In conclusion, this study showed 10 ng/ml VIP promotes follicular survival and caprine preantral follicle growth after 7 days of in vitro culture and not the number of growing follicles. These findings suggest that VIP plays an important role in early follicular

development and could have significant utility in improving the quality of oocytes used for in vitro maturation and fertilization.

Acknowledgments

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References

Ahmed, C.E., W.L. Dees, S.R. Ojeda (1986) The immature rat ovary is innervated by vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. Endocrinology *118:* 1682–1689.

Amorim, C.A., C.M. Lucci, A.P.R. Rodrigues, F.C.A. Carvalho, J.R. Figueiredo, D. Rondina, R. Cecchi, A. Giorgetti, A. Martini, P. B.D. Gonçalves (2000) Quantitative and qualitative analysis of the effectiveness of the mechanical method for the isolation of preantral follicles from ovines ovaries. Theriogenology *53:* 1251–1262.

Burden, H.W. (1985) The adrenergic innervations of mammalian ovaries in: Ben Jonathan, N., M.M. Bahr, R.I. Weiner (eds): Catecholamines as Hormone Regulators. New York, Raven Press, pp 261–278.

Cecconi, S., G. Rossi, M. Barberi, L. Scaldaferri, R. Canipari, (2004) Effect of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide on mouse preantral follicle development in vitro. Endocrinology *145:* 2071–2079.

Celestino, J.J.H., J.B. Bruno, I.B. Lima-Verde, M.H.T. Matos, M.V.A. Saraiva, R.N. Chaves, F.S. Martins, L.F. Lima, K.P.O. Name, C.C. Campello, J.R.V. Silva, S.N. Báo, J.R. Figueiredo (2009) Recombinant epidermal growth factor maintains follicular ultrastructure and promotes the transition to primary follicles in caprine ovarian tissue cultured in vitro. Reprod Sci *16:* 239–246.

Chaves, R.N., F.S. Martins, M.V.A. Saraiva, J.J.H. Celestino, C.A.P. Lopes, J.C. Correia, I.B. Lima-Verde, M.H.T. Matos, S.N. Báo, K.P.O. Name, C.C. Campello, J.R.V. Silva, J.R.

Figueiredo (2008) Chilling ovarian fragments during transportation improves viability and growth of goat preantral follicles cultured in vitro. Reprod Fertil Dev *20:* 640–647.

Davoren, J.B., A.J.W. Hsueh (1985) Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. Biol Reprod *33:* 37–52.

Delgado, M., E. Garrido, C. Martinez, J. Leceta, R.P. Gomariz (1996) Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptides (PACAP27) and (PACAP38) protect CD4+CD8+ thymocytes from glucocorticoid-induced apoptosis. Blood *87:* 5152–5161.

Flaws, J.A., A. Desanti, K.I. Tilly, R.0. Javid, K. Kugu, A.L. Johnson, A.N. Hirshfield, J.L. Tilly (1995) Vasoactive intestinal peptide mediated suppression of apoptosis in the ovary: potential mechanisms of action and evidence of a conserved antiatretogenic role through evolution. Endocrinology *136:* 4351–4359.

Fraser, A., G. Evan (1996) A license to kill. Cell *85:* 781–784.

Gougeon, A., G.B.N. Chainy (1987) Morphometric studies of small follicles in ovaries of women at different ages. J. Reprod Fert *81:* 433–442.

Greenwald, G.S., R.M. Moor (1989) Isolation and preliminary characterization of pig primordial follicles. J Reprod Fertil *87:* 561–571.

Gupta, P.S.P., S. Nandi, B.M. Rvindranatha, P.V. Sarma (2002) In vitro culture of buffalo *(Bubalus bubalis)* preantral follicles. Theriogenology *57:* 1839–1854.

Gutierrez-Cañas, I., N. Rodriguez-Henche, O. Bolanos, M.J. Carmena, J.C. Prieto, M.G. Juarranz (2003) VIP and PACAP are autocrine factors that protect the androgen independent prostate cancer cell line PC-3 from apoptosis induced by serum withdrawal. Br J Pharmacol *139:* 1050–1058.

Hulshof, S.C.J., G. Dijkstra, E.M. Van Der Beek, M.M. Bevers, J.R. Figueiredo, J.F. Beckers, R. Van Den Hurk (1994) Immunocytochemical localization of vasoactive intestinal peptide and neuropeptide y in the bovine ovary. Biol Reprod *50:* 553–560.

Hulshof, S.C.J. (1995) Bovine preantral follicles and their development in vitro; thesis, Utrecht. Johnson, A.L., J.L. Tilly (1988) Effects of vasoactive intestinal peptide on steroid secretion and plasminogen activator activity in granulose cells of the hen. Biol Reprod *38:* 296–303.

Johnson, A.L., Z. Li, J.A. Gibney, S. Malamed (1994) vasoactive intestinal peptide induced expression of cytochrome p450 cholesterol side-chain cleavage and 17a hydroxylase enzyme activity in hen granulosa cells. Biol Reprod *51:* 327–333.

Lucci, C.M., C.A. Amorim, A.P.R. Rodrigues, J.R. Figueiredo, S.N. Báo, J.R.V. Silva, P.B.D. Gonçalves (1999) Study of preantral follicles population in situ and after mechanical isolation from caprine ovaries at different reproductive stages. Anim Reprod Sci *56:* 223–236.

Lucci, C.M., R.V. Silva, C.A. Carvalho, R. Figueiredo, N. Báo (2001) Light microscopical and ultrastrutural characterization of goat preantral follicles. Small Rum Res *41:* 61–69.

Matos, M.H.T., I.B. Lima-Verde, M.C.A. Luque, J.E. Maia Jr, J.R. Silva, J.J.H. Celestino, F.S. Martins, S.N. Báo, C.M. Lucci, J.R. Figueiredo (2007a) Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability in vitro. Zygote *15:* 173–182.

Matos, M.H.T., R. van den Hurk, I.B. Lima- Verde, M.C.A. Luque, K.D.B. Santos, F.S. Martins, S.N. Báo, C.M. Lucci, J.R. Figueiredo (2007b) Effects of fibroblast growth factor-2 on the in vitro culture of caprine preantral follicles. Cells Tissues Organs *186:* 112–120.

Mayerhofer, A., G.A. Dissen, M. Costa, S.R. Ojeda (1997) A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. Endocrinology *138:* 3320–3329.

Nilsson, E.E., M.K. Skinner (2004) Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. Mol Cell Endocrinol *214:* 19–25.

Ojeda, S.R., H.E. Lara (1989) The role of the sympathetic nervous system in the regulation of ovarian function; in Pirke, K.M., W. Wuttke, U. Schweiger (eds): The Menstrual Cycle and Its Disorders. Berlin, Springer-Verlag, pp 26–32.

Ojeda, S.R., H.E. Lara, C.E. Ahmed (1989) The potential relevance of vasoactive intestinal peptide to ovarian physiology; in Adashi, E. (ed) Putative Intraovarian Regulators. New York, Thieme Inc., pp 52–60.

Oktay, K., D. Briggs, R.G. Gosde (1997) Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. J Clinic Endocrinol Metabol *82:* 3748–3751.

Parra, C., J.L. Fiedler, S.L. Lunal, M. Greiner, V. Padmanabhan, H.E. Lara (2007) Participation of vasoactive intestinal polypeptide in ovarian steroids production during the rat estrous cycle and in the development of estradiol valerate-induced polycystic ovary. Reproduction *133:* 147–154.

Said, S.I. (1996) Molecules that protect: the defense of neurons and other cells. J Clin Invest *97:* 2163–2164.

Said, S.I., K. Dickman (2000) Pathways of inflammation and cell death in the lung: modulation by vasoactive intestinal peptide. Regul Pept *93:* 21–29.

Sastry, K.S.R., A.J. Smith, Y. Karpova, S.R. Datta, G. Kulik (2006) Diverse antiapoptotic signaling pathways activated by vasoactive intestinal polypeptide, epidermal growth factor, and phosphatidylinositol 3-kinase in prostate cancer cells converge on bad. J Biol Chem *281:* 20891–20901.

Silva, J.R.V., M.A.L. Ferreira, S.H.F. Costa, R.R. Santos, F.C.A. Carvalho, A.P.R. Rodrigues, C.M. Lucci, S.N. Báo, J.R. Figueiredo (2002) Degeneration rate of preantral follicles in the ovaries of goats. Small Rum Res *43:* 203–209.

Silva, J.R., R. Van Den Hurk, M.H.T. Matos, R.R. Santos, C. Pessoa, M.O. Moraes, J.R. Figueiredo (2004) Influences of FSH and EGF on primordial follicles during in vitro culture of caprine ovarian cortical tissue. Theriogenology *61:* 1691–1704.

Steel, R.G.D., J.H. Torrie, D. Dickey (1997) Principles and Procedures of Statistics: A Biometrical Approach, ed 3. New York, Mc-Graw-Hill.

Tornell, J., B. Carlsson, T. Hillensjo (1988) Vasoactive intestinal peptide stimulates oocyte maturation, steroidogenesis, and cyclic adenosine 3’ , 5’ –monophosphate production in isolated preovulatory rat follicles. Biol Reprod *39:* 213–220.

Vaccari, S., S. Latini, M. Barberi, A. Teti, M. Stefanini, R. Canipari (2006) Characterization and expression of different pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptors in rat ovarian follicles. J Endocrinol *191:* 287–299.

Van Wezel, I., R.J. Rodgers (1996) Morphological characterization of primordial follicles and their environment in vivo. Biol Reprod *55:* 1003–1010.

11. CAPÍTULO 6

Níveis de peptídeo intestinal vasoativo em ovários de cabras e seu efeito sobre o desenvolvimento in vitro de folículos pré-antrais isolados

(Steady-state level of Vasoactive Intestinal Peptide in goat ovaries and its effect on in vitro development of isolated preantral follicles)

Periódico: Molecular and Cellular Endocrinology (Submetido em outubro de 2010).

Resumo

Os objetivos deste estudo foram investigar o nível de RNAm para Peptídeo Intestinal Vasoativo (VIP) em ovários de cabra e avaliar os efeitos do VIP e / ou FSH sobre o desenvolvimento in vitro e os níveis de mRNA para o ligante VIP e receptor do FSH (FSHR). Para esse fim, folículos primordiais, primários e secundários de cabras, bem como folículos antrais pequenos e grandes foram obtidos e os níveis de mRNA para VIP foram quantificados por PCR em tempo real. Os efeitos do VIP e / ou FSH sobre o desenvolvimento dos folículos pré-antrais e sobre a expressão de mRNA para VIP e FSHR foram avaliados após 6 dias de cultivo em meio de controle (α-MEM) ou em α-MEM suplementado com FSH (100 ng / mL), VIP (10 ng / mL) ou VIP+FSH. A RT-PCR demonstrou que os níveis de mRNA para VIP em folículos secundários foi significativamente maior do que no estágio de folículo primordial. Em pequenos e grandes folículos antrais, níveis de mRNA para VIP em complexo cumulus oócito (CCOs) foram significativamente maiores do que suas respectivascélulas da granulosa / teca. A adição do VIP e / ou FSH ao meio de cultivo não teve efeito sobre a sobrevivência, a formação de antro e diâmetro folicular. No entanto, a presença de FSH, VIP ou ambos, em meio de cultivo reduziu significativamente os níveis de mRNA para a VIP, mas não alterou os níveis de mRNA para FSHR. Em conclusão, o mRNA para a VIP foi detectado em todas as categorias e tipos celulares de cabra. Além disso, VIP e / ou FSH não afetou o desenvolvimento dos folículos secundários e reduziu os níveis de mRNA para VIP após curto período de cultivo de folículos pré-antrais caprinos.

Palavras-chave: VIP. FSH. Folículos pré-antrais. Caprino. Cultivo in vitro.

Steady-state levels of Vasoactive Intestinal Peptide mRNA in goat ovaries and its effect on the in vitro development of isolated preantral follicles

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Abstract

The aims of this study were to investigate the levels of vasoactive intestinal peptide (VIP) mRNA in goat ovaries and to evaluate the effects of VIP and/or follicle-stimulating hormone (FSH) on follicular development and mRNA levels of VIP and FSH receptor (FSHR). Preantral follicles were cultured for 6 days in α-MEM$^+$ or in α-MEM$^+$ supplemented with FSH and/or VIP. RT-PCR demonstrated that levels of VIP mRNA in secondary follicles were significantly higher than in primordial follicle. The addition of VIP and/or FSH to the culture medium had no effect on follicular development. However, the presence of FSH and/or VIP in the culture medium significantly reduced VIP mRNA levels, but did not alter FSHR mRNA levels. In conclusion, VIP mRNA was detected in all goat follicular categories and cellular types, VIP and/or FSH did not affect the development of secondary follicles and reduce the expression of VIP mRNA levels.

Keywords: VIP, FSH, Preantral Follicles, Culture, Caprine.

1. Introduction

Ovarian activity is regulated not only by gonadotropins and steroids but also by a number of neural inputs and paracrine regulatory mechanisms. The mammalian ovary is innervated by extrinsic nerves, which are both catecholaminergic and peptidergic in nature (Burden, 1985; Ojeda and Lara, 1989; Ojeda et al., 1989). Peptidergic innervation of the ovary was verified, among other ways, by the presence of vasoactive intestinal peptide (VIP) (Ahmed et al., 1986).

Some findings suggest that VIP may play a vital role in several ovarian functions, such as the regulation of steroidogenesis (Ahmed et al., 1986; Tornell et al., 1988), cAMP accumulation (Tornell et al., 1988, Vaccari et al., 2006), plasminogen activator production (Johnson and Tilly, 1988) and oocyte maturation (Tornell et al., 1988). VIP has been shown to stimulate androgen and estradiol release in vitro (Davoren and Hsueh, 1985; Parra et al., 2007). Hulshof et al. (1994) demonstrated that the bovine ovary is innervated by VIP-positive nerve fibers beginning at the onset of follicular development and increasingly with age.

Furthermore, this neuropeptide is produced by nerve fibers innervating follicles at all stages of development in rodent (Ahmed et al., 1986) and avian (Johnson et al., 1994) ovaries. However, VIP mRNA levels in goat ovarian follicles have not yet been described. Previous in vitro studies also demonstrated that VIP inhibits the apoptosis of rat and mice follicles (Flaws et al., 1995; Vaccari et al., 2006) and stimulates the development of isolated bovine primary and early secondary follicles (Hulshof, 1995). Moreover, we recently reported that the addition of VIP to the in vitro culture medium maintained the survival and increases the diameter of the early preantral follicles enclosed in caprine ovarian tissue (Bruno et al., 2010).

Follicle-stimulating hormone (FSH) acts through binding to the FSH receptor (FSHR), a G protein-coupled receptor superfamily member that is located exclusively in granulosa cells (Gudermann et al., 1995), from the primary follicle developmental stage onwards (Méduri et al., 2002). FSHR mRNA was detected in the preantral and antral follicles of goats (Saraiva et al., unpublished data). FSH increased the in vitro survival and proliferation of granulosa cells, antrum formation and steroidogenesis in preantral follicles isolated from several species (mouse: Cortvrindt et al., 1997, sheep: Cecconi et al., 1999; buffalo: Sharma et al., 2009). In caprine species, FSH maintained the ultrastructural integrity, promoted the activation of primordial follicles and furthered the growth of preantral follicles enclosed in ovarian tissue (Matos et al., 2007; Magalhães et al., 2009). Moreover, the addition of FSH to the culture medium in a sequential way maintained viability, stimulated antrum formation and reduced oocyte extrusion in isolated caprine secondary follicles (Saraiva et al., unpublished data). Finally, FSH interacts with several intraovarian growth factors, which mediate its effect in regulating cellular interactions by autocrine and paracrine mechanisms, thus inducing follicular growth (Erickson and Shimasaki, 2001). Therefore, several growth and endocrine factors locally produced by the ovary are able to amplify or attenuate FSH action. However, among these factors, it is not known whether VIP, with or without an association with FSH, has an effect on the in vitro development of isolated caprine preantral follicles, as well as on VIP and FSHR mRNA levels.

Therefore, this study aimed to accomplish the following: (1) to determine the steady-state levels of VIP mRNA during different follicular stages in goat ovaries; (2) to investigate the influence of VIP and/or FSH on the development of isolated caprine preantral follicles

after 6 days of in vitro culture; and (3) to verify the effects of VIP and/or FSH on VIP and FSHR mRNA levels in isolated caprine preantral follicles cultured for 6 days.

2. Material and Methods

2.1. Source of ovaries

Ovaries (n=70) from 35 adult mixed-breed goats (*Capra hircus*, one to three years old) were collected at a local slaughterhouse, with 30 ovaries used for experiment 1 and 40 ovaries used for experiment 2. Immediately postmortem, the ovaries were washed in 70% alcohol followed by two washes in minimum essential medium (MEM). The ovaries were placed into tubes containing 15 ml of MEM plus HEPES (MEM HEPES) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin, and they were transported to the laboratory at 4°C (Chaves et al., 2008) within 1 h. Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, USA).

2.2. Experiment 1: Steady-state levels of VIP mRNA in goat ovarian follicles

To evaluate steady-state mRNA levels, ovaries (n=10) were used for the isolation of primordial, primary and secondary follicles using a mechanical procedure, as previously described (Lucci et al., 1999). After isolation, these follicles were washed twice to completely remove the stromal cells, and the follicles were then placed by category into separate Eppendorf tubes in groups of 10. This procedure was completed within 2 h, and all samples were stored at –80°C until the RNA was extracted.

The remaining ovaries were used for the collection of COCs, mural granulosa cells and thecal cells from small and large antral follicles. Compact COCs aspirated from small (1–3 mm) and large (3–6 mm) antral follicles were recovered from the ovaries (n=15). Thereafter, groups of 10 COCs were stored at –80°C until RNA extraction. To collect mural granulosa and theca cell complexes, 10 small and large antral follicles were isolated from ovaries (n=5) and dissected from stromal tissue as previously described (Van Tol and Bevers,

1998). The follicles were then bisected and granulosa and theca cell complexes were collected and stored at –80°C.

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

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

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

Table 1: Primer pairs used for real-time PCR analyses.

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
GAPDH	TGTTTGTGATGGGCGTGAACCA	s	287-309	GI:27525390
	ATGGCGTGGACAGTGGTCATAA	as	440-462	
β-actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI:28628620
	TCCTTGATGTCACGGACGATTTCC	as	386-410	
UBQ	GAAGATGGCCGCACTCTTCTGAT	s	607-631	GI:57163956
	ATCCTGGATCTTGGCCTTCACGTT	as	756-780	
VIP	ACCAATCAAACGCCACTCAGATGC	s	360-384	GI:340253
	AGACTCTCCTTCACTGCTTCGCTT	as	483-507	
FSH-R	AGGCAAATGTGTTCTCCAACCTGC	s	250-274	GI:95768228
	TGGAAGGCATCAGGGTCGATGTAT	as	316-340	

s,sense; as, antisense

2.3. Experiment 2 - Effect of VIP and/or FSH on the follicular development and steady-state levels of VIP and FSHR mRNA

2.3.1. Isolation and selection of caprine preantral follicles

In the laboratory, the surrounding fat tissue and ligaments were stripped from the ovaries. Ovarian cortical slices (1-2 mm in diameter) were cut from the ovarian surface using a surgical blade under sterile conditions. Then, the ovarian cortex was placed in fragmentation medium consisting of MEM plus HEPES. Secondary follicles ≥ 200 μm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the strips of ovarian cortex using 27.5 gauge (27.5 G) needles. After isolation, follicles were transferred to 100 μL drops containing fresh medium under mineral oil to further evaluate the follicular quality. Follicles with a visible oocyte, surrounded by granulosa cells, an intact basement membrane and with no antral cavity, were selected for culture.

2.3.2. Caprine preantral follicle culture

After selection, follicles were individually cultured in 100 µL drops of culture medium under mineral oil in Petri dishes (60x15 mm, Corning, USA). Control culture medium, called α-MEM^{+}, consisted of α-MEM (pH 7.2-7.4) supplemented with 3.0 mg/mL bovine serum albumin (BSA), ITS (insulin 10 µg/mL, transferrin 5.5 µg/mL and selenium 5 ng/mL), 2 mM glutamine, 2 mM hypoxantine and 50 µg/mL of ascorbic acid under mineral oil. Incubation was conducted at 39°C with 5% CO_2 in the air for 6 days. Fresh culture medium was prepared and incubated for 1 h prior to use. Preantral follicles obtained from each animal were randomly distributed in the following treatments: α-MEM^{+} alone or supplemented with 100 ng/mL of recombinant FSH (rFSH: Tecnopec, Brazil), 10 ng/mL of VIP or both, constituting the treatments α-MEM^{+}, FSH, VIP and VIP+FSH, respectively. Every other day, 60 µL of the culture media were replaced with fresh medium. The culture was replicated four times and a minimum of 35 follicles was used per treatment.

2.3.3. Morphological evaluation of follicle development

Follicles were classified according to their morphology, and those showing morphological signs of degeneration, such as darkness of oocytes and surrounding cumulus cells or those with misshapen oocytes, were classified as degenerated. Follicular diameter was measured only in healthy follicles in the *x* and *y* dimensions (90^{o}), by using an ocular micrometer (100 x magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) every other day of culture. To measure follicular growth, the mean increase in follicular diameter was calculated as follows: the diameter of viable follicles at day 6 minus the diameter of viable follicles at day 0 divided by the total number of viable follicles at day 6. In addition, the percentages of secondary follicles that reached the antrum formation in vitro were determined. Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers.

2.3.4. Steady-state levels of VIP and FSHR mRNA in goat ovarian follicles cultured in vitro

To evaluate the effect of VIP on the expression of VIP and FSHR mRNA after a 6 day culture period, groups of 10 follicles were collected at the end of culture period and stored at -80°C until the extraction of total RNA. Quantification of mRNA was performed as previously described, and the primers for VIP and FSHR are shown in Table 1. β-actin and ubiquitin (UBQ) were used as endogenous controls to normalize gene expression (Table 1).

2.4 Statistical analysis

mRNA expression data from primordial, primary and secondary follicles were analyzed by the Kruskal-Wallis non-parametric test, while the *t* test was used for paired comparisons of mRNA expression in the small and large antral follicles ($P<0.05$). For the cell culture experiments, follicles were considered to be the experimental unit, following the same approach as reported by Silva et al. (2010). Data from the follicular survival and antrum formation for each treatment were compared using the Chi-square test, with the results expressed as percentages. Follicular diameter data were submitted to the Kolmogorov-Smirnov and Bartlett tests to confirm normal distribution and homoscedasticity, respectively. Because of the heterogeneity of the variances, the follicular diameter, growth rate, and VIP and FSHR mRNA levels after culture were compared using the Kruskal-Wallis non-parametric test (SAS, 1999). The results were expressed as the mean ± standard error of the mean (SEM), and differences were considered to be significant when $P<0.05$.

3. Results

3.1. Experiment 1 - Steady-state levels of VIP mRNA in goat ovarian follicles

Quantification of mRNA demonstrated that secondary follicles had significantly higher levels of VIP mRNA compared to the primordial follicle ($P<0.05$). When the levels of VIP mRNA in primordial and primary follicles were compared, no significant difference was observed ($P>0.05$; Figure 1 A). In addition, no significant difference was observed between

COCs collected from small and large antral follicles (P>0.05; Figure 1 B), but granulosa/theca cells from large antral follicles had higher levels of VIP mRNA than small antral follicles (P<0.05; Figure 1 C). Real time PCR showed that COCs from both small and large antral follicles had significantly higher levels of VIP mRNA than their respective granulosa/theca cells (P<0.05; Figure 1 D, E).

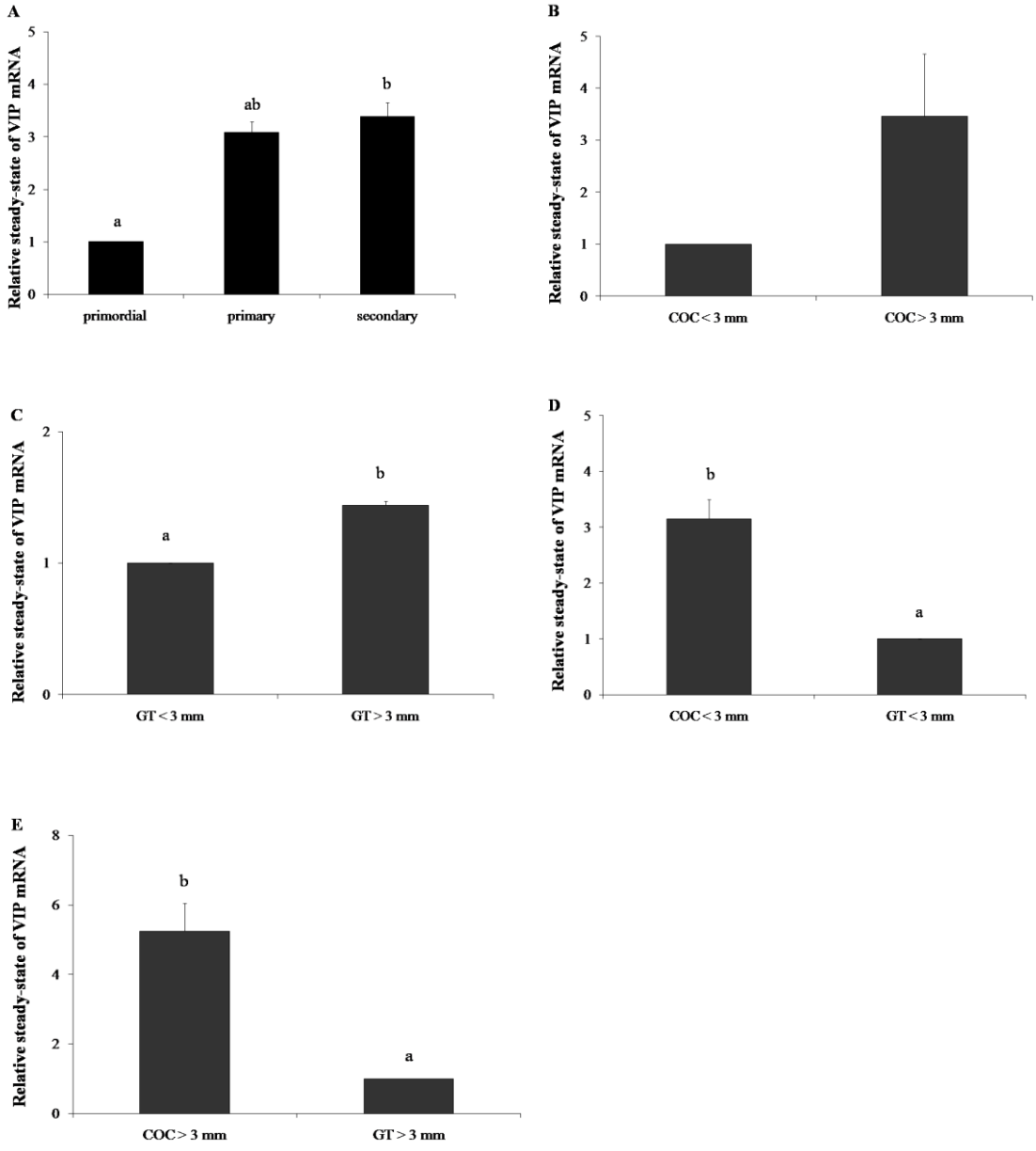

Figure 1. Steady-state levels of VIP mRNA in goat ovarian follicles (means ± SEM). A) Primordial, primary and secondary follicles. B) COCs from small and large antral follicles. C)

Granulosa/theca cells from small and large antral follicles. D) COCs and granulosa/theca cells from small antral follicles. E) COCs and granulosa/theca cells from large antral follicles. [a,b] ($P<0.05$).

3.2. Experiment 2 - Effect of VIP and/or FSH on follicular development and steady-state levels of VIP and FSHR mRNA

3.2.1. Follicular survival and growth after in vitro culture

The rates of follicular survival were greater than 90% at day 6 of culture in all treatments tested. However, no significant differences were observed among the treatments (Table 2; $P>0.05$). Figure 2 shows normal follicles before and after 6 days of in vitro culture. From as early as day 2 of culture, all treatments had follicles with an antral cavity. Furthermore, as the culture progressed from day 0 to day 6, a significant increase in the percentage of antral follicles was observed (data not shown; $P<0.05$). However, when the treatments were compared with each other, no significant differences were observed ($P>0.05$).

The follicles in the in vitro culture showed an initial mean diameter of 221.59±0.22, 237.73±0.21, 223.94±0.21 and 245.34±0.22 μm for the α-MEM^{+}, FSH, VIP and VIP+FSH treatments, respectively (Table 2; $P>0.05$). With the progression of the culture, an increase in follicular diameter was observed from day 0 to day 6 in all treatment groups ($P<0.05$); however, no significant differences were observed among the treatments ($P>0.05$). In addition, follicles cultured in α-MEM^{+}, FSH, VIP and VIP+FSH increased 16.94±0.09, 19.22±0.09, 18.89±0.09 and 20.89±0.08 μm/day, respectively. Similar to the diameter, no significant differences were observed when the treatments were compared to each other ($P>0.05$).

3.2.2. Steady-state levels of VIP and FSHR mRNA in goat ovarian follicles cultured in vitro

Figure 3 shows VIP mRNA levels after 6 days of culture with the different treatments that were tested. Culture with FSH, VIP and VIP+FSH promoted a significant reduction in

VIP mRNA levels in caprine preantral follicles after 6 days when compared with the control ($P<0.05$). With regard to FSHR mRNA levels, no significant differences were observed among the various treatments ($P>0.05$; Figure 4).

Table 2: Survival (%), antrum (%) e diameter (μm) of goat preantral follicles cultured for 6 days in VIP and/or FSH.

Treatments	Survival (%) Day 6	Antrum (%) Day 6	Diameter ± SEM	
			Day 0	Day 6
α-MEM	100 (37/37)	72.97 (27/37)	221.59 ± 66.43 b	323.22 ± 101.30 a
FSH	91.89 (34/37)	72.97 (27/37)	237.73 ± 58.12 b	358.04 ± 100.61 a
VIP	97.30 (36/37)	75.68 (28/37)	223.94 ± 60.47 b	338.72 ± 84.88 a
VIP + FSH	100 (35/35)	85.71 (30/35)	245.34 ± 68.66 b	370.70 ± 91.96 a

Values with different letters between a column differ significantly (a, b; $P<0.05$)

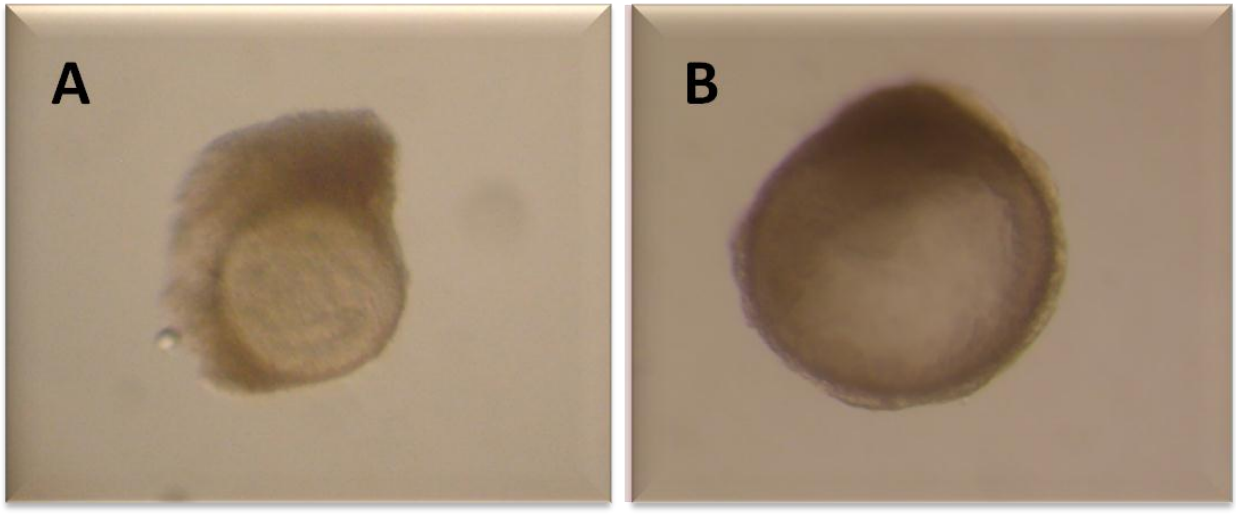

Figure 2. Preantral follicles from goats at day 0 (A) and antral follicles after 6 days of in vitro culture with 10 ng/mL VIP (B).

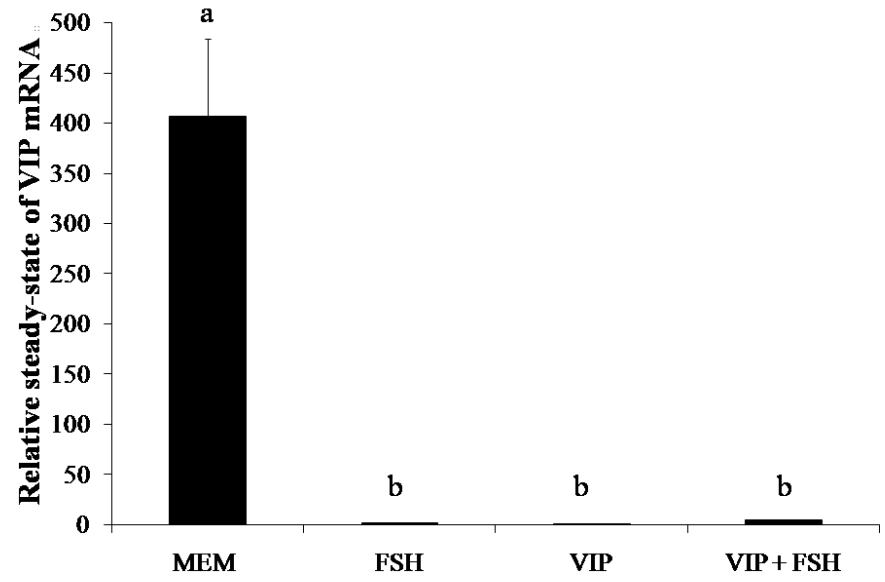

Figure 3. Steady state levels of VIP mRNA in goat preantral follicles cultured for 6 days in α-MEM^{+} supplemented with FSH, VIP or both. ^{a,b} ($P<0.05$).

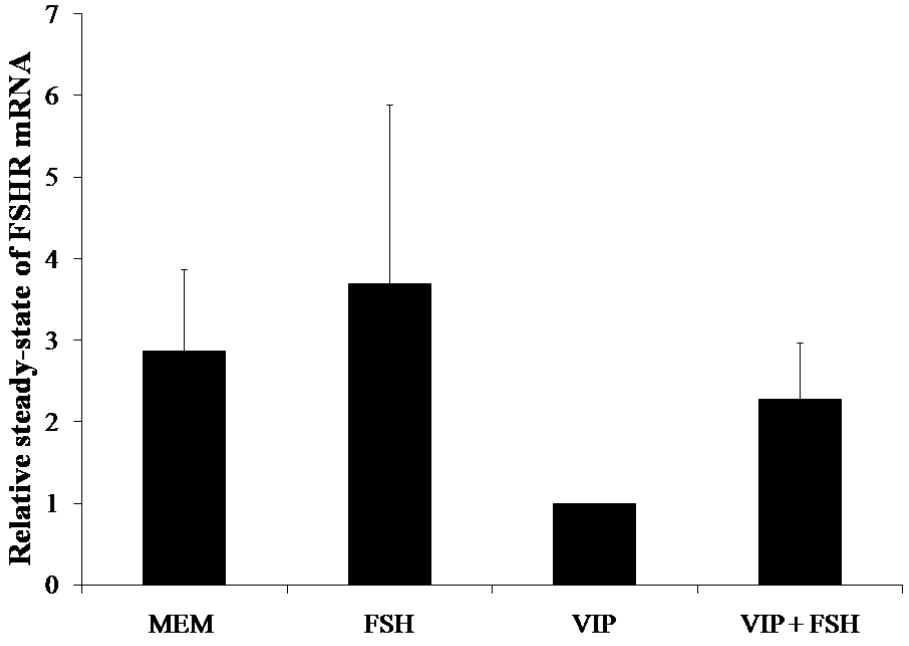

Figure 4. Steady state levels of FSHR mRNA in goat preantral follicles cultured for 6 days in α-MEM^{+} supplemented with FSH, VIP or both.

4. Discussion

The current study demonstrates the presence of VIP mRNA in all follicular categories studied in goat ovaries, showing an increase in VIP mRNA levels during the transition from the primordial to secondary follicle. The presence of VIP mRNA and its receptors was also detected in rat and mouse ovaries (Gozes and Tsafriri, 1986; Cecconi et al., 2004; Vaccari et al., 2006; Barberi et al., 2007). VIP protein was also reported to increase with the appearance of secondary and antral follicles in bovine ovaries (Hulshof et al., 1994). These results suggest a role for this peptide in follicular growth.

Granulosa/theca cells from large antral follicles had higher levels of VIP mRNA than small antral follicles, suggesting that VIP acts in a stage-dependent way because the mRNA expression levels increase with the progression of follicular development. In addition, COCs from either small or large antral follicles had higher VIP mRNA levels than their respective granulosa/theca cells. In rats, some studies have shown that VIP stimulates maturation in follicle-enclosed oocytes but could transiently inhibit, or not affect, the spontaneous maturation of cumulus-enclosed oocytes (Tornell et al., 1988; Apa et al., 1997). Furthermore, other studies have shown that VIP increases the levels of cAMP, which regulates oocyte maturation (Eppig and Downs, 1984) in preovulatory follicles rats (Tornell et al., 1988, Apa et al., 1997) and stimulates ovulation in perfused rat ovaries (Schmidt et al., 1990). In addition, VIP stimulates the production of plasminogen activator (Johnson and Tilly, 1988), which converts plasminogen into plasmin that acts in the follicular wall releasing the COCs.

In the current study, high rates of survival and antrum formation were observed after the in vitro culture period, and there was an increase in the follicular diameter after all treatments. These results occurred independently of the addition of VIP and/or FSH to the culture medium. Previous in vitro studies in different species have demonstrated that VIP (rat: Flaws et al., 1995; Vaccari et al., 2006; bovine: Hulshof, 1995) inhibits apoptosis in preantral follicles and stimulates the development of isolated primary and/or secondary follicles. Recently, a study performed by our research team has demonstrated that VIP is an important factor that promotes the growth of small preantral follicles enclosed in caprine ovarian tissue (Bruno et al., 2010). However, despite the fact that VIP mRNA that was detected in caprine secondary follicles, no additional effect of this factor was observed after the culture of

isolated follicles in this category when compared to the follicles cultured in α-MEM$^+$. This result may be due to the fact that α-MEM$^+$ is a medium that is rich in nutrients (amino acids, carbohydrates, B-vitamin complexes, vitamins C and D, inorganic salts and pyruvate) and DNA precursors, all of which promote cell division (Hartshorne, 1997). Another possible explanation for this finding is that VIP may need to interact with other substances and in other concentrations to promote the in vitro growth of isolated secondary follicles in the caprine species. Cecconi et al. (2004) demonstrated that VIP did not affect mouse follicular development and caused the inhibition of follicular growth, antrum formation, granulosa cell proliferation, and estradiol production. Moreover, other studies demonstrated that FSH inhibits follicle apoptosis (murine: Cortvrindt et al., 1998; bovine: Itoh et al., 2002), maintains the ultrastructural integrity of goat preantral follicles after culturing cortical tissue (Saraiva et al., 2008), and promotes survival and antrum formation after 18 days culture of isolated caprine secondary follicles (Saraiva et al., unpublished data). However, in the present study, no additional influence of this hormone was observed after 6 days of culture when compared with the control medium. It is likely that the use of the different concentrations of FSH associated with a longer culture period of isolated follicles could improve the beneficial effects of FSH on follicular survival and viability.

In this study, the presence of VIP in the culture medium reduces the mRNA levels for itself. It is possible that the use of exogenous VIP, associated with its endogenous production during the culture, might have caused a down-regulation in the expression of VIP mRNA. Similarly, the addition of FSH to the culture medium reduces VIP mRNA levels. Flaws et al (1995) verified that VIP can prevent follicular atresia in the absence of the influence of gonadotropin. Analysis of VIP receptor (VPAC1-R) mRNA levels in whole mouse ovaries showed that transcripts were present in untreated 22-day-old immature animals and were significantly down-regulated after gonadotropin stimulation (Barberi et al., 2007). These findings raise the possibility that VIP can promote the survival of follicles that have not yet acquired dependence upon gonadotropins for continued development. This proposal would be consistent with the fact that VIP can induce aromatase activity in immature follicles that are not yet responsive to FSH (George and Ojeda, 1987). Many of the effects of VIP on ovarian granulosa cells are similar to the action of gonadotropins (Ahmed et al., 1986; Johnson and

Tilly, 1988; Ojeda et al., 1989; Johnson et al., 1994), suggesting that VIP may be important for regulating gonadotropin-independent development and ovarian follicle survival.

Although VIP stimulates the synthesis of cAMP, a key intracellular messenger involved in the formation of FSHR (Knecht et al., 1983; Tano et al., 1997), in the present study, the presence of VIP had no influence on FSHR mRNA levels in caprine preantral follicles cultured in vitro. Evidence also suggests the existence of cAMP-independent mechanisms, such as activin, that regulate the formation of FSHR in immature granulosa cells (Tano et al., 1997; Nakamura et al., 1995; Xiao et al., 1992). In contrast to our findings, VIP stimulation induced aromatase and FSHR expression at both the transcript and protein levels in rat neonatal ovaries several days before the ovaries became responsive to gonadotropins (Mayerhofer et al., 1997). In addition, there was no increase in FSHR mRNA expression when the medium was supplemented with FSH. It is possible that gonadotropins are not required for their own receptor expression during initial folliculogenesis, as shown in hpg/hpg mice, in which the development of FSHR mRNA levels was normal (O'Shaughnessy et al., 1997). Moreover, Saraiva et al. (unpublished data) used a sequential culture system with FSH and showed no increase in the expression of FSHR after 6 and 12 days of isolated caprine secondary preantral follicle culture. However, most studies show that gonadotropins stimulate the expression of their own receptors (Smith and Ojeda, 1986; Minegishi et al., 1997; Guglielmo et al., in press). A second possible explanation for the limited response could be that FSH may have promoted a transcriptional down-regulation or decreased the stability of receptor mRNA induced by high FSH concentrations (Tilly et al., 1992; Tisdall et al., 1995; Xu et al., 1995).

In conclusion, the present study demonstrated that VIP mRNA was detected in all follicular categories and cellular types in caprine species. In the culture conditions used here, the presence of VIP and/or FSH did not affect follicular survival and development after 6 days of in vitro culture, in addition to reduces the mRNA levels for VIP, however without influences the mRNA levels for FSHR. More detailed studies of the mechanisms of action of VIP on follicular development in vivo and in vitro are essential to better understand folliculogenesis. Furthermore, future applications of in vitro follicle culture systems would improve fertility preservation for humans and rare animal species.

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References

Ahmed, C.E., Dees, W.L., Ojeda, S.R., 1986. The immature rat ovary is innervated by vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. Endocrinology 118, 1682–1689.

Apa, R., Lanzone, A., Mastrandrea, M., Miceli, F., Macchione, E., Fulghesu, A.M., Caruso, A., Canipari, R., 1997. Effect of pituitary adenylate cyclase activating peptide on meiotic maturation in follicle-enclosed, cumulus-enclosed, and denuded rat oocytes. Biol. Reprod. 57, 1074-1079.

Barberi, M., Muciaccia, B., Morelli, M.B., Stefanini, M., Cecconi, S., Canipari, R., 2007. Expression localisation and functional activity of pituitary adenylate cyclase activating polypeptide, vasoactive intestinal polypeptide and their receptors in mouse ovary. Reproduction 134, 281-292.

Bruno, .JB., Celestino, J.J.H., Lima-Verde, I.B., Matos, M.H.T., Lima, L.F., Name, K.P.O., Araújo, V.R., Saraiva, M.V.A., Martins, F.S., Campello, C.C., Silva, J.R.V., Báo, S.N., Figueiredo, J.R., 2010. Vasoactive intestinal peptide improves the survival and development of caprine preantral follicles after in vitro tissue culture. Cells Tissues Organs 191, 414-21.

Burden, H.W., 1985. The adrenergic innervations of mammalian ovaries, in: Ben-Jonathan, N., Bahr, M.M., Weiner, R.I. (Eds.), Catecholamines as Hormone Regulators. Raven Press, New York, pp. 261-278.

Cecconi, S., Barboni, B., Coccia, M., Mattioli, M., 1999. *In vitro* development of sheep preantral follicles. Biol. Reprod. 60, 594-601.

Cecconi, S., Rossi, G., Barberi, M., Scaldaferri, L., Canipari, R., 2004. Effect of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide on mouse preantral follicle development in vitro. Endocrinology 145, 2071-2079.

Chaves, R.N., Martins, F.S., Saraiva, M.V.A., Celestino, J.J.H., Lopes, C.A.P., Correia, J.C., Lima-Verde, I.B., Matos, M.H.T., Báo, S.N., Name, K.P.O., Campello, C.C., Silva, J.R.V., Figueiredo, J.R., 2008. Chilling ovarian fragments during transportation improves viability and growth of goat preantral follicles cultured in vitro. Reprod. Fertil. Dev. 20, 640-647.

Cortvrindt, R., Smitz, J., Steirteghem, A.C., 1997. Assessment of the need for follicle stimulating hormone in early pre antral mouse follicle culture in vitro. Hum. Reprod. 12, 759-768.

Cortvrindt, R.G., Hu, Y., Liu, J., Smitz, J.E., 1998. Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotrophin. Fertil. Steril. 70, 1114-1125.

Davoren, J.B., Hsueh, A.J., 1985. Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. Biol. Reprod. 33, 37-52.

Eppig, J.J., Downs, S.M., 1984. Chemical signals that regulate mammalian oocyte maturation. Biol. Reprod. 30, 1-11.

Erickson, G.F., Shimasaki, S., 2001. The physiology of folliculogenesis: the role of novel growth factors. Fertil. Steril. 76, 943-949.

Flaws, J.A., Desanti, A., Tilly, K.I., Javid, R.0., Kugu, K., Johnson, A.L., Hirshfield, A.N., Tilly, J.L., 1995. Vasoactive intestinal peptide mediated suppression of apoptosis in the ovary: potential mechanisms of action and evidence of a conserved antiatretogenic role through evolution. Endocrinology 136, 4351-4359.

George, F.W., Ojeda, S.R., 1987. Vasoactive intestinal peptide induces aromatase activity before development of primary follicles or responsiveness to follicle-stimulating hormone. Proc. Natl. Acad. Sci. U.S.A. 84, 5803-5807.

Gozes, I., Tsafriri, A., 1986. Detection of vasoactive intestinal peptide encoding messenger ribonucleic acid in the rat ovaries. Endocrinology 119, 2606-2610.

Gudermann, T., Nürnberg, B., Schultz, G., 1995. Receptors and G proteins as primary components of transmembrane signal transduction. I. G-protein-coupled receptors: structure and function. J. Mol. Med. 73, 51-63.

Guglielmo, M.C., Ricci, G., Catizone, A., Barberi, M., Galdieri, M., Stefanini, M., Canipari, R., in press. The effect of hepatocyte growth factor on the initial stages of mouse follicle development. J. Cell. Physiol.

Hartshorne, G., 1997. *In vitro* culture of ovarian follicles. Rev. Reprod. 2, 94-104.

Hulshof, S.C.J., Dijkstra, G., Van der Beek, E.M., Bevers, M.M., Figueiredo, J.R., Beckers, J.F., Van den Hurk, R., 1994. Immunocytochemical localization of vasoactive intestinal peptide and neuropeptide Y in the bovine ovary. Biol. Reprod. 50, 553-560.

Hulshof, S.C.J., 1995. Bovine preantral follicles and their development in vitro; thesis, Utrecht.

Itoh, T., Kacchi, M., Abe, H., Sendai, Y., Hoshi, H., 2002. Growth, antrum formation, and estradiol production of bovine preantral follicles cultured in a serum-free medium. Biol. Reprod. 67, 1099-1105.

Johnson, A.L., Tilly, J.L., 1988. Effects of vasoactive intestinal peptide on steroid secretion and plasminogen activator activity in granulose cells of the hen. Biol. Reprod. 38, 296-303.

Johnson, A.L., Li, Z., Gibney, J.A., Malamed, S., 1994. Vasoactive intestinal peptide-induced expression of cytochrome p450 cholesterol side-chain cleavage and 17a-hydroxylase enzyme activity in hen granulosa cells. Biol. Reprod. 51, 327-333.

Knecht, M., Ranta, T., Catt, K.J., 1983. Granulosa cell differentiation *in vitro*: induction and maintenance of follicle-stimulating hormone receptors by adenosine 3_,5_-monophosphate. Endocrinology 113, 949-956.

Lucci, C.M., Amorim, C.A., Rodrigues, A.P.R., Figueiredo, J.R., Báo, S.N., Silva, J.R.V., Gonçalves, P.B.D., 1999. Study of preantral follicles population in situ and after mechanical isolation from caprine ovaries at different reproductive stages. Anim. Reprod. Sci. 56, 223-236.

Magalhães, D.M., Araujo, V.R., Lima-Verde, I.B., Matos, M.H.T, Silva, R.C., Lucci, C.M., Báo, S.N., Campello, C.C., Figueiredo, J.R., 2009. Different Follicle-Stimulating Hormone (FSH) sources influence caprine preantral follicle viability and development *in vitro* Braz. J. Vet. Res. Anim. Sci. 46, 378-386.

Matos, M.H.T., Lima-Verde, I.B., Luque, M.C.A., Maia Jr, J.E., Silva, J.R.V., Celestino, J.J.H., Martins, F.S., Báo, S.N., Lucci, C.M., Figueiredo, J.R., 2007. Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability *in vitro.* Zygote 15, 173-182.

Mayerhofer, A., Dissen, G.A., Costa, M., Ojeda S.R., 1997. A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. Endocrinology 138, 3320-3329.

Méduri, G., Charnaux, N., Driancourt, M.A., Combettes, L., Granet, P., Vannier B, Loosfelt, H., Migrom, E., 2002. Follicle-stimulating hormone receptors in oocytes? J. Clin. Endocrinol. Metab. 87, 2266-2276.

Minegishi, T., Tano, M., Kishi, H., Kameda, T., Miyamoto, K., 1997. Follicle-stimulating hormone regulation on its receptor messenger ribonucleic acid levels in cultured rat granulosa cells. Biochim. Biophys. Acta. 1359, 165-173.

Nakamura, M., Nakamura, K., Igarashi, S., Tano, M., Miyamoto, K., 1995. Interaction between activin A and cAMP in the induction of FSH receptor in cultured rat granulosa cells. J. Endocrinol. 147, 103-110.

Ojeda, S.R., Lara, H.E., 1989. The role of the sympathetic nervous system in the regulation of ovarian function, in: Pirke, K.M., Wuttke, W., Schweiger, U. (Eds.), The Menstrual Cycle and Its Disorders. Springer-Verlag, Berlin, pp. 26-32.

Ojeda, S.R., Lara, H.E., Ahmed, C.E., 1989. The potential relevance of vasoactive intestinal peptide to ovarian physiology, in: Adashi, E. (Ed.), Putative Intraovarian Regulators. Thieme Inc., New York, pp. 52-60.

O'Shaughnessy, P.J., McLelland, D., McBride, M.W., 1997. Regulation of luteinizing hormone-receptor and follicle stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. Biol. Reprod. 57, 602-608.

Parra, C., Fiedler, J.L., Luna, S.L., Greiner, M., Padmanabhan, V., Lara, H.E., 2007. Participation of vasoactive intestinal polypeptide in ovarian steroids production during the rat estrous cycle and in the development of estradiol valerate-induced polycystic ovary. Reproduction 133, 147–154.

Saraiva, M.V.A., Celestino, J.J.H., Chaves, R.N., Martins, F.S., Bruno, J.B., Lima-Verde, I.B., Matos, M.H.T., Silva, G.M., Porfirio, E.P., Báo, S.N., Campello. C.C., Silva, J.R.V., Figueiredo, J.R., 2008. Influence of different concentrations of LH and FSH on *in vitro* caprine primordial ovarian follicle development. Small Rum. Res. 78, 87-95.

Schmidt, G., Jörgensen, J., Kannisto, P., Liedberg, F., Ottesen, B., Owman, C., 1990.

Vasoactive intestinal polypeptide in the PMSG-primed immature rat ovary and its effect on ovulation in the isolated rat ovary perfused in vitro. J. Reprod. Fertil. 90, 465-472.

Sharma, G.T., Dubey, P.K., Meur, S.K., 2009. Survival and developmental competence of buffalo preantral follicles using three dimensional collagen gel culture system. Anim. Reprod. Sci. 114, 115-124.

Silva, C.M.G., Matos, M.H.T., Rodrigues, G.Q., Faustino, L.R., Pinto, L.C., Chaves, R.N., Araújo, V.R., Campello, C.C., Figueiredo J.R., 2010. In vitro survival and development of goat preantral follicles in two different oxygen tensions. Anim. Reprod. Sci. 117, 83-89.

Smith, S.S., Ojeda, S.R., 1986. Neonatal release of gonadotropins is essential for development of ovarian follicle-stimulating hormone receptor. Biol. Reprod. 34. 219-227.

Tano, M., Minegishi, T., Nakamura, K., Karino, S., Ibuki, Y., Miyamoto, K., 1997. Transcriptional and post-transcriptional regulation of FSH receptor in rat granulosa cells by cyclic AMP and activin. J. Endocrinol. 153, 465-473.

Tilly, J.L., Kowalski, K.I., Schomberg, D.W., Hsueh, A.J.W., 1992. Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 aromatase. Endocrinology 131, 1670-1676.

Tisdall, D.J., Watanabe, K., Hudson, N.L., Smith, P., McNatty, K.P., 1995. FSH receptor gene expression during ovarian follicle development in sheep. J. Endocrinol. 15, 273-281.

Tornell, J., Carlsson, B., Hillensjo, T., 1988. Vasoactive intestinal peptide stimulates oocyte maturation, steroidogenesis, and cyclic adenosine 3 _ , 5 _ -monophosphate production in isolated preovulatory rat follicles. Biol. Reprod. 39, 213-220.

Vaccari, S., Latini, S., Barberi, M., Teti, A., Stefanini, M., Canipari, R., 2006. Characterization and expression of different pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptors in rat ovarian follicles. J. Endocrinol. 191, 287–299.

Van Tol HT, Bevers MM, 1998. Theca cells and theca-cell conditioned medium inhibit the progression of FSH-induced meiosis of bovine oocytes surrounded by cumulus cells connected to membrana granulosa. Mol Reprod Dev 51, 315–321.

Xiao, S., Robertson, D.M., Findlay, J.K., 1992. Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131, 1009-1016.

Xu, Z., Garverick, H.A., Smith, G.W., Smith, M.F., Hamilton, S.A., Youngquist, R.S., 1995. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol. Reprod. 53, 951-957.

12. CONCLUSÕES

- A adição de 10 ng/mL de ANG II manteve a viabilidade e a ultraestrutura de folículos pré-antrais caprinos após sete dias de cultivo *in vitro*. Os RNAm para os receptores AGTR1 e AGTR2 foram detectados em todas as categorias foliculares, sendo expressos de forma diferenciada nos diferentes compartimentos de folículos antrais.
- O VEGF na concentração de 200 ng/mL manteve a viabilidade e a ultraestrutura e na concentração de 10 ng/mL promoveu o crescimento de folículos pré-antrais caprinos após sete dias de cultivo *in vitro*. Além disso, o VEGFR-2 foi expresso em oócitos de folículos ovarianos caprinos em todos os estágios de desenvolvimento e em células da granulosa de folículos em desenvolvimento.
- A utilização de 10 ng/mL VIP assegurou a manutenção da integridade folicular e estimulou o crescimento *in vitro* de folículos pré-antrais caprinos após sete dias de cultivo *in vitro*.
- O RNAm para a VIP foi detectado em todas as categorias e tipos celulares de folículos caprinos. Além disso, o VIP e/ou o FSH não afetaram o desenvolvimento dos folículos secundários e reduziram os níveis de RNAm para VIP após 6 dias de cultivo de folículos pré-antrais caprinos.

13. PERSPECTIVAS

A eficiência dos programas de fecundação *in vitro* e transferência de embriões visando à multiplicação de animais de alto valor zootécnico ou em via de extinção a partir de oócitos provenientes dos folículos pré-antrais, depende ainda da completa compreensão dos mecanismos que controlam a foliculogênese inicial, uma vez que os folículos primordiais representam a grande reserva dos oócitos que poderão ser destinados para as diferentes biotécnicas reprodutivas.

Os resultados deste projeto evidenciaram a participação da ANG II, VEGF e VIP no desenvolvimento de folículos pré-antrais caprinos cultivados *in situ*. Entretanto, estudos complementares poderão ser realizados visando à utilização destas substâncias sobre o desenvolvimento de folículos pré-antrais isolados após ativação e crescimento destes folículos *in situ.*

Diante das conclusões deste trabalho, as informações obtidas poderão ser utilizadas para aperfeiçoar a elaboração e o fornecimento de meios de cultivo capazes de propiciar ótimas condições para um completo crescimento folicular, preservando a viabilidade celular e revolucionando a produção *in vitro* de embriões.

14. REFERÊNCIAS BIBLIOGRÁFICAS

ABIR, R.; FISCH, B.; JIN, S.; BARNNET, M.; FREIMANN, S.; VAN DEN HURK, R.; FELDBERG, D.; NITKE, S.; KRISSI, H.; AO, A. Immunocytochemical detection and RT-PCR expression of leukaemia inhibitory factor and its receptor in human fetal and adult ovaries. *Molecular Human Reproduction***,** v. 10, n.5, p. 313-319, 2004.

ABIR, R.; NITKE, A.; BEN-HAROUSH, A.; FISCH, B. *In vitro* maturation of primordial ovarian follicles: Clinical significance, progress in mammals, and methods for growth evaluation. *Histology and Histopatology*, v. 21, p. 887-898, 2006.

ABRAMOVICH, D.; CELIN, A. R.; HERNANDEZ, F.; TESONE, M.; PARBOREL, F. Spatiotemporal analysis of the protein expression of angiogenic factors and their related receptors during folliculogenesis in rats with and without hormonal treatment. *Reproduction*, v. 137, p. 309- 320, 2009.

ABRAMOVICH, D.; PARBOREL, F.; TESONE, M. Effect of a vascular endothelial growth factor (VEGF) inhibitory treatment on the folliculogenesis and ovarian apoptosis in gonadotropin-treated prepubertal rats. *Biology of Reproduction*, v. 75, p. 434-441, 2006.

ACOSTA, T. J.; HAYASHI, K. G.; OHTANI, M.; MIYAMOTO, A. Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. *Reproduction*, v, p. 125:759-767, 2003.

ACOSTA, T. J.; BERISHA, B.; OZAWA, T.; SATO, K.; SCHAMS, D.; MIYAMOTO, A. Evidence for a local endothelin-angiotensin-atrial natriuretic peptide system in bovine mature follicles *in vitro*: effects on steroid hormones and prostaglandin secretion. *Biology of Reproduction*, v. 61, n. 6, p. 1419-1425, 1999.

AHMED, C. E.; DEES, W. L.; OJEDA, S. R. The immature rat ovary is innervated by vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. *Endocrinology*, v. 118, p. 1682–1689, 1986.

AMORIM, C. A.; LUCCI, C. M.; RODRIGUES, A. P. R.; CARVALHO, F. C. A.; FIGUEIREDO, J. R.; RONDINA, D.; CECCHI, R.; GIORGETTI, A.; MARTINI, A.; GONÇALVES, P. B. D. Quantitative and qualitative analysis of the effectiveness of the mechanical method for the isolation of preantral follicles from ovines ovaries. *Theriogenology*, v. 53, p. 1251–1262, 2000.

ANDERSON, E.; LEE, G. Y. The participation of growth factors in stimulating the quiescent, proliferative, and differentiative stages of rat granulosa cells grown in a serum-free medium. *Tissue Cell*, v. 25, p. 49-72, 1993.

ANDERSON, R. A.; ROBINSON, L. L. L.; BROOKS, J.; SPEARS N. Neurotropins and their receptors are expressed in the human fetal ovary. *Journal of Clinical Endocrinology and Metabolism*, v. 87, p. 890-897, 2002.

APA, R.; LANZONE, A.; MASTRANDREA, M.; MICELI, F.; MACCHIONE, E.; FULGHESU, A.M.; CARUSO, A.; CANIPARI, R. Effect of pituitary adenylate cyclase activating peptide on meiotic maturation in follicle-enclosed, cumulus-enclosed, and denuded rat oocytes. *Biology of Reproduction*, v. 57, p. 1074-1079, 1997.

ARAÚJO, V. R.; CHAVES, R. N.; DUARTE, A. B. G.; CELESTINO, J. J. H.; SILVA, G. M.; FERNANDES, D. D.; MATOS, M. H. T.; CAMPELLO, C. C.; FIGUEIREDO, J. R. Effect of culture medium replacement protocol on the in vitro development of isolated caprine secondary follicles. *Small Ruminant Research*, 2010b. [no prelo].

ARAÚJO, V. R.; SILVA, C. M. G.; MAGALHÃES, D. M.; SILVA, G. M.; BÁO, S. N.; SILVA, J. R. V.; FIGUEIREDO, J. R.; RODRIGUES, A. P. R. Effect of Bone Morphogenetic Protein-7 (BMP-7) on *in vitro* survival of caprine preantral follicles. *Pesquisa Veterinária Brasileira*, v. 30, n. 4, p. 305-310, 2010a**.**

ARIMURA, A. Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research. *Regulatory Peptides*, v. 37, p. 287-303, 1992.

ARUNAKUMARI, G.; SHANMUGASUNDARAM, N.; RAO, V. H. Development of morulae from the oocytes of cultured sheep preantral follicles. *Theriogenology*, v. 74, n. 5, p. 884-94, 2010.

ASAHARA, T.; BAUTERS, C.; ZHENG, L.P.; TAKESHITA, S.; BUNTING, S.; FERRARA, N. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation*, v. 91, p. 365-371, 1995.

ASAKAI, R.; SONG, S.; ITOH, N.; YAMAKUNI, T.; TAMURA, K.; OKAMOTO, R. Differential gene expression of fibroblast growth factor receptor isoforms in rat ovary. *Molecular and Cellular Endocrinology*, v. 114, p. 223, 1995.

ASAKAI, R.; TAMURA, K.; EISHI, Y.; IWAMOTO, M.; KATO, Y.; OKAMO, R. Basic fibroblast growth factor (bFGF) receptors decrease with luteal age in rat ovarian luteal cells: colocalization of bFGF receptors and bFGF in luteal cells. *Endocrinology*, v. 133, p. 1074-1084, 1993.

BARBERI, M.; MUCIACCIA, B.; MORELLI, M. B.; STEFANINI, M.; CECCONI, S.; CANIPARI, R. Expression localization and functional activity of pituitary adenylate cyclase activating polypeptide, vasoactive intestinal polypeptide and their receptors in mouse ovary. *Reproduction*, v.134, p. 281-292, 2007.

BARBONI, B.; TURRIANI, M.; GALEATI, G.; SPINACI, M.; BACCI, M. L.; FORNI, M.; MATTIOLI, M. Vascular endothelial growth factor production in growing pig antral follicles. *Biology of Reproduction*, v. 63, p. 858-864, 2000.

BARNETT, K. R.; SCHILLING, C.; GREENFELD, C. R.; TOMIC, D.; FLAWS, J. A. Ovarian follicle development and transgenic mouse models. *Human Reproduction Update*, v. 12, p. 537-55, 2006.

BEERS W. H. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. *Cell*, v. 6, p. 379-386. 1975.

BEKER, A. R. C. L.; IZADYAR, F.; COLENBRANDER, B.; BEVERS, M. M. Effect of growth hormone releasing hormone (GHRH) and vasoactive intestinal peptide (VIP) on *in vitro* bovine oocyte maturation. *Theriogenology*, v, 53, p. 1771-1782. 2000.

BEN-HAROUSH, A.; ABIR, R.; AO, A.; JIN, S.; KESSLER-ICEKSON, G.; FELDBERG, D.; FISCH, B. Expression of basic fibroblast growth factor and its receptors in human ovarian follicles from adults and fetuses. *Fertility and Sterility*, v.84, p. 1257-1268, 2005.

BERISHA, B.; SCHAMS, D.; KOSMANN, M.; AMSELGRUBER, W.; EINSPANIER, R. Expression and tissue concentration of vascular endothelial growth factor, its receptors and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biology of Reproduction*, v. 63, p. 1106-1114, 2000.

BERISHA, B.; SCHAMS, D.; MIYAMOTO, A. The mRNA Expression of Angiotensin and Endothelin System Members in Bovine Ovarian Follicles During Final Follicular Growth. *Journal Reproduction and Development*, v. 48, p. 573-582, 2002.

BODENSTEINER, K. J.; CLAY, C. M.; MOELLER, C. L.; SAWYER, H. R. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. *Biology of Reproduction*, v. 60, p. 381-386, 1999.

BOONYAPRAKOB, U.; GADSBY, J. E.; HEDGPETH, V.; ROUTH, P.; ALMOND, G. W. Expression and localization of vascular endothelial growth factor and its receptors in pig corpora lutea during the oestrous cycle. *Reproduction*, v. 126, p. 393-405, 2003.

BRAW-TAL, R.; YOSSEFI, S. Studies *in vivo* and *in vitro* on the initiation of follicle growth in the bovine ovary. *Journal of Reproduction Fertility*, v. 109, p.165-71, 1997.

BRISTOL-GOULD, S.; WOODRUFF, T. K. Folliculogenesis in the domestic cat (*Felis catus*). *Theriogenology*, v. 66, p. 5-13, 2006.

BRUNO, J. B.; CELESTINO, J. J. H.; LIMA-VERDE, I. B.; MATOS, M. H. T.; LIMA, L. F.; NAME, K. P. O.; ARAÚJO, V. R.; SARAIVA, M. V. A.; MARTINS, F. S.; CAMPELLO, C. C.; SILVA, J. R. V.; BÁO, S. N.; FIGUEIREDO, J. R. Vasoactive intestinal peptide improves the survival and development of caprine preantral follicles after in vitro tissue culture. *Cells Tissue Organs*, v. 191, n. 5, p. 414-21, 2010.

BRUNO, J. B.; LIMA-VERDE, I. B.; MARTINS, F. S.; MATOS, M. H. T.; LOPES, C. A. P.; MAIA-JR, J. E.; BÁO, S. N.; NOBRE-JUNIOR, H. V.; MAIA F. D.; PESSOA, C.; MORAES, M. O.; SILVA, J. R. V., FIGUEIREDO, J. R.; RODRIGUES, A.P.R. Característica histológica, ultra-estrutural e produção de nitrito de folículos pré-antrais caprinos cultivados *in vitro* na ausência ou presença de soro. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v. 60, n. 6, p. 1329-1337, 2008.

BURATINI, J. J.; GLAPINSK, V. F.; GIOMETTI, I. C.; TEIXEIRA, A. B.; COSTA, I. B. Expression of fibroblast growth factor-8 and its cognate receptor, fibroblast growth factor receptor (FGFR) -3c and -4, in fetal bovine preantral follicles. *Molecular Reproduction and Development*, v. 70, p. 255-261, 2005.

BURDEN, H. W. The adrenergic innervations of mammalian ovaries. *In*: BEN-JONATHAN, N.; BAHR, M. M.; WEINER, R. I. (Eds.), *Catecholaminesas Hormone Regulators*, New York: Raven Press, 1985, p. 261–278.

BUSTIN, S. A. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology*. v. 29, p. 23-39, 2002.

BUTCHER, L.; ULLMANN, S. L. Culture of Preantral Ovarian Follicles in the Grey, Shorttailed Opossum, Monodelphis domestica. *Reproduction, Fertility and Development*, v. 8, p. 535-539, 1996.

CAIN, L.; CHATTERJEE, S.; COLLINS, T. J. *In vitro* folliculogenesis of rat preantral follicles. *Endocrinology*, v. 136, p. 3369-3377, 1995.

CAMPBELL, B. K. The endocrine and local control of ovarian follicle development in the ewe. *Animal Reproduction*, v. 6, n. 1, p. 159-171, 2009.

CARLSSON, I. B.; SCOTT J. E.; VISSER, J. A.; RITVOS, O.; THEMMEN, A. P. N.; HOVATTA, O. Anti-Mullerian hormone inhibits initiation of growth of human primordial ovarian follicles *in vitro*. *Human Reproduction*, v. 21, p. 2223-27, 2006.

CARMELIET, P.; JAIR, R. K. Angiogenesis in cancer and other diseases. *Nature*, v. 407, p. 249-257, 2000.

CECCONI, S.; BARBONI, B.; COCCIA, M.; MATTIOLI, M. *In vitro* development of sheep preantral follicles. *Biology of Reproduction*. v. 60, p. 594-601, 1999.

CECCONI, S.; ROSSI, G.; BARBERI, M.; SCALDAFERRI, L.; CANIPARI, R. Effect of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide on mouse preantral follicle development *in vitro*. *Endocrinology*, v. 145, p. 2071–2079, 2004.

CELESTINO, J. J. H.; BRUNO, J. B.; LIMA-VERDE I. B.; MATOS, M. H. T.; SARAIVA, M. V. A.; CHAVES, R. N.; MARTINS, F. S.; LIMA, L. F.; NAME, K. P. O.; CAMPELLO, C. C.; SILVA, J. R. V.; BÁO, S. N.; FIGUEIREDO, J. R. Recombinant Epidermal Growth Factor maintains follicular ultrastructure and promotes the transition to primary follicles in caprine ovarian tissue cultured *in vitro*. *Reproductive Sciences*. v. 16, n. 3, p. 239-246, 2009.

CELESTINO, J. J. H.; BRUNO, J. B.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SARAIVA, M. V. A.; CHAVES, R. N.; MARTINS, F. S.; ALMEIDA, A. P.; CUNHA, R. M. S.; LIMA, L. F.; NAME, K. P. O.; CAMPELLO, C. C.; SILVA, J. R. V.; BAO, S. N.; FIGUEIREDO, J. R. Steady-State Level of Kit Ligand mRNA in Goat Ovaries and the Role of Kit Ligand in Preantral Follicle Survival and Growth In Vitro. *Molecular Reproduction & Development*, v. 77, p. 231-240, 2010.

CELIK-OZENCI, C.; AKKOYUNHLU, G.; KAYISLI, U. A.; ARICI, A.; DEMIR, R.; Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. *Histochemistry and Cell Biology*, v. 120, p. 383-390, 2003.

CHAVES, R. N.; ALVES, A. M. C. V.; DUARTE, A. B. G.; ARAÚJO, V. R.; CELESTINO, J. J. H.; MATOS, M. H. T.; LOPES, C. A. P.; CAMPELLO, C. C.; NAME, K. P. O.; BÁO, S. N.; FIGUEIREDO, J. R. Nerve growth factor promotes the survival of goat preantral follicles cultured *in vitro*. *Cells Tissues Organs*, v.192, n. 4, p. 272-82, 2010a.

CHAVES, R. N.; LIMA-VERDE, I. B.; CELESTINO, J. J. H.; DUARTE, A. B. G.; ALVES, A. M. C. V.; MATOS, M. H. T.; CAMPELLO, C. C.; NAME, K. P. O.; BÁO, S. N.; BURATINI JR, J.; FIGUEIREDO, J. R. Fibroblast growth factor-10 maintains the survival and promotes the growth of cultured goat preantral follicles. *Domestic Animal Endocrinology*, v. 39, p. 249-258, 2010b.

CHAVES, R. N.; MARTINS, F. S.; SARAIVA, M. V. A.; CELESTINO, J. J. H.; LOPES, C. A. P.; CORREIA, J. C.; LIMA-VERDE, I. B.; MATOS, M. H. T.; BÁO, S. N.; NAME, K. P. O.; CAMPELLO, C. C.; SILVA, J. R. V.; FIGUEIREDO, J. R. Chilling ovarian fragments during transportation improves viability and growth of goat preantral follicles cultured *in vitro*. *Reproduction Fertility and Development*, v. 20, p. 640–647, 2008.

CLARK, D. E.; TISDALL, D. J.; FIDLER, A. E.; MCNATTY, K. P. Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. *Journal of Reproduction and Fertility*, v. 106, p. 329-335, 1996.

CORNER, G. W. J. The histological dating of the human corpus luteum of menstruation. *The American Journal of Anatomy*, v.9, p. 377-401, 1956.

CORTVRINDT, R. G.; HU, Y.; LIU, J.; SMITZ, J. E. Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotrophin. *Fertility and Sterility*, v. 70, p.1114-1125, 1998.

CORTVRINDT, R. G.; SMITZ, J. E. J. Fluorescent probes allow rapid and precise recording of follicle density and staging in human ovarian cortical biopsy samples. *Fertility and Sterility*, v. 75, p. 588-593, 2001b.

CORTVRINDT, R.; SMITZ, J. E. J. *In vitro* follicle growth: Achievements in mammalian species. *Reproduction in Domestic Animals*, v. 36, p. 3-9, 2001a.

CORTVRINDT, R.; SMITZ, J. E.; VAN STEIRTEGHEM, A. C. Assesment of the need for follicle stimulating hormone in early preantral mouse follicle culture *in vitro*. *Human Reproduction*, v. 12, p. 759-768, 1997.

COSTA, A. P. R.; FAGUNDES-MOURA, C. R.; PEREIRA, V. M.; SILVA, L. F. M.; VIEIRA, A. R.; SANTOS, R. A. S; REIS, A. M. Angiotensin-(1–7): A novel peptide in the ovary. *Endocrinology*, v. 144, p. 1942-1948, 2003.

DANFORTH, D. R.; ARBOGAST, L. K.; GHOSH, S.; DICKERMAN, A.; ROFAGHA, R.; FRIEDMAN, C. I. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. *Biology of Reproduction*, v. 68, p. 1736-1741, 2003.

DAVOREN, J. B.; HSUEH, A. J. W. Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. *Biology of Reproduction*, v. 33, p. 37–52, 1985.

DELGADO, M.; GARRIDO, E.; MARTINEZ, C.; LECETA, J.; GOMARIZ, R. P. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptides (PACAP27) and (PACAP38) protect CD4+CD8+ thymocytes from glucocorticoid-induced apoptosis. *Blood*, v. 87, p. 5152–5161, 1996.

DEMEESTERE, I.; GERVY, C.; CENTNER, J.; DEVREKER, F.; ENGLERT, Y.; DELBAERE, A. Effect of insulin-like growth factor-I during preantral follicular culture on steroidogenesis, in vitro oocyte maturation, and embryo development in mice. *Biology of Reproduction*, v. 70, p. 1664-1669, 2004.

DISSEN, G. A.; ROMERO, C.; HIRSHFIELD, A. N.; OJEDA, S. R. Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology*, v. 142, p. 2078-2086, 2001.

DOLE, G.; NILSSON, E. E.; SKINNER, M. K. Glial-derived neurotrophic factor promotes ovarian primordial follicle development and cell–cell interactions during folliculogenesis. *Reproduction*, v. 135, p. 671-682, 2008.

DONG, J.; ALBERTINI, D. F.; NISHIMORI, K.; KUMAR, T. R.; LU, N.; MATZUK, M. M. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, v. 383, p. 531-535, 1996.

DRUMMOND, A. E. The role of steroids in follicular growth. *Reproductive Biology and Endocrinology*, v. 4, p. 1-11, 2006.

DRUMMOND, A. E.; LE, M. T.; ETHIER, J. F.; DYSON, M.; FINDLAY, J. K. Expression and localization of activin receptors, Smads, and beta glycan to the postnatal rat ovary. *Endocrinology*, v. 143, p. 1423-1433, 2002.

DUARTE, A. B. G.; CHAVES, R. N.; ARAÚJO, V. R.; CELESTINO. J. J. H.; SILVA, G. M.; LOPES C. A. P.; TAVARES L. M. T.; CAMPELLO, C. C.; FIGUEIREDO, J. R. Follicular interactions affect the *in vitro* development of isolated goat preantral follicles. *Zygote*, 2010. [no prelo].

DURLINGER, A. L. L.; VISSER, J. A.; THEMMEN, A. P. N. Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction*, v. 124, p. 601-609, 2002.

DURLINGER, A. L.; KRAMER, P.; KARELS, B.; DE JONG, F. H.; UILENBROEK, J. T.; GROOTEGOED, J. A.; THEMMEN, A. P. Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology*, v. 140, p. 5789-5796, 1999.

EKHOLM, C.; HIILENSJÖ, T.; MAGNUSSON, C.; ROSBERG, S. Stimulation and inhibition of rat oocyte meiosis by forskolin. *Biology of Reproduction*, v, 30, p. 537-43. 1984.

EPPIG, J. J.; SCHROEDER, A. C. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. *Biology of Reproduction*, v. 41, p. 268-276, 1989.

EPPIG, J. J.; DOWNS, S. M. Chemical signals that regulate mammalian oocyte maturation. *Biology of Reproduction*, v. 30, p. 1-11, 1984.

EPPIG, J. J. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, v. 122, n. 6, p. 829-838, 2001.

ERICKSON, G. F.; SHIMASAKI, S. The spatiotemporal expression pattern of the bonemorphogenetic proteinfamily inratovary cell types during the estrous cycle. *Reproductive Biology and Endocrinology*, v. 1, p. 9, 2003.

ERICKSON, G. F.; SHIMASAKI, S. The physiology of folliculogenesis: the role of novel growth factors. *Fertility and Sterility*, v. 76, p. 943-949, 2001.

ESPINOSA-CERVANTES, M. C.; ROSADO-GARCIA, A. Angiogenesis in reproductive physiology: follicular development, formation and maintenance of the corpus luteum. *Ginecología y Obstetricia de México*, v. 70, p. 17-27, 2002.

FATEHI, A. N.; VAN DEN HURK, R.; COLENBRANDER, B.; DAEMEN, A. J. J. M.; VAN TOL, H. T. A.; MONTEIRO, R. M.; ROELEN, B. A. J.; BEVERS, M. M. Expression of bone morphogenetic protein2 (BMP2), BMP4 and BMP receptors in the bovine ovary but absence of effects of BMP2 and BMP4 during IVM on bovine oocyte nuclear maturation and subsequent embryo development. *Theriogenology*, v. 63, p. 872-889, 2005.

FENG, P.; KNECHT, M.; CATT, K. Hormonal control of epidermal growth factor receptors by gonadotropins during granulosa cell differentiation. *Endocrinology*, v. 120, p. 1121-1126, 1987.

FERAL, C.; LEGALL, S.; LEYMARIE, P. Angiotensin II modulates steroidogenesis in granulosa and theca in the rabbit ovary: Its possible involvement in atresia. *European Journal of Endocrinology*, v. 133, p. 747-753, 1995.

FERNANDEZ, L.A.; TWICKLER, J., MEAD, A. Neovascularization produced by angiotensin II. *The Journal of Laboratory and Clinical Medicine*, v. 105, p. 141-145, 1985.

FERRARA, N. Vascular endothelial growth factor: basic science and clinical progress. *Endocrine Reviews*, v. 25, p. 581-611, 2004.

FERRARA, N.; DAVIS-SMYTH, T. The biology of vascular endothelial growth factor. *Endocrine Reviews*, v. 18, p. 4–25, 1997.

FERRARA, N.; FRANTZ, G.; LECOUTER, J.; DILLART-TELM, L.; PHAM, T.; DRAKSHARAPU, A.; GIORDANO, T.; PEALE, F. Differential expression of the angiogenic factor genes VEGF and EG-VEGF in normal and polycystic human ovaries. *The American Journal of Pathology*, v. 162, p. 1881-1893, 2003.

FIGUEIREDO, J.R.; RODRIGUES, A. P. R.; AMORIM, C. A.; SILVA, J. R. V. Manipulação de oócitos inclusos em folículos ovarianos pré-antrais – MOIFOPA. In: GONÇALVES, P. B. D.; FIGUEIREDO, J. R.; FREITAS, V. J. F (Eds.) *Biotécnicas aplicadas à reprodução animal*, São Paulo: Editora Roca, 2008, p. 303-327.

FILIPPATOS, G. S.; GANGOPADHYAY, N.; LALUDE, O.; PARAMESWARAN, N.; SAID, S. I.; SPIELMAN, W.; UHAL, B. D. Regulation of apoptosis by vasoactive peptides. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, v, 281 p. 749-761. 2001.

FLAWS, J. A.; DESANTI, A.; TILLY, K. I.; JAVID, R. 0.; KUGU, K.; JOHNSON, A. L.; HIRSHFIELD, A. N.; TILLY, J. L. Vasoactive intestinal peptide mediated suppression of apoptosis in the ovary: potential mechanisms of action and evidence of a conserved antiatretogenic role through evolution. *Endocrinology*, v. 136, p. 4351–4359, 1995.

FORNERIS, M. L.; AGUADO, L. I. Neonatal superior nerve transection disturbs the cyclic activity of the female rats. *The Journal of Steroid Biochemistry and Molecular Biology*, v, 82 p. 75-82. 2002.

FORTUNE, J. E. The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Animal Reproduction Science*, v. 78, p. 135-163, 2003.

FORTUNE, J. E.; KITO, S.; WANDJI, S. A.; SRSEN, V. Activation of bovine and baboon primordial follicles in vitro. *Theriogenology*, v. 49, p. 441-449, 1998.

FRASER, A.; EVAN, G. A license to kill. *Cell*, v. 85, p. 781–784, 1996.

FRASER, H. M.; LUNN, S. F. Angiogenesis and its control in the female reproductive system. *British Medical Bulletin*, v. 56, p. 787-797, 2000.

FREDERICKS, C. M.; LUNDQUIST, L. E.; MATHUR, R. S.; ASHTON, S. H.; LANDGREBE, S. C. Effects of vasoactive intestinal polypeptide upon ovarian steroids, ovum transport and fertility in the rabbit. *Biology of Reproduction*, v, 28 p. 1052-1060. 1983.

GALEATI, G.; SPINACI, M.; GOVONI,N.; ZANNONI, A.; FANTINATI, P.; SEREN, E.; TAMANINI, C. Stimulatory effects of fasting on vascular endothelial growth factor (VEGF) production by growing pig ovarian follicles. *Reproduction*, v. 126, p. 647–652, 2003.

GALLOWAY, S. M.; MCNATTY, K. P.; CAMBRIDGE, L. M.; LAITINEN, M. P. E.; JUENGEL, J. L.; JOKIRANTA, T. S.; MCLAREN, R. J.; LUIRO, K.; DODDS, K. G.; MONTGOMERY, G. W.; BEATTIE, A. E.; DAVIS, G. H.; RITVOS, O. Mutations in an oocyte-derived growth factor gene (BMP-15) cause increased ovulation rate and infertility in a dosagesensitive manner. *Nature Genetics*, v. 25, p. 279-83, 2000.

GAROR, R.; ABIR, R.; ERMAN, A.; FELZ, C.; NITKE, S.; FISCH, B. Effects of basic fibroblast growth factor on in vitro development of human ovarian primordial follicles. *Fertility and Sterility*, v. 91, p. 1967-1975, 2009.

GEORGE, F. W.; OJEDA, S. R. Vasoactive intestinal peptide induces aromatase activity before development of primary follicles or responsiveness to follicle-stimulating hormone. *Proceedings of the National Academy of Sciences of the United States of America*. v. 84, p. 5803-5807, 1987.

GIGLI, I.; CUSHMAN, R. A.; WAHL, C. M.; FORTUNE, J. E. Evidence for a role for anti-Mullerian hormone in the suppression of follicle activation in mouse ovaries and bovine ovarian cortex grafted beneath the chick chorioallantoic membrane. *Molecular Reproduction and Development*, v. 71, p. 480-488, 2005.

GIOMETTI, I. C.; BERTAGNOLLI, A. C.; ORNES, R. C.; COSTA, L. F. S.; CARAMBULA, S. F.; REIS, A. M,; OLIVEIRA, J. F.; EMANUELLI, I. P.; GONÇALVES, P. B. Angiotensin II reverses the inhibitory action produced by theca cells on bovine oocyte nuclear maturation. *Theriogenology*, v. 63, p. 1014-1025, 2005.

GLINKA, A.; WU, W.; DELIUS, H.; MONAGHAN, A. P.; BLUMENSTOCK, C.; NIEHRS, C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature*, v. 391, p. 357-362, 1998.

GOSPODAROWICZ, D.; BIALECKI, H. Fibroblast and epidermal growth factors are mitogenic agents for cultured granulose cells of rodent, porcine and human origin. *Endocrinology*, v. 104, p. 757-764, 1979.

GOSPODAROWICZ, D.; CHENG, J.; LUI, G.M.; BAIRD, A.; ESCH, F.; BOHLEN, P. Corpus luteum angiogenic factor isrelated to fibroblast growth factor. *Endocrinology*, v. 117, p. 2383-2391, 1985.

GOUGEON, A.; CHAINY, G. B. N. Morphometric studies of small follicles in ovaries of women at different ages. *Journal of Reproduction and Fertility*, v. 81, p. 433–442, 1987.

GOZES, I.; FRIDKINB, M.; HILL, J. M.; BRENNEMAN, D. E. Pharmaceutical VIP: prospects and problems. *Current Medicinal Chemistry*, v. 6, p. 1019-34. 1999.

GOZES, I.; TSAFRIRI, A. Detection of vasoactive intestinal peptide encoding messenger ribonucleic acid in the rat ovaries. *Endocrinology,* v. 119, p. 2606-2610; 1986.

GRAS, S.; HANNIBAL, J.; FAHRENKRUG, J. Pituitary adenylate cyclaseactivating polypeptide is an auto/paracrine stimulator of acute progesterone accumulation and subsequent luteinization in cultured periovulatory granulosa/lutein cells. *Endocrinology,* v, 140 p. 2199-2205, 1999.

GRASSELLI, F.; BASINI, G.; BUSSOLATI, S.; TAMANINI, C. Effects of VEGF and bFGF on proliferation and production of steroids and nitric oxide in porcine granulosa cells. *Reproduction in Domestic Animals*, v. 37, p. 362-368, 2002.

GRASSELLI, F.; BASINI, G.; TIRELLI, M.; CAVALLI, V.; BUSSOLATI, S.; TAMANINI, C. Angiogenic activity of porcinegranulosa cells cocultured with endothelial cells in amicrocarrier based three-dimensional fibrin gel. *Journal of Physiology and Pharmacology: an Official Journal of the Polish Physiological Society*, v. 54, p. 361-370, 2003.

GREENAWAY, J.; CENTRY, P. A.; FEIGE, J. J.; LAMARRE, J.; PETRIK, J. J. Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. *Biology of Reproduction*, v. 72, p. 1071-1078, 2005.

GREENAWAY, J.; CONNOR, K.; PEDERSEN, H. G.; COOMBER, B. L.; LAMARRE, J.; PETRIK, J. Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. *Endocrinology*, v. 145, p. 2896-2905, 2004.

GREENWALD, G. S.; MOOR, R. M. Isolation and preliminary characterization of pig primordial follicles. *Journal of Reproduction and Fertility*, v. 87, p. 561–571, 1989.

GUDERMANN, T.; NÜRNBERG, B.; SCHULTZ, G. Receptors and G proteins as primary components of transmembrane signal transduction. I. G-protein-coupled receptors: structure and function. *Journal of Molecular Medicine*. v. 73, p. 51-63, 1995.

GUGLIELMO, M. C.; RICCI, G.; CATIZONE, A.; BARBERI, M.; GALDIERI, M.; STEFANINI, M.; CANIPARI, R. The effect of hepatocyte growth factor on the initial stages of mouse follicle development. *Journal of Cellular Physiology*, 2010.[no prelo].

GULYAS, B. J.; HODGEN, G. D.; TULLNER, W. W.; ROSS, G. T. Effects of fetal or maternal hypophysectomy on endocrine organs and bodyweight in infant rhesusmonkeys (Macaca mulatta): with particular emphasis on oogenesis. *Biology of Reproduction,* v. 16, p. 216-27, 1977.

GUPTA, P. S. P.; NANDI, S.; RVINDRANATHA, B. M.; SARMA, P. V. *In vitro* culture of buffalo(*Bubalus bubalis*) preantral follicles. *Theriogenology*, v. 57, p. 1839–1854, 2002.

GUPTA, P. S. P.; RAMESH, H. S.; MANJUNATHA, B. M.; NANDI, S.; RAVINDRA, J. P. Production of buffalo embryos using oocytes from in vitro grown preantral follicles. *Zygote*, v. 16, p. 57-63, 2008.

GUTIERREZ, C. G.; RALPH, J. H.; TELFER, E. E.; WILMUT, I.; WEBB, R. Growth and antrum formation of bovine preantral follicles in long-term culture in vitro. *Biology of Reproduction*, v. 62, p. 1322-1328, 2000.

GUTIERREZ-CAÑAS, I.; RODRIGUEZ-HENCHE, N.; BOLANOS, O.; CARMENA, M. J.; PRIETO, J. C.; JUARRANZ, M. G. VIP and PACAP are autocrinefactors that protect the androgen-independentprostate cancer cell line PC-3 fromapoptosis induced by serum withdrawal. *British journal of pharmacology*, v. 139, p. 1050–1058, 2003.

HALPIN, D. M.; JONES, A.; FINK, G.; CHARLTON, H. M. Postnatal ovarian follicle development in hypogonadal (hpg) and normal mice and associated changes in the hypothalamic-pituitary ovarian axis**,** *Journal of Reproduction and Fertility,* v. 77, p. 287-296, 1986.

HANAHAN, D. Signaling vascular morphogenesisand maintenance. *Science*, v. 277, p. 55-60, 1997.

HANRAHAN, J. P.; GREGAN, S. M.; MULSANT, P.; MULLEN, M.; DAVIS, G. H.; POWELL, R.; GALLOWAY, S. M. Mutations in the Genes for Oocyte-Derived Growth Factors GDF9 and BMP15 Are Associated with Both Increased Ovulation Rate and Sterility in Cambridge and Belclare Sheep (*Ovis aries*). *Biology of Reproduction,* v. 70, p. 900-909, 2004.

HARATA, T.; ANDO, H.; IWASE, A.; NAGASAKA, T.; MIZUTANI, S.; KIKKAWA, F. Localization of angiotensin II, theAT1 receptor, angiotensin-converting enzyme, aminopeptidase A, adipocyte-derived leucineaminopeptidase, and vascular endothelial growth

factorin the human ovary throughout the menstrual cycle. *Fertility and Sterility*, v. 86, p. 433-439, 2006.

HARTSHORNE, G. *In vitro* culture of ovarian follicles. *Reviews of Reproduction,* v. 2, p. 94-104, 1997.

HAZZARD, T. M.; MOLSKNESS, T. A.; CHAFFIN, C. L.; STOUFFERRL. Vascular endothelial growth factor (VEGF) and angiopoiet in regulation by gonadotropin and steroids in macaque granulosa cells during the periovulatory interval. *Molecular Human Reproduction*, v.5, p.1115-1121, 1999.

HAZZARD, T. M.; STOUFFER, R. L. Angiogenesis in ovarian follicular and luteal development. *Baillière's best practice & research. Clinical Obstetrics & Gynaecology*, v. 4, p. 883-900, 2000.

HILL, J. L.; HAMMAR, K.; SMITH, P. J.; GROSS, D. J.; Stage-dependent effects of epidermal growth factor on Ca2þ efflux in mouse oocytes. *Molecular Reproduction and Development*, v. 53, p. 244-253, 1999.

HILLENSJÖ, T.; EKHOHN, C.; AHRÉN, K. Role of cyclic AMP in oocyte maturation and glycolysis in the pre-ovulatory rat follicle. *Acta Endocrinologica*, v, 87 p. 377-88. 1978.

HILLIER, S. G. Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Human Reproduction*, v. 9, p. 188–91, 1994.

HIRAO, Y.; NAGAI, T.; KUBO, M.; MIYANO, T.; MIYAKE, M.; KATO, S. *In vitro* growth and maturation of pig oocytes. *Journal of Reproduction and Fertility*, v. 100, p. 333–339, 1994.

HIRSHFIELD, A. N. Development of follicles in mammalian ovary. *International Review of Cytology*, v. 124, p. 43–101, 1991.

HOVATTA, O.; SILYE, R.; ABIR, R.; KRAUSZ, T.; WINSTON, R. M. L. Extracellular matriz improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Human Reproduction*, v. 12, p. 1032-1036, 1997.

HREINSSON, J. G.; SCOTT, J. E.; RASMUSSEN, C.; SWAHN, M. L.; HSUEH, A. J.; HOVATTA, O. Growth differentiation factor-9 promotes the growth, development, and

survival of human ovarian follicles in organ culture. *Journal of Clinical Endocrinology and Metabolism*, v. 87, p. 316-321, 2002.

HU, D. E.; HILEY, C. R.; FAN, T. P. Comparative studies of the angiogenic activity of vasoactive intestinal peptide, endothelins-1 and -3 and angiotensin II in a rat sponge model. *British Journal of Pharmacology*, v. 117, n. 3, p. 545-551, 1996.

HULSHOF, S. C. J.; FIGUEIREDO, J. R.; BECKERS, J. F.; BEVERS, M. M.; VANDERSTICHELE, H.; VAN DEN HURK, R. Bovine preantral follicles and activin: immunohistochemistry for activin and activin receptor and the effect of bovine activin A *in vitro*. *Theriogenology*, v. 48, p. 133–142, 1997.

HULSHOF, S. C. J. Bovine preantral follicles and their development *in vitro*; thesis, Utrecht, 1995.

HULSHOF, S. C. J.; DIJKSTRA, G.; VAN DER BEEK, E. M.; BEVERS, M. M.; FIGUEIREDO, J. R.; BECKERS, J. F.; VAN DEN HURK, R. Immunocytochemical localization of vasoactive intestinal peptide and neuropeptide y in the bovine ovary. *Biology of Reproduction*, v. 50, p. 553–560, 1994.

HULSHOF, S. C. J.; FIGUEIREDO, J. R.; BEKERS, J. F.; BEVERS, M. M.;VAN DEN HURK, R. Isolation and Characterization of preantral follicles from foetal bovine ovaries. *The Veterinary Quarterly*, v. 2, p. 78-80, 1994.

HUSAIN, A.; BUMPUS, F. M.; SILVA, P.; SPETH, R. C. Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries. *Proceedings of the National Academy of Sciences of the United States of America*, v. 84, n. 8, p. 2489-2493, 1987.

HUTT, K. J.; MCLAUGHLIN, E. A.; HOLLAND, M. K. KIT/KIT ligand in mammalian oogenesis and folliculogenesis: roles in rabbit and murine ovarian follicle activation and oocyte growth. *Biology of Reproduction*, v. 75, p. 421–433, 2006.

IIJIMA, K.; JIANG, J. Y.; SHIMIZU,T.; SASADA, H.; SATO, E. Acceleration of follicular development by administration of vascular endothelial growth factor in cycling female rats. *The Journal of Reproduction and Development*, v. 51, p. 161–168, 2005.

ITOH, H.; MUKOYAMA, M.; PRATT, R. E.; GIBBONS, G. H.; DZAU, V. J. Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. *The Journal of Clinical Investigation*, v. 91, p. 2268-2274, 1993.

ITOH, T.; KACCHI, M.; ABE, H.; SENDAI, Y.; HOSHI, H. Growth, antrum formation, and estradiol production of bovine preantral follicles cultured in a serum-free medium. *Biology of Reproduction*, v. 67, p. 1099-1105, 2002.

JANA, B.; DZIENIS, A.; PAŃCZYSZYN, J.; ROGOZIŃSKA, A.; WOJTKIEWICZ, J.; SKOBOWIAT, C.; MAJEWSKI, M. Denervation of the porcine ovaries performed during the early luteal phase influenced morphology and function of the gonad. *Reproductive Biology*, v. 5, p. 69-82. 2005.

JEWGENOW, K. Impact of peptide growth factors on the culture of small preantral follicles of domestic cats. *Theriogenology*, v. 45, p. 889-895, 1996.

JEWGENOW, K.; STOLTE, M. Isolation of preantral follicles from nondomestic cats - viability and ultrastructural investigations. *Animal Reproduction Science*, v. 44, p. 183-193, 1996.

JIANG, J. Y.; MACCHIARELLI, G.; TSANG, B. K.; SATO, E. Capillary angiogenesis and degeneration in bovineovarian antral follicles. *Reproduction*, v. 125, p. 211-223, 2003.

JOHNSON, A. L.; LI, Z.; GIBNEY, J. A.; MALAMED, S. Vasoactive intestinal peptide-induced expression of cytochrome p450 cholesterol side-chain cleavage and 17a-hydroxylase enzyme activity in hen granulosa cells. *Biology of Reproduction*, v. 51, p. 327–333, 1994.

JOHNSON, A. L.; TILLY, J. L. Effects of vasoactive intestinal peptide on steroid secretion and plasminogen activator activity in granulose cells of the hen. *Biology of Reproduction*, v. 38, p. 296–303, 1988.

JOUBERT, F. J.; STRYDOM, D. J. Snake venom. Theamino acid sequence of protein A from Dendroaspis polylepis polylepis (black mamba) venom. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, v. 361, p. 1787-1794, 1980.

JUENGEL, J. L.; BODENSTEINER, K. J.; HEATH, D. A.; HUDSONA, N. L.; MOELLER, C. L.; SMITH, P.; GALLOWAY, S. M.; DAVIS, G. H.; SAWYER, H. R.; MCNATTY, K. P.

Physiology of GDF9 and BMP15 signalling molecules. *Animal Reproduction Science*, v. 82-83, p. 447-460, 2004.

JUENGEL, J. L.; HUDSON, N. L.; HEATH, D. A.; SMITH, P.; READER, K. L.; LAWRENCE, S. B.; O'CONNELL, A. R.; LAITINEN, M. P.; CRANFIELD, M.; GROOME, N. P.; RITVOS, O.; MCNATTY, K. P. Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biology of Reproduction*, v. 67, p. 1777-1789, 2002.

JUENGEL, J. L.; MCNATTY, K. P. The role of proteins of the transforming growth factor-b superfamily in the intraovarian regulation of follicular development. *Human Reproduction Update*, v. 1, p. 144-161, 2005.

JUNQUEIRA, L. C.; CARNEIRO, J. *Biologia celular e molecular*. Oitava edição. Rio de Janeiro: Guanabara Koogan 332p, 2005.

KASSON, B. G.; MEIDAN, R.; DAVOREN, J. B.; HSUEH, A. J. W. Identification of subpopulations of rat granulosa cells: sedimentation properties and hormonal responsiveness. *Endocrinology*, v, 7 p. 1027-1034. 1985.

KEZELE, P.; NILSSON, E. E.; SKINNER, M. K. Insulin but not insulin-like growth factor-1 promotes the primordial to primary follicle transition. *Molecular and Cellular Endocrinology*, v. 192, p. 37-43. 2002.

KEZELE, P. R.; AGUE, J. M.; NILSSON, E.; SKINNER, M. K. Alteration in the ovarian transcriptome during primordial follicle assembly and development. *Biology of Reproduction*, v. 72, p. 241-255, 2005.

KIDDER, G. M.; MHAWI, A. A. Gap junctions and ovarian folliculogenesis. *Reproduction*, v. 123, p. 613-620, 2002.

KIKUCHI, N.; ANDOH, K.; ABE, Y.; YAMADA, K.; MIZUNUMA, H.; IBUKI, Y. Inhibitory action of leptin on early follicular growth differs in immature and adult female mice. *Biology of Reproduction*, v. 65, p. 66–71, 2001.

KLAGSBRU, N. M.; D`AMORE, P. A. Vascular endothelial growth factor and its receptors. *Cytokine & Growth Factor Reviews*, v. 7, p. 259-270, 1996.

KNECHT, M.; RANTA, T.; CATT, K. J. Granulosa cell differentiation *in vitro*: induction and maintenance of follicle-stimulating hormone receptors by adenosine 3_,5_-monophosphate. *Endocrinology*, v.113, p. 949-956, 1983.

KNIGHT, P. G.; GLISTER, C. TGF-b superfamily members and ovarian follicle development. *Reproduction*, v. 132, p. 191-206, 2006.

KOBAYASHI, J.; MIZUNUMA, H.; KIKUCHI, N.; LIU, X.; ANDOH, K.; ABE, Y.; YOKOTA, H.; YAMADA, K.; IBUKI, Y.; HAGIWARA, H**.** Morphological assessment of the effect of growth hormone on preantral follicles from 11-day-old mice in an in vitro culture system. *Biochemical and Biophysical Research Communications*, v. 268, p. 36–41, 2000.

KOWALEWSKI, M. P.; DYSON, M. T.; BOOS, A.; STOCCO, D. M. Vasoactive intestinal peptide (VIP)-mediated expression and function of steroidogenic acute regulatory protein (StAR) in granulosa cells. *Molecular and Cellular Endocrinology*, v, 328, p. 93-103. 2010.

KREUZER, H.; MASSEY, A. *Engenharia genética e biotecnologia*, 434p. 2. ed. Porto Alegre: Artmed, 2002.

KUO, T. C.; ENDO, K.; DHARMARAJAN, A. M.; MIYAZAKI, T.; ATLAS, S. J.; WALLACH, E. E. Direct effect of angiotensin II on *in vitro* perfused rabbit ovary. *Journal of Reproduction and Fertility*, v. 92, p. 469-474, 1991.

LACOMBE, A.; LELIEVRE, V.; ROSELLI, C. E.; MULLER, J. M.; WASCHEK, J. A.; VILAIN, E. Lack of vasoactive intestinal peptide reduces testosterone levels and reproductive aging in mouse testis. *The Journal of Endocrinology*, v, 194, n. 1, p. 153-160. 2007.

LARA, H. E.; MCDONALD, J. K.; AHMED, C. E.; OJEDA, S. R. Guanethidine-mediated destruction of ovarian sympathetic nerves disrupts ovarian development and function in rats. *Endocrinology*, v, 127 p. 2199-2209. 1990.

LATINI, S.; CHIARPOTTO, M.; MUCIACCIA, B.; VACCARI, S.; BARBERI, M.; GUGLIELMO, M. C.; STEFANINI, M.; CECCONI, S.; CANIPARI, R. Inhibitory effect of pituitary adenylate cyclase activating polypeptide on the initial stages of rat follicle development. *Molecular and Cellular Endocrinology*, v, 320 p. 34-44. 2010.

LECOUTER, J.; FERRARA, N. Endocrine gland-derivedVEGF and the emerging hypothesis of organ-specific regulation of angiogenesis. *Nature Medicine*, v. 8, p. 913-917, 2002.

LECOUTER, J.; KOWALSKI, J.; FOSTER, J.; HASS, P.; ZHANG, Z.; DILLARD-TELM, L.; FRANTZ, G.; RANGELL, L.; DEGUZMAN, L.; KELLER, G. A.; PEALE, F.; GURNEY, A.; HILLAN, K. J.; FERRARA, N. Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature*, v. 412, p. 877-884, 2001.

LECOUTER, J.; LIN, R.; FRANTZ, G.; TEJADA, M.; PEALE, F.; HILLAN, K.J.; FERRARA, N. The EG-VEGF homologue, Bv8, is a potent angiogenic factor. Localization of Bv8 receptors to endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America, v.* 100, p. 2685-2690, 2003.

LEDDA, S.; BOGLIOLO, L.; LEONI, G.; CALVIA, P.; NAITANA, S. Influence of vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP) and insulin-like growth factor-I (IGF-I) on *in vitro* maturation of prepubertal and adult sheep oocytes. *Zygote,* v, 4 p. 343-348, 1996.

LEE, J.; PARK, H. J.; CHOI, H. S.; KWON, H. B.; ARIMURA, A.; LEE, B. J.; CHOI, W. S.; CHUN, S. Y. Gonadotropin stimulation of pituitary adenylate cyclase-activating polypeptide (PACAP) messenger ribonucleic acid in the rat ovary and the role of PACAP as a follicle survival factor. *Endocrinology,* v, 140 p. 818-826, 1999.

LEE, W. S.; OTSUKA, F.; MOORE, R. K.; SHIMASAKI, S. Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biology of Reproduction*, v. 65, p. 994-999, 2001.

LEE, W. S.; YOON, S. J.; YOON, T. K.; CHA, K. Y.; LEE, S. H.; SHIMASAKI, S.; LEE, S.; LEE, K. A. Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. *Molecular Reproduction and Development*, v. 69, p. 159-163, 2004.

LI, H. K.; KUO, T. Y.; YANG, H. S.; CHEN, L. R.; LI, S. S.; HUANG, H. W. Differential gene expression of bone morphogenetic protein 15 and growth differentiation factor 9 during *in vitro* maturation of porcine oocytes and early embryos. *Animal Reproduction Science,* v. 103, p. 312-322, 2008.

LI, X. F.; AHMED, A. Dual role of angiotensin II in the human endometrium. *Human Reproduction*, v. 11, p. 95-108, 1996.

LI, Y. H.; JIAO, L. H.; LIU, R. H.; CHEN, X. L.; WANG, H.; WANG, W. H. Localization of angiotensin II in pig ovary and its effects on oocyte maturation *in vitro*. *Theriogenology*, v. 61, p. 447-459, 2004.

LIMA-VERDE, I. B.; MATOS, M. H. T.; BRUNO, J. B.; MARTINS, F. S.; SANTOS R. R.; BÁO, S. N.; LUQUE, M. C. A.; VIEIRA, G. A. B.; SILVEIRA, E. R.; RODRIGUES, A. P. R.; FIGUEIREDO, J. R.; OLIVEIRA, M. A. L.; LIMA, P. F. Effects of α-tocopherol and ternatin antioxidants on morphology and activation of goat preantral follicles *in vitro* cultured. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v. 61, n. 1, p. 57-65, 2009.

LIMA-VERDE, I. B.; ROSSETTO, R.; MATOS, M. H. T.; CELESTINO, J. J. H.; BRUNO, J. B.; SILVA, C. M. G.; FAUSTINO, L. R.; MORORÓ, M. B. S.; ARAÚJO, V. R.; CAMPELLO, C. C.; FIGUEIREDO, J. R. Androstenedione and follicle stimulating hormone involvement in the viability and development of goat preantral follicles *in vitro*. *Animal reproduction*, v. 7, p. 80-89, 2010b.

LIMA-VERDE, I. B.; SARAIVA, M. V. A.; MATOS, M. H. T.; BRUNO, J. B.; TENÓRIO, S. B.; MARTINS, F. S.; CUNHA, L. D.; NAME, K. P. O.; BÁO, S. N., CAMPELLO, C. C.; FIGUEIREDO, J. R. Interaction between estradiol and follicle stimulating hormone promotes *in vitro* survival and development of caprine preantral follicles. *Cells Tissues Organs*, v. 191, p. 240-247, 2010a.

LIN, R.; LECOUTER, J.; KOWALSKI, J.; FERRARA, N. Characterization of EGVEGF signaling in adrenal cortex capillary endothelial cells. *The Journal of Biological Chemistry*, v. 277, p. 8724-8729, 2002.

LIU, K.; RAJAREDDY, S.; LIU, L.; JAGARLAMUDI, K.; BOMAN, K.; SELSTAM, G.; REDDY, P. Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer. *Developmental Biology*, v. 299, p. 1-11, 2006.

LIU, X.; ANDO, H. K.; YOKOTA, H.; KOBAYASHI, J.; ABE, Y.; YAMADA, K.; MIZUNUMA, H.; IBUKI, Y.. Effects of growth hormone, activin, and follistatin on the development of preantral follicle from immature female mice. *Endocrinology*, v. 139, p. 2342-2347, 1998.

LIU, Y. X.; KASSON, B. G.; DAHL, K. D.; HSUEH, A. J. Vasoactive intestinal peptide stimulates plasminogen activator activity by cultured rat granulosa cells and cumulus-oocyte complexes. *Peptides*, v, 8 p. 29-33. 1987.

LIU, Y. X.; NY, T.; SARKAR, D.; LOSKUTOFF, D.; HSUEH, A. J. W. Identification and regulation of tissue plasminogen activator activity in rat cumulus-oocyte complexes. *Endocrinology*, v, 119 p. 1578-1587. 1986.

LONERGAN, P.; CAROLAN, C.; VAN LANGENDONCKT, A.; DONNAY, I.; KHATIR, H.; MERMILLOD, P. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. *Biology of Reproduction,* v. 54, p. 1420-1429, 1996.

LOPES, C. A. P.; SANTOS, R. R.; CELESTINO, J. J. H.; MELO, M. A.; CHAVES, R. N.; CAMPELLO, C. C.; SILVA, J. R.; BÁO, S. N.; JEWGENOW, K.; FIGUEIREDO, J. R.; Short-term preservation of canine preantral follicles: Effects of temperature, medium and time. *Animal Reproduction Science*, v. 115, p. 201–14, 2009.

LOUHIO, H.; HOVATTA, O.; SJÖBERG, J.; TUURI, T. The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. *Molecular Human Reproduction*, v. 6, p. 694-698, 2000.

LUCCI, C. M.; AMORIM, C. A.; BÁO, S. N.; FIGUEIREDO, J. R.; RODRIGUES, A. P. R.; SILVA, J. R.; GONÇALVES, P. B. D. Effect of the interval of serial sections of ovarian in the tissue chopper on the number of isolated caprine preantral follicles. *Animal Reproduction Science*, v. 56, p.39–49, 1999.

LUCCI, C. M.; AMORIM, C. A.; RODRIGUES, A. P. R.; FIGUEIREDO, J. R.; BÁO, S. N.; SILVA, J. R. V.; GONÇALVES, P. B. D. Study of preantral follicles population in situ and after mechanical isolation from caprine ovaries at different reproductive stages. *Animal Reproductive Sciences*, v. 56, p. 223–236, 1999.

LUCCI, C. M.; SILVA, R. V.; CARVALHO, C. A.; FIGUEIREDO, R.; BÁO, N. Light microscopical and ultrastrutural characterization of goat preantral follicles. *Small Ruminant Research : the Journal of the International Goat Association*, v. 41, p. 61–69, 2001.

LUTZ, E. M.; SHEWARD, W. J.; WEST, K. M.; MORROW, J. A.; FINK, G.; HARMAR, A. J. The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Letters*, v. 334, p. 3-8, 1993.

MACCHIARELLI, G.; NOTTOLA, S. A.; VIZZA, E.; FAMILIARI, G.; KIKUTA, A.; MURAKAMI, T.; MOTTA, P. M. Microvasculature of growing and atretic follicles in the

rabbit ovary: a SEM study of corrosion casts. *Archives of Histology and Cytology*, v. 56, p. 1-12, 1993.

MAGALHÃES, D. M.; ARAÚJO, V. R.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SILVA, R. C.; LUCCI, C. M.; BÁO, S. N.; CAMPELLO, C. C.; FIGUEIREDO, J. R.. Different Follicle-Stimulating Hormone (FSH) sources influence caprine preantral follicle viability and development *in vitro*. *Brazilian Journal of Veterinary Research and Animal Science*, v. 46, p. 378-386, 2009a.

MAGALHÃES, D. M.; ARAÚJO, V. R.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SILVA, R. C.; LUCCI, C. M.; BÁO, S. N.; CAMPELLO, C. C.; FIGUEIREDO, J. R., Impact of pituitary FSH purification on *in vitro* early folliculogenesis in goats. *Biocell*, v. 33, p. 91-97, 2009b.

MAGALHÃES, D. M.; DUARTE, A. B. G.; ARAÚJO, V. R.; BRITO, I. R.; SOARES, T. G.; LIMA, I. M. T.; LOPES, C. A. P.; CAMPELLO, C. C.; RODRIGUES, A. P. R.; FIGUEIREDO, J. R. In vitro production of a caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. *Theriogenology*, v. 75, p. 182-188, 2011.

MAGALHÃES, D. M.; FERNANDES; D. D., MORORÓ, M. B. S.; SILVA, C. M. G.; RODRIGUES, G. Q.; BRUNO, J. B.; MATOS, M. H. T.; CAMPELLO, C. C.; FIGUEIREDO, J. R. Effect of the medium replacement interval on the viability, growth and *in vitro* maturation of isolated caprine and ovine pre-antral follicles. *Reproduction in Domestic Animals*, 2010. [no prelo].

MAO, J.; SMITH, M. F.; RUCKER, E. B.; WU, G. M.; MCCAULEY, T. C.; CANTLEY, J. T. C.; PRATHER, R. S.; DIDION, B. A.; DAY, B. N. Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosal cell proliferation and suppression of apoptosis in vitro. *Journal of animal science*, v. 82, p. 1967-1975, 2004.

MARTELLI, A.; BERNABÒ, N.; BERARDINELLI, P.; RUSSO, V.; RINALDI, C.; DI GIACINTO, O.; MAURO, A.; BARBONI, B. Vascular supply as a discriminating for pig preantral follicle selection. *Reproduction*, v. 137, p. 45-58, 2009.

MARTINS, F. S.; CELESTINO, J. J. H.; SARAIVA, M. V. A.; CHAVES, R. N.; ROSSETTO, R.; SILVA, C. M. G.; LIMA-VERDE, I. B.; LOPES, C. A. P.; CAMPELLO, C. C.; FIGUEIREDO, J. R. Interaction between growth differentiation factor 9, insulin-like growth factor I and growth hormone on the *in vitro* development and survival of goat

preantral follicles. *Brazilian Journal of Medical and Biological Research*, v. 43, p. 728-736, 2010.

MARTINEZ-MADRID, B.; CAMBONI, A.; DOLMANS, M. M.; NOTTOLA, S.; LANGENDONCKT, A. V.; DONNEZ, J. Apoptosis and ultrstructural assessment after cryopreservation of whole human varies with their vascular pedicle. *Fertility and Sterility*, v. 87, p.1153-1165, 2007.

MARTINS, F. S.; CELESTINO, J. J. H.; SARAIVA, M. V. A.; MATOS, M. H. T.; BRUNO, J. B.; ROCHA-JUNIOR, C. M. C.; LIMA-VERDE, I. B.; LUCCI, C. M.; BÁO, S. N.; FIGUEIREDO, J. R. Growth and differentiation factor-9 stimulates activation of goat primordial follicles *in vitro* and their progression to secondary follicles. *Reproduction Fertility and Development*, v. 20, p. 916-924, 2008.

MARTINS, F. S.; VAN DEN HURK, R.; SANTOS, R. R.; SILVA, J. R. V.; MATOS, M. H. T.; CELESTINO, J. J. H.; RODRIGUES, A. P. R.; PESSOA, C.; FERREIRA, F. V. A.; FIGUEIREDO, J. R. Development of goat primordial follicles after in vitro culture of ovarian tissue in Minimal Essential Medium supplemented with coconut water. *Animal Reproduction*, v. 2, n. 2, p. 106-113, 2005.

MASUDA, Y.; TAKATSU, Y.; TERAO, Y.; KUMANO, S.; ISHIBASHI, Y.; SUENAGA, M.; ABE, M.; FUKUSUMI, S.; WATANABE, T.; SHINTANI, Y.; YAMADA, T.; HINUMA, S.; INATOMI, N.; OHTAKI, T.; ONDA, H.; FUJINO, M. Isolation and identification of EG-VEGF/ prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochemical and Biophysical Research Communications*, v. 293, p. 396-402, 2002.

MATOS, M. H. T.; LIMA-VERDE, I. B.; BRUNO, J. B.; LOPES, C. A. P.; MARTINS, F. S.; SANTOS, K. D. B.; ROCHA, R. M. P.; SILVA, J. R. V.; BÁO, S. N.; FIGUEIREDO, J. R. Follicle stimulating hormone and fibroblast growth factor-2 interact and promote goat primordial follicle development *in vitro*. *Reproduction Fertility and Development*, v. 19, p. 677-684, 2007c.

MATOS, M. H. T.; LIMA-VERDE, I. B.; LUQUE, M. C. A.; MAIA JR, J. E.; SILVA, J. R.; CELESTINO, J. J. H.; MARTINS, F. S.; BÁO, S. N.; LUCCI, C. M.; FIGUEIREDO, J. R. Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability *in vitro*. Zygote, v. 15, p. 173–182, 2007a.

MATOS, M. H. T.; SILVA, J. R. V.; RODRIGUES, A. P. R.; FIGUEIREDO, J. R.; Técnicas para avaliação da qualidade de folículos ovarianos pré-antrais cultivados *in vitro*. *Revista Brasileira de Reprodução Animal*, v.31, n.4, p.433-442, 2007d.

MATOS, M. H. T.; VAN DEN HURK, R.; LIMA-VERDE, I. B.; LUQUE, M. C. A.; SANTOS, K. D. B.; MARTINS, F. S.; BÁO, S. N.; LUCCI, C. M.; FIGUEIREDO, J. R. Effects of fibroblast growth factor-2 on the *in vitro* culture of caprine preantral follicles. *Cell Tissues Organs*, v. 186, p. 112-120, 2007b.

MATOS, M. H. T.; VAN DEN HURK, R.; MARTINS, F. S.; SANTOS, R. R.; LUQUE, M. C. A.; SILVA, J. R. V.; CELESTINO, J. J. H.; BÁO, S. N.; FIGUEIREDO, J. R. Histological and ultrastructural features of caprine preantral follicles after *in vitro* culture in the presence or absence of indole-3-acetic acid. *Animal Reproduction*, v. 3, n. 4, p. 415-422, 2006.

MATTIOLI, M.; BARBONI, B.; TURRIANI, M.; GALEATI, G.; ZANNONI, A.; CASTELLANI, G.; BERARDINELLI, P.; SCAPOLO, P. A. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. *Biology of Reproduction*, v. 65, p. 1014-1019, 2001.

MAYERHOFER, A.; DISSEN, G. A.; COSTA, M.; OJEDA, S. R. A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. *Endocrinology*, v. 138, p. 3320–3329, 1997.

MCGEE, E. A.; CHUN, S. Y.; LAI, S.; HE, Y.; HSUEH, A. J. Keratinocyte growth factor promotes the survival, growth, and differentiation of preantral ovarian follicles. *Fertility and Sterility*, v. 71, p. 732-738, 1999.

MCGHEE, E. A.; HSUEH, A. J. Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews*, v. 21, p. 200–214, 2000.

MCNATTY, K. P.; FIDLER, A. E.; JUENGEL, J. L.; QUIRKE, L. D.; SMITH, P. R.; HEATH, D. A.; LUNDY, T.; OCONNELL, A.; TIDDALL, D. J. Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology*, v. 163, p. 11–20, 2000.

MÉDURI, G.; CHARNAUX, N.; DRIANCOURT, M. A.; COMBETTES, L.; GRANET, P.; VANNIER, B.; LOOSFELT, H.; MIGROM, E. Follicle-stimulating hormone receptors in oocytes? *Journal of Clinical Endocrinology & Metabolism*, v. 87, p. 2266-2276, 2002.

MERY, L.; LEFEVRE, A.; BENCHAIB, M.; DEMIRCI, B.; SALLE, B.; GUERIN, J-F.; LORNAGE, J. Follicular growth *in vitro*: Detection of growth differentiation factor 9 (GDF9) and Bone morphogenetic protein 15 (BMP15) during *in vitro* culture of ovine cortical slices. *Molecular Reproduction and Development*, v. 74, p. 767-774, 2007.

MINEGISHI, T.; TANO, M.; KISHI, H.; KAMEDA, T.; MIYAMOTO, K. Follicle-stimulating hormone regulation on its receptor messenger ribonucleic acid levels in cultured rat granulosa cells. *Biochimica et Biophysica Acta*, v. 1359, p. 165-173, 1997.

MITSUBE, K.; MIKUNI, M.; MATOUSEK, M.; ZACKRISSON, U.; BRANNSTROM, M. Role of the angiotensin II system in regulation of ovulation and blood flow in the rat ovary. *Reproduction*, v. 125, p. 425-435, 2003.

MIYATA, A.; ARIMURA, A.; DAHL, R. R.; MINAMINO, N.; UEHARA, A.; JIANG, L.; CULLER, M. D.; COY, D. H. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochemical and Biophysical Research Communications*, v, 164 p. 567-574. 1989.

MOLLAY, C.; WECHSELBERGER, C.; MIGNOGNA, G.; NEGRI, L.; MELCHIORRI, P.; BARRA, D.; KREIL, G. Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats. *European Journal of Pharmacology*, v. 374, p. 189-196, 1999.

MOORE, R. K.; SHIMASAKI, S. Molecular biology and physiological role of the oocyte factor, BMP-15. *Molecular and Cellular Endocrinology*, v. 234, p. 67-73, 2005.

MORBECK, D. E.; FLOWERS, W. L.; BRITT, J. H. Response of porcine granulosa cells isolated from primary and secondary follicles to FSH, 8-bromo-cAMP and EGF *in vitro*. *Journal of Reproduction and Fertility*, v. 99, p. 577-584, 1993.

MOTRO, B.; BERNSTEIN, A. Dynamic changes in ovarian c-kit and syeel expression during the estrous reproductive cycle. *Developmental Dynamics*, v. 197, p. 69-79, 1993.

MURUVI, W.; PICTON, H. M.; RODWAY, R. G.; JOYCE, I. M. *In vitro* growth of oocytes from primordial follicles isolated from frozen-thawed lamb ovaries. *Theriogenology*, v. 64, p. 1357-1370, 2005.

NAKAMURA, M.; NAKAMURA, K.; IGARASHI, S.; TANO, M.; MIYAMOTO, K. Interaction between activin A and cAMP in the induction of FSH receptor in cultured rat granulosa cells. *Journal of Endocrinology*, v. 147, p. 103-110, 1995.

NANDI, S.; RAVINDRANATHA, B. M.; GUPTA, P. S. P.; RAGHU, H. M.; SARMA, P. V. Developmental competence and post-thaw survivability of buffalo embryos produced *in vitro*: effect of growth factors in oocyte maturation medium and of embryo culture system. *Theriogenology*, v. 60, p. 1621-1631, 2003.

NIELSEN, A. H.; HAGEMANN, A.; SVENSTRUP, B.; NIELSEN, J.; POULSEN, K. Angiotensin-II receptor density in bovine ovarian follicles relates to tissue renin and follicular size. *Clinical and Experimental Pharmacology & Physiology*, v. 21, p. 463-469, 1994.

NILSSON, E. E.; KEZELE, P.; SKINNER, M. K. Leukemia inhibiting factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Molecular and Cellular Endocrinology*, v. 188, p. 65–73, 2002.

NILSSON, E. E.; PARROTT, J. A.; SKINNER, M. K. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Molecular and Cellular Endocrinology*, v. 175, p. 123–130, 2001.

NILSSON, E. E.; SKINNER, M. K. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biology of Reproduction*, v. 69, p. 1265-1272, 2003.

NILSSON, E. E.; SKINNER, M. K. Growth and differentiation factor-9 stimulates progression of early primary but not primordial rat ovarian follicle development. *Biology of Reproduction*, v. 67, p. 1018–1024, 2002.

NILSSON, E. E.; SKINNER, M. K. Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Molecular and Cellular Endocrinology*, v. 214, p. 19–25, 2004.

NUTTINCK, F.; COLLETTE, L.; MASSIP, A.; DESSY, F. Histologic and autoradiographic study of the *in vitro* effects of FGF-2 and FSH on isolated bovine preantral follicles: preliminary investigation. *Theriogenology*, v. 45, p. 1235-1245, 1996.

O'BRIEN, M. J.; PENDOLA, J. K.; EPPIG, J. J. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biology of Reproduction*, v. 68, p. 1682-1686, 2003.

O'SHAUGHNESSY, P. J.; DUDLEY, K.; RAJAPAKSHA, W. R. Expression of follicle stimulating hormone-receptor mRNA during gonadal development. *Molecular and Cellular Endocrinology*, v. 125, 169-175, 1996.

O'SHAUGHNESSY, P. J.; MCLELLAND, D.; MCBRIDE, M. W. Regulation of luteinizing hormone-receptor and follicle stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. *Biology of Reproduction*, v. 57, p. 602-608, 1997.

OBERMÜLLER, N.; SCHLAMP, D.; HOFFMANN, S.; GENTILI, M.; INAGAMI, T.; GRETZ, N.; WEIGEL, M. Localization of the mRNA for the angiotensin II receptor subtype 2 (AT2) in follicular granulosa cells of the rat ovary by nonradioactive in situ hybridization. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, v. 46, p. 865-870, 1998.

OJEDA, S. R.; LARA, H. E. The role of the sympathetic nervous system in the regulation of ovarian function. *In*: PIRKE, K. M.; WUTTKE, W.; SCHWEIGER, U. (Eds), *The Menstrual Cycle and Its Disorders*. Berlin: Springer-Verlag, 1989, p.26–32.

OJEDA, S. R.; LARA, H. E.; AHMED, C. E. The potential relevance of vasoactive intestinal peptide to ovarian physiology. *In*: ADASHI, E. (Eds), *Putative Intraovarian Regulators*. NewYork: Thieme Inc., 1989, p 52–60.

OJEDA, S. R.; ROMERO, C.; TAPIA, V.; DISSEN, G. A. Neurotrophic and cell-cell dependent control of early follicular development. *Molecular and Cellular Endocrinology*, v. 163, p. 67-71, 2000.

OJEDA, S. R.; WHITE, S. S.; AGUADO, L. I.; ADVIS, J. P.; ANDERSEN, J. M. Abdominal vagotomy delays the onset of puberty and inhibits ovarian function in the female rat. *Neuroendocrinology*, v, 36 p. 261-267. 1983.

OKTAY, K.; BRIGGS, D.; GOSDE, R.G. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *The Journal of Clinical Endocrinology and Metabolism*, v. 82, p. 3748–3751, 1997.

ORISAKA, M.; ORISAKA, S. J.; JIN-YI, C. J.;WANG,Y.; KOTSUJI, F.; ANDTSANG, B. K. Growth differentiation factor-9 is anti-apoptotic during follicular development from preantral to early antral stage. *Molecular Endocrinology*, v. 20, p. 2456–2468, 2006.

OTANI, A.; TAKAGI, H.; OH, H.; SUZUMA, K.; MATSUMURA, M.; IKEDA, E.; HONDA, Y. Angiotensin II stimulated vascular endothelial growth factor expression in bovine retinal pericytes. *Investigative Ophthalmology & Visual Science*, v. 41, p. 1192- 1199, 2000.

OTANI, N.; MINAMI, S.; YAMOTO, M.; SHIKONE, T.; OTANI, H.; NISHIYAMA, R.; OTANI, T.; NAKANO, R. The vascular endothelial growth factor/fms-like tyrosine kinase system in human ovary during the menstrual cycle and early pregnancy. *The Journal of Clinical Endocrinology and Metabolism*, v. 84, p. 3845- 3851, 1999.

OTSUKA, F.; YAO, Z.; LEE, T. H.; YAMAMOTO, S.; ERICKSON, G. F.; SHIMASAKI, S. Bone morphogenetic protein-15: identification of target cells and biological functions. *The Journal of Biological Chemistry*, v. 275, p. 39523-39528, 2000.

OTTESEN, B; FAHRENKRUG, J. Vasoactive intestinal polypeptide and other preprovasoactive intestinal polypeptide-derived peptides in the female and male genital tract: localization, biosynthesis, and functional and clinical significance. *American journal of Obstetrics and Gynecology,* v, 172 p. 1615–1631. 1995.

PANGAS, S. A.; RADEMAKER, A. W.; FISHMAN, D. A.; WOODRUFF, T. K. Localization of the activin signal transduction components in normal human ovarian follicles: implications for autocrine and paracrine signaling in the ovary. *Journal of Clinical Endocrinology and Metabolism,* v. 87, p. 2644–2657, 2002.

PARK, H. J.; LEE, J.; WANG, L.; PARK, J. H.; KWON, H. B.; ARIMURA, A.; CHUN, S. Y. Stage-specific expression of pituitary adenylate cyclase-activating polypeptide type I receptor messenger ribonucleic acid during ovarian follicle development in the rat. *Endocrinology*, v. 141, p. 702-709, 2000.

PARRA, C.; FIEDLER, J. L.; LUNAL, S. L.; GREINER, M.; PADMANABHAN, V.; LARA, H. E. Participation of vasoactive intestinal polypeptide in ovarian steroids production during the rat estrous cycle and in the development of estradiol valerate-induced polycystic ovary. *Reproduction*, v. 133, p. 147–154, 2007.

PARROT, J. A.; SKINNER, M. K. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinology*, v. 140, p. 4262-4271, 1999.

PAUL, M.; MEHR, A. P.; KREUTZ, R. Physiology of Local Renin-Angiotensin Systems. *Physiological Reviews*, v. 86, p. 747-803, 2006.

PELLICER, A.; PALUMBO, A.; DECHERNEY, A. H.; NAFTOLIN, F. Blockage of ovulation by an angiotensin antagonist. *Science*, v. 240, p. 1660-1661, 1988.

PEPLING, M. E.; SPRADLING, A. C. Female mouse germ cells form synchronously dividing cysts. *Development*, v. 125, p. 3323-3328, 1998.

PEPPER, M. S.; FERRARA, N.; ORCI, L.; MONTESANO, R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochemical and Biophysical Research Communications*, v. 189, p. 824-831, 1992.

PEROLLET, C.; HAN, Z.C.; SAVONA, C.; CAEN, J.P.; BIKFALVI, A. Platelet factor 4 modulates fibroblast growth factor 2 (FGF-2) activity and inhibits FGF-2 dimerization. *Blood*, v. 91, p. 3289-3299, 1998.

PETRIK, J. J.; GENTRY, P. A.; FEIGE, J. J.; LAMARRE, J. Expression and localization of thrombospondin-1 and -2 and their cell surface receptor, CD36, during rat follicular development and formation of the corpus luteum. *Biology of Reproduction*, v. 67, p. 1522-1531, 2002.

PICTON, H. M.; HARRIS, S. E.; MURUVI, W.; CHAMBERS, E. L. The *in vitro* growth and maturation of follicles. *Reproduction*, v. 136, p. 703–715, 2008.

PICTON, H.; BRIGGS, D.; GOSDEN, R. The molecular basis of oocyte growth and development. *Molecular and Cellular Endocrinology*, v. 145, p. 27–37, 1998.

PLENDL, J. Angiogenesis and vascular regression in the ovary. *Anatomia, Histologia, Embryologia*, v. 29, p. 257–66, 2000.

PORTELA, V. M.; GONÇALVES, P. B. D.; VEIGA, A. M.; NICOLA, E.; BURATINI, J. J.; PRICE, C. A. Regulation of angiotensin type 2 receptor in bovine granulosa cells. *Endocrinology*, v. 149, p. 5004–5011, 2008.

PUCELL, A. G.; HODGES, J. C.; SEN, I.; BUMPUS, F. M.; HUSAIN, A. Biochemical properties of the ovarian granulosa type 2- angiotensin II receptor. *Endocrinology*, v. 128, p. 1947-1959, 1991.

QU, J.; GODIN, P. A.; NISOLLE, M.; DONNEZ, J. Distribution of epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation. *Human Reproduction*, v. 15, p. 302-310, 2000.

QUINTANA, R.; KOPCOW, L.; SUELDO, C.; MARCONI, G.; RUEDA, N. G.; BARAÑAO, R. I. Direct injection of vascular endothelial growth factor into the ovary of mice promotes follicular development. *Fertility and Sterility*, v. 82, p. 1101-1105, 2004.

RABINOVICI, J.; GOLDSMITH, P. C.; ROBERTS, V. J.; VAUGHAN, J.; VALE, W.; JAFFE, R. B. Localization and secretion of inhibin/activin subunits in the human and subhuman primate fetal gonads. *Journal of Clinical Endocrinology and Metabolism*, v. 73, p. 1141–1149, 1991.

REDMER, D. A.; DORAISWAMY, V.; BORTNEM, B. J.; FISHER, K.; JABLONKA-SHARIFF, A.; GRAZUL-BILSKA, A. T.; REYNOLDS, L. P. Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. *Biology of Reproduction*, v. 65, p. 879-889, 2001.

REDMER, D.A.; REYNOLDS, L.P. Angiogenesis in the ovary. *Reviews of Reproduction*, v. 1, p. 182-192, 1996.

REICH, R.; MISKIN, R.; TSAFRIRI, A. Follicular plasminogen activator: involvement in ovulation. *Endocrinology*, v, 116 p. 516-521, 1985.

REYNOLDS, L. P.; REDMER, D. A. Expression of the angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in the ovary. *Journal of animal science*, v. 76, p. 1671–81, 1998.

REYNOLDS, L.P.; REDMER, D.A. Utero-placental vascular development and placental function. *Journal of Animal Science*, v. 73, p. 1839-1851, 1995.

ROBERTS, A.E.; ARBOGAST, L.K.; FRIEDMAN, C.I.; COHN, D.E.; KAUMAYA, P.T.; DANFORTH, D.R. Neutralization of endogenous vascular endothelial growth factor depletes primordial follicles in the mouse ovary. *Biology of Reproduction*, v. 76, p. 218-223, 2007.

ROBERTS, R. D.; ELLIS, R. C. L. Mitogenic effects of fibroblast growth factors on chicken granulosa and theca cells in vitro. *Biology of Reproduction*, v. 61, p. 1387-92, 1999.

ROBINSON, R. S.; WOAD, K. J.; HAMMOND, A. J.; LAIRD, M.; HUNTER, M. G.; MANN, G. E. Angiogenesis and vascular function in the ovary Focus on Vascular Function in Female Reproduction. *Reproduction*, v. 138, p. 869–881, 2009.

RODRIGUES, G. Q.; SILVA, C. M. G.; FAUSTINO, L. R.; BRUNO, J. B.; PINTO, L. C.; LOPES, C. A. P.; CAMPELLO, C. C.; FIGUEIREDO, J. R. Efeito de diferentes concentrações de hormônio folículo-estimulante recombinante sobre o desenvolvimento in vitro de folículos pré-antrais caprinos e ovinos isolados. *Acta Veterinaria Brasilica*, v.4, p. 144-152, 2010.

ROMANO, M.; KRAUS, E. R.; BOLAND, C. R.; COFFEY, R. J. Comparison between transforming growth factor alpha and epidermal growth factor in the protection of rat gastric mucosa against drug-induced injury. *The Italian Journal of Gastroenterology*, v. 26, p. 223-228, 1994.

ROSSETTO, R.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SARAIVA, M. V. A.; MARTINS, F. S.; FAUSTINO, L. R.; ARAÚJO, V. R.; SILVA, C. M. G.; NAME, K. P. O.; BÁO, S. N.; CAMPELLO, C. C.; FIGUEIREDO, J. R.; BLUME, H. Interaction between ascorbic acid and follicle-stimulating hormone maintains follicular viability after long-term *in vitro* culture of caprine preantral follicles. *Domestic Animal Endocrinology*, v. 37, p. 112-123, 2009.

ROY, S. K.; KOLE, A. R. Ovarian transforming growth factor-beta (TGF-beta) receptors: in vitro effects of follicle stimulating hormone, epidermal growth factor and TGF beta on receptor expression in human preantral follicles. *Molecular Human Reproduction*, v. 4, p. 207-214, 1998.

ROY, S. K.; TREACY, B. J. Isolation and long-term culture of human preantral follicles. *Fertility and Sterility*, v.59, p. 783-791, 1993.

RÜSSE, I. Oogenesis in cattle and sheep. *Bibliotheca Anatomica*, v. 24, p. 77-92, 1983.

SAID, S.I. Molecules that protect: the defenseof neurons and other cells. *The Journal of Clinical Investigation*, v. 97, p. 2163–2164, 1996.

SAID, S.I.; DICKMAN, K. Pathways of inflammation and cell death in the lung: modulation by vasoactive intestinal peptide. *Regulatory Peptides*, v. 93, p. 21–29, 2000.

SALEHNIA, M.; MOGHADAM, E. A.; VELOJERDI, M. R. Ultrastructure of follicles after vitrification of mouse ovarian tissue. *Fertility and Sterility*, v.78, p.644-645, 2002.

SARAIVA, M. V. A.; CELESTINO, J. J. H.; ARAÚJO, V. R.; CHAVES, R. N.; ALMEIDA, A. P.; LIMA-VERDE, I. B.; DUARTE, A. B. G.; SILVA, G. M.; MARTINS, F. S.; BRUNO, J. B.; MATOS, M. H. T.; CAMPELLO, C. C.; SILVA, J. R. V.; FIGUEIREDO, J. R. Expression of follicle-stimulating hormone receptor (FSH-R) in goat ovarian follicles and the impact of sequential culture medium on *in vitro* development of caprine preantral follicles. *Zygote*, 2010a. [no prelo].

SARAIVA, M. V. A.; CELESTINO, J. J. H.; CHAVES, R. N.; MARTINS, F. S.; BRUNO, J. B.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SILVA, G. M.; PORFIRIO, E. P.; BÁO, S. N.; CAMPELLO, C. C.; SILVA, J. R. V.; FIGUEIREDO, J. R.. Influence of different concentrations of LH and FSH on *in vitro* caprine primordial ovarian follicle development. *Small Ruminant Research*, v. 78, p. 87-95, 2008.

SARAIVA, M. V. A.; ROSSETTO, R.; BRITO, I. R.; CELESTINO, J. J. H.; SILVA, C. M. G.; FAUSTINO. L. R.; ALMEIDA, A. P.; BRUNO, J. B.; MAGALHÃES, D. M.; RODRIGUES, G. Q.; MATOS, M. H. T.; CAMPELLO, C. C.; SILVA, J. R. V.; FIGUEIREDO, J. R. Dynamic medium produces caprine embryo from preantral follicles grown in vitro. *Reproductive Sciences*, v. 17, p. 1135-1143, 2010b.

SASTRY, K. S. R.; SMITH, A. J.; KARPOVA, Y.; DATTA, S. R.; KULIK, G. Diverse antiapoptotic signaling pathways activated by vasoactive intestinal polypeptide, epidermal growth factor, and phosphatidylinositol 3-kinase in prostate cancer cells converge on bad. *The Journal of Biological Chemistry*, v. 281, p. 20891–20901, 2006.

SCHAMS, D.; AMSELGRUBER, W.; EINSPANIER, R.; SINOWATZ, F.; GOSPODAROWICZ, D. Localization and tissue concentration of basic fibroblast growth factor in the bovine corpus luteum. *Endocrine*, v. 2, p. 907-912, 1994.

SCHAMS, D.; BERISHA, B.; KOSMANN, M.; AMSELGRUBER, W. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal

tissue during different stages of estrous cycle and pregnancy. *Domestic Animal Endocrinology*, v. 22, p. 51-72, 2002.

SCHAMS, D.; KOSMANN, M.; BERISHA, B.; AMSELGRUBER, W. M.; MIYAMOTO, A. Stimulatory and synergistic effects of luteinising hormone and insulin-like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulose cells. *Experimental and Clinical Endocrinology & Diabetes: Official Journal, German Society of Endocrinology and German Diabetes Association*, v. 109, p. 155-162, 2001.

SCHAUSER, K. H.; NIELSEN, A. H.; WINTHER, H.; DANTZER, V.; POULSEN, K. Localization of the renin-angiotensin system in the bovine ovary: cyclic variation of the angiotensin II receptor expression. *Biology of Reproduction*, v. 65, p. 1672-1680, 2001.

SCHECTERSON, L. C.; MCKNIGHT, G. S. Role of cyclic adenosine 3',5'- monophosphate-dependent protein kinase in hormone-stimulated /3-endorphin secretion in AtT20 cells. *Molecular Endocrinology*, v. 5, p. 170-178. 1991.

SCHMIDT, G.; JÖRGENSEN, J.; KANNISTO, P.; LIEDBERG, F.; OTTESEN, B.; OWMAN, C. Vasoactive intestinal polypeptide in the PMSG-primed immature rat ovary and its effect on ovulation in the isolated rat ovary perfused in vitro. *Journal of Reproduction and Fertility*, v. 90, p. 465-472, 1990.

SCHOTANUS, K.; HAGE, W. J.; VANDERSTICHELE, H.; VAN DEN HURK, R. Effects of conditioned media from murine granulosa cell lines on the growth of isolated bovine preantral follicles. *Theriogenology*, v. 48, p. 471–483, 1997.

SCHWEITZ, H.; PACAUD, P.; DIOCHOT, P.; MOINIER, D.; LADZUNSKI, M. MIT(1), a black mamba intestinal toxin with a new and highly potent activity on intestinal contraction. *FEBS Letters*, v. 461, p. 183-188, 1999.

SERAFIM, M. K.; ARAUJO, V. R.; SILVA, G. M.; DUARTE, A. B.; ALMEIDA, A. P.; CHAVES, R. N.; CAMPELLO, C. C.; LOPES, C. A.; FIGUEIREDO, J. R.; SILVA, L. D. Canine preantral follicles cultured with various concentrations of follicle-stimulating hormone (FSH). *Theriogenology*, v. 74, n. 5, p. 749-55, 2010.

SHARMA, G. T.; DUBEY, P. K.; MEUR, S. K. Survival and developmental competence of buffalo preantral follicles using three dimensional collagen gel culture system. *Animal Reproduction Science*, v. 114, p. 115-124, 2009.

SHIBUYA, M. Role of VEGF–Flt receptor system in normal and tumor angiogenesis. *Advances in Cancer Research*, v. 67, p. 281–316, 1995.

SHIKONE, T.; YAMOTO, M.; NAKANO, R. Follicle-stimulating hormone induces functional receptors for basic fibroblast growth factor in rat granulosa cells. *Endocrinology*, v. 131, p. 1063-1068, 1992.

SHIMASAKI, S.; MOORE, R. K.; OTSUKA, F.; ERICKSON, G. F. The bone morphogenetic protein system in mammalian reproduction. *Endocrine Reviews*, v. 25, p. 72-101, 2004.

SHIMIZU, T. Promotion of ovarian follicular development by injecting vascular endothelial growth factor (VEGF) and growth differentiation factor 9 (GDF-9) genes. *The Journal of Reproduction and Development*, v. 52, p. 23-32, 2006.

SHIMIZU, T.; JIANG, J. Y.; IIJIMA, K.; MIYABAYASHI, K.; OGAWA,Y.; SASADA, H.; SATO, E. Induction of follicular development by direct single injection of vascular endothelial growth factor gene fragments into the ovary of miniature gilts. *Biology of Reproduction*, v. 69, p. 1388–1393, 2003.

SHIMIZU, T.; JIANG, J. Y.; SASADA, H.; SATO, E. Changes of messenger RNA expression of angiogenic factors and related receptors during follicular development in gilts. *Biology of Reproduction*, v. 67, p. 1846-1852, 2002.

SHUTTLEWORTH, G.; PIPKIN, F. B.; HUNTER, M. G. *In vitro* development of pig preantral follicles cultured in a serum-free medium and the effect of angiotensin II. *Reproduction*, v. 123, p. 807-818, 2002.

SILVA, C. M. G.; MATOS, M. H. T.; RODRIGUES, G. Q.; FAUSTINO, L. R.; PINTO, L. C.; CHAVES, R. N.; ARAÚJO, V. R.; CAMPELLO, C. C.; FIGUEIREDO, J. R. *In vitro* survival and development of goat preantral follicles in two different oxygen tensions. *Animal Reproduction Science*, v.117, p. 83-89, 2010.

SILVA, J. R. V. Growth factors in gota ovarios and the role of ativina-A in the development of esrly-staged follicles. *Phd Thesis*. Utrecht University, Faculty of Veterinary Medicine, 142, 2005.

SILVA, J. R. V.; THARASANIT, T.; TAVERNE, M. A. M.; VAN DER WEIJDEN, G. C.; SANTOS, R. R.; FIGUEIREDO, J. R.; VAN DEN HURK, R. The activin-follistatin system

and *in vitro* early follicle development in goats. *Journal of Endocrinology*, v. 189, p. 113-125, 2006a

SILVA, J. R. V.; VAN DEN HURK, R.; COSTA, S. H. F.; ANDRADE, E. R.; NUNES, A. P. A.; FERREIRA, F. V. A.; LÔBO, R. N. B.; FIGUEIREDO, J. R. Survival and growth of goat primordial follicles after *in vitro* culture of ovarian cortical slices in media containing coconut water. *Animal Reproduction Science*, v. 81, p. 273-286, 2004a.

SILVA, J. R. V.; VAN DEN HURK, R.; FIGUEIREDO, J. R. Expression of mRNA and protein localization of epidermal growth factor and its receptor in goat ovaries. *Zygote*, v. 14, p. 107-117, 2006c.

SILVA, J. R. V.; VAN DEN HURK, R.; MATOS, M. H. T.; SANTOS, R. R.; PESSOA, C.; MORAES, M. O.; FIGUEIREDO, J. R. Influences of FSH and EGF on primordial follicles during *in vitro* culture of caprine ovarian cortical tissue. *Theriogenology*, v. 61, p. 1691–1704, 2004c.

SILVA, J. R. V.; VAN DEN HURK, R.; VAN TOL, H. T. A.; ROELEN, B. A. J.; FIGUEIREDO, J. R. The Kit ligand/c-Kit receptor system in goat ovaries: gene expression and protein localization. *Zygote*, v. 14, p. 317–328, 2006b.

SILVA, J. R. V.; VAN DEN HURK, R.; VAN TOL, H. T. A.; ROELEN, B. A. J.; FIGUEIREDO, J. R. Expression of Growth Differentiation Factor 9 (GDF9), Bone Morphogenetic Protein 15 (BMP15), and BMP Receptors in the Ovaries of Goats. *Molecular Reproduction and Development*, v. 70, p. 11–19, 2004b.

SILVA, J. R.; FERREIRA, M. A. L.; COSTA, S. H. F.; SANTOS, R. R.; CARVALHO, F. C. A.; RODRIGUES, A. P. R.; LUCCI, C. M.; BÁO, S. N.; FIGUEIREDO, J. R. Degeneration rate of preantral follicles in the ovaries of goats. *Small Ruminant Research: the Journal of the International Goat Association*, v. 43, p. 203–209, 2002.

SINGH, B.; RUTLEDGE, J. M.; ARMSTRONG, D. T. Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles. *Molecular Reproduction and Development*, v. 40, p. 391-399, 1995.

SMITH, S. K. Angiogenesis and reproduction. *British Journal of Obstetrics and Gynaecology*, v. 108, p. 777-783, 2001.

SMITH, S. S.; OJEDA, S. R. Neonatal release of gonadotropins is essential for development of ovarian follicle-stimulating hormone receptor. *Biology Reproduction* v. 34. p. 219-227, 1986.

SMITZ, J.; CORTVRINDT, R.; HU, Y.; VANDERSTICHELE, H. Effects of recombinant activin A on in vitro culture of mouse preantral follicles. *Molecular Reproduction and Development*, v. 50, p. 294–304. 1998.

SPEARS, N.; MURRAY, A. A.; ALISSON, V.; BOLAND, N. I.; GOSDEN, R. G. Role of gonadotrofins and ovarian steroids in the development of mouse follicles in vitro. *Journal of Reproduction and Fertility*, v. 113, p. 19–26, 1998.

SPENGLER, D.; WAEBER, C.; PANTALONI, C.; HOLSBOER, F.; BOCKAERT, J.; SEEBURG, P. H.; JOURNOT, L. Differential signal transduction by five splice variants of the PACAP receptor. *Nature*, v. 365, p. 170-175, 1993.

STALDEMANN, C.; LASSMANN, H. Detection of apoptosis in tissue sections. *Cell and Tissue Research*, v. 301, p. 19-31, 2000

STECKELINGS, U. M.; KASCHINA, E.; UNGER, Th. The AT2 receptor- A matter of love and hate. *Peptides*, v. 26, p. 1401-1409, 2005.

STEEL, R. G. D.; TORRIE, J. H.; DICKEY, D. A. 'Principles and Procedures of Statistics: A Biometrical Approach, 3rd edn. NewYork: McGraw- Hill, 1997.

STEFANELLO, J. R.; BARRETA, M. H.; PORCIUNCULA, P. M.; ARRUDA, J. N.; OLIVEIRA, J. F.; OLIVEIRA, M. A.; GONÇALVES, P. B. Effect of angiotensin II with follicle cells and insulin-like growth factor-I or insulin on bovine oocyte maturation and embryo development. *Theriogenology*, v. 66, p. 2068-2076, 2006.

STOUFFER, R. L.; MARTÍNEZ-CHEQUER, J. C.; MOLSKNESS, T. A.; XU, F. HAZZARD, T. M. Regulation and action of angiogenic factors in the primate ovary. *Archives of Medical Research*, v.32, p. 567-575, 2001.

TAMANINI, C.; DE AMBROGI, M. Angiogenesis in developing follicle and corpus luteum. *Reproduction in Domestic Animals*, v.39, p. 206-216, 2004.

TANO, M.; MINEGISHI, T.; NAKAMURA, K.; KARINO, S.; IBUKI, Y.; MIYAMOTO, K. Transcriptional and post-transcriptional regulation of FSH receptor in rat granulosa cells by cyclic AMP and activin. *Journal of Endocrinology*, v. 153, p. 465-473, 1997.

TAYLOR, P. D.; HILLIER, S. G.; FRASER, H. M. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. *The Journal of Endocrinology*, v.183, p. 1-17, 2004.

TEIXEIRA FILHO, F. L.; BARACAT, E. C.; LEE, T. H.; SUH, C. S.; MATSUI, M.; CHANG, R. J.; SHIMASAKI, S.; ERICKSON, G. F. Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology and Metabolism*, v. 87, p. 1337-1344, 2002.

TELFER, E. E. The development of methods for isolation and culture of preantral follicles from bovine and porcine ovaries. *Theriogenology*, v. 45, p. 101–110, 1996.

THOMAS, F. H.; CAMPBELL, B. K.; ARMSTRONG, D. G.; TELFER, E. E. Effects of IGF-I bioavailability on bovine preantral follicular development in vitro. *Reproduction*, v.133, p. 1121-1128, 2007.

TILLY, J. L.; BILLIG, H.; KOWALSKI, K. I.; HSUEH, A. J. Epidermal growth factor and basic fibroblast growth factor supress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine-kinase-depedent mechanism. *Molecular Endocrinology*, v. 6, p. 1942-1950, 1992.

TILLY, J. L.; KOWALSKI, K. I.; SCHOMBERG, D. W.; HSUEH, A. J. W. Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 aromatase. *Endocrinology*, v. 131, p. 1670-1676, 1992.

TISDALL, D. J.; WATANABE, K.; HUDSON, N. L.; SMITH, P.; MCNATTY, K. P. FSH receptor gene expression during ovarian follicle development in sheep. *Journal of Endocrinology*. v. 15, p. 273-281, 1995.

TORNELL, J.; CARLSSON, B.; HILLENSJO, T. Vasoactive intestinal peptide stimulates oocyte maturation, steroidogenesis, and cyclic adenosine 3',5'-monophosphate production in isolated preovulatory rat follicles. *Biology of Reproduction*, v. 39, p. 213–220, 1988.

TOYODA, T.; NAKAMURA, K.; YAMADA, K.; THANSEEM, I.; ANITHA, A.; SUDA, S.; TSUJII, M.; IWAYAMA, Y.; HATTORI, E.; TOYOTA, T.; MIYACHI, T.; IWATA, Y.; SUZUKI, K.; MATSUZAKI, H.; KAWAI, M.; SEKINE, Y.; TSUCHIYA, K.; SUGIHARA, G.; OUCHI, Y.; SUGIYAMA. T.; TAKEI, N.; YOSHIKAWA, T.; MORI, N. SNP analyses of growth factor genes EGF, TGF-b1, and HGF reveal haplotypic association of EGF with autism. *Biochemical and Biophysical Research Communications*, v. 360, p. 715-720, 2007.

TRZECIAK, W. H.; SCHMID, C. E.; SIMPSON, E.; OJEDA, S. R. Vasoactive intestinal peptide induces the synthesis of the cholesterol side-chain cleavage enzyme complex in cultured rat ovarian granulosa cells. *Proceedings of the National Academy of Sciences of the United States of America*, v. 83, p. 7490-7494. 1986.

VACCARI, S.; LATINI, S.; BARBERI, M.; TETI, A.; STEFANINI, M.; CANIPARI, R. Characterization and expression of different pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptors in rat ovarian follicles. *The Journal of Endocrinology*, v. 191, p. 287–299, 2006.

VAN DEN HURK, R.; ABIR, R.; TELFER, E. E.; BEVERS, M. M. Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. *Human Reproduction Update*, v. 6, p. 457–74, 2000.

VAN DEN HURK, R.; BEVERS, M. M.; BECKERS, J. F. *In vivo* and *in vitro* development of preantral follicles. *Theriogenology*, v. 47, p.73–82, 1997.

VAN DEN HURK, R.; BEVERS, M. M.; DIELEMAN, S. J. In: JOY, K. P.; KRISHNA, A.; HALDAR C. (Eds.), *Comparative endocrinology and reproduction*. New Delhi: Narosa Publishing House, 1999, p. 296–312.

VAN DEN HURK, R.; SPEK, E. R.; HAGE, W. J.; FAIR, T.; RALPH, J. H.; SCHOTANUS, K. Ultrastructure and viability of isolated bovine preantral follicles. *Human Reproduction Update*, v. 4, p. 833–841, 1998.

VAN DEN HURK, R.; ZHAO, J. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology*, v. 63, p. 1717-1751, 2005.

VAN TOL, H. T.; BEVERS, M. M. Theca cells and theca-cell conditioned medium inhibit the progression of FSH-induced meiosis of bovine oocytes surrounded by cumulus cells

connected to membrana granulosa. *Molecular Reproduction and Development*, v. 51, p. 315–321, 1998.

VAN WEZEL, I. L.; UMAPATHYSIVAM, K.; TILLEY, W. D.; RODGERS, R. J. Immunohistochemical localization of basic fibroblast growyh factor in bovine ovarian follicles. *Molecular and Cellular Endocrinology*, v. 115, p. 133-140, 1995.

VAN WEZEL, I.; RODGERS, R. J. Morphological characterization of primordial follicles and their environment *in vivo*. *Biology of Reproduction*., v. 55, p. 1003–1010, 1996.

VAUDRY, D.; GONZALEZ, B. J.; MAGALI, B.; PAMANTUNG, T. F.; FONTAINE, M.; FOURNIER, A.; VAUDRY, H. The neuroprotective effect of pituitary adenylate cyclase-activating polypeptide on cerebellar granule cells is mediated through inhibition of the CED3-related cysteine protease caspase-3/CPP32. *Proceedings of the National Academy of Sciences of the United States of America*, v. 97, p. 13390-13395, 2000.

VERNON, R. K.; SPICER, L. J. Effects of basic fibroblast growth factor and heparin on follicle-stimulating hormone-induced streroidogenesis by bovine granulosa cells. *Journal of Animal Science*, v. 72, p. 2696-702, 1994.

WALSH, D. A.; HU, D. E.; WHARTON, J.; CATRAVAS, J. D.; BLAKE, D. R.; FAN, T. P. Sequencial development of angiotensin receptors and angiotensin I converting enzyme during angiogenesis in the rat subcutaneous sponge granuloma. *British Journal of Pharmacology*, v. 120, p. 1302-1311, 1997.

WALTON, M. R.; DRAGUNOW, M. Is CREB a key to neuronal survival?. *Trends Neurosci*, v. 23, p. 48-53, 2000.

WANDJI, S. A.; EPPIG, J. J.; FORTUNE, J. E. FSH and growth factors affect the growth and endocrine function in vitro of granulosa cells of bovine preantral follicles. *Theriogenology*, v. 45, p. 817-832, 1995.

WANDJI, S. A.; SRSEN, V.; NATHANIELSZ, P. W.; EPPIG, J. J.; FORTUNE, J. E. Initiation of growth of baboon primordial follicles *in vitro*. *Human Reproduction*, v. 12, p. 1993- 2001, 1997.

WANDJI, S. A.; SRSEN, V.; VOSS, A. K.; EPPIG, E. E.; FORTUNE, J. E. Initiation *in vitro* of growth of bovine primordial follicles. *Biology of Reproduction*, v. 55, p. 942-948, 1996.

WANDJI, S.; FORTIER, M. A.; SIRARD, M. Differential response to gonadotrophins and prostaglandin E2 in ovarian tissue during prenatal and postnatal development in cattle. *Biology of Reproduction*, v. 46, p. 1034-1041, 1992.

WANG, C.; LEUNG, A. Gonadotropins regulate plasminogen activator production by rat granulosa cells. *Endocrinology*, v, 112 p. 1201-1207. 1983.

WANG, J.; ROY, S. K. Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: modulation by follicle-stimulating hormone. *Biology of Reproduction*, v. 70, p. 577–585, 2004.

WU, J.; BENJAMIN, R. E.; CARRELL, D. T. *In vitro* growth, maturation, fertilization, and embryonic development of oocytes from porcine preantral follicles. *Biology of Reproduction*, v. 64, p. 375-381, 2001.

WU, J.; TIAN, Q. Role of follicle stimulating hormone and epidermal growth factor in the development of porcine preantral follicle *in vitro*. *Zygote*, v. 15, p. 233–240, 2007.

WULFF, C.; WIEGAND, S. J.; SAUNDERS, P. T.; SCOBIE, G. A.; FRASER, H. M.. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40). *Endocrinology*, v. 142, p. 3244–3254, 2001.

XIAO, S.; ROBERTSON, D. M.; FINDLAY, J. K. Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. *Endocrinology*, v. 131, p. 1009-1016, 1992.

XU, Z.; GARVERICK, H. A.; SMITH, G. W.; SMITH, M. F.; HAMILTON, S. A.; YOUNGQUIST, R. S. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biology Reproduction*, v. 53, p. 951-957, 1995.

YAMAMOTO, S.; KONISHI, I.; NANDU, K.; KOMATSU, T.; MANDAI, M. KURODA, H.; MATSUSHITA, K.; MORI, T. Immunohistochemical localization of basic fibroblast growth factor (bFGF) during folliculogenesis in the human ovary. *Gynecological Endocrinology*, v. 11, p. 223-230, 1997.

YAMAMOTO, S.; KONISHI, I.; TSURUTA, Y.; NANBU, K.; MANDAI, M.; KURODA, H.; MATSUSHITA, K.; HAMID, A.A.; YURA, Y.; MORI, T. Expression of vascular

endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary. *Gynecological endocrinology: the Official Journal of the International Society of Gynecological Endocrinology*, v. 11, p. 371-381, 1997.

YANG, M. Y.; FORTUNE, J. E. Vascular endothelialgrowth factor stimulates the primary to secondary follicle transition in bovine *in vitro*. *Molecular Reproduction and Development*, v. 74, p. 1095-1104, 2007.

YOON, S-J.; KIM, K-H.; CHUNG, H-M.; CHOI, D-H.; LEE, W-S.; CHA, K-Y.; LEE, K-A. Gene expression profiling of early follicular development in primordial, primary, and secondary follicles. *Fertility and Sterility*, v. 85, p. 193-203, 2006.

YOSHIDA, H.; TAKAKURA, N.; NATAOKA, H.; KUNISADA, T.; OKAMURA, H.; NISHIKAWA, S. I. Stepwise requirement of c-Kit tyrosine kinase in mouse ovarian follicle development. *Developmental Biology*, v. 184, p. 122-137, 1997.

YOSHIMURA, Y. The ovarian renin-angiotensin system in reproductive physiology. *Frontiers in Neuroendocrinology*, v. 18, n. 3, p. 247-291, 1997.

YOSHIMURA, Y.; KARUBC, M.; KOYAMA, N.; SHIOKAWA, S.; NANNO, T.; NAKAMURA, Y. Angiotensin II directly induces follicle rupture and oocyte maturation in the rabbit. *FEBS Letters*, v. 307, n. 3, p. 305-308, 1992.
YOSHIMURA, Y.; KARUBE, M.; AOKI, H.; ODA, T.; KOYAMA, N.; NAGAI, A.; AKIMOTO, Y.; HIRANO, H.; NAKAMURA, Y. Angiotensin II induces ovulation and oocyte maturation in rabbit ovaries via the AT2 receptor subtype. *Endocrinology*, v. 137, p. 1204-1211, 1996.

YOSHIMURA, Y.; KARUBE, M.; ODA, T.; KOYAMA, N.; SHIOKAWA, S.; AKIBA, M.; YOSHINAGA, A.; NAKAMURA, Y. Locally produced angiotensin II induces ovulation by stimulating prostaglandin production *in vitro* perfused rabbit ovaries. *Endocrinology*, v. 133, p. 1609-1616, 1993.

ZAMORANO, P. L.; MAHESH, V. B.; BRANN, D. W. Quantitative RT-PCR for neuroendocrine studies. A minireview. *Neuroendocrinology*, v. 63, p. 397–407, 1996.

ZHAO, J.; TAVERNE, M. A. M.; VAN DER WEIJDEN, G. C.; BEVERS, M. M.; VAN DEN HURK, R. Effect of activin A on *in vitro* development of rat preantral follicles and localisation of activin A and activin receptor II. *Biology of Reproduction*, v. 65, p. 967–977, 2001.

ZHAO, J.; TAVERNE, M. A.; VAN DE WEIJDEN, G. C.; BEVERS, M. M.; VAN DEN HURK, R. Immunohistochemical localisation of growth hormone (GH), GH receptor (GHR), insulin-like growth factor I (IGF-I) and type I IGF-I receptor, and gene expression of GH and GHR in rat pre-antral follicles. *Zygote*, v. 10, p.85–94, 2002.

ZHAO, J.; TAVERNE, M. A. M.; VAN DER WEIJDEN, G. C.; BEVERS, M. M.; VAN DEN HURK, R. Insulin-like growth factor-I (IGF-I) stimulates the development of cultured rat preantral follicle. *Molecular Reproduction and Development*, v. 58, p. 287- 296, 2001.

ZHOU, H.; ZHANG, Y. *In vitro* development of caprine ovarian preantral follicles. *Theriogenology*, v. 54, p. 641-650, 2000.

ZHOU, H.; ZHANG, Y. Regulation of *in vitro* growth of preantral follicles by growth factors in goats. *Domestic Animal Endocrinology*, v. 28, p.235– 242, 2005.

ZIMMERMANN, R. C.; HARTMAN, T.; KAVIC, S.; PAULI, S. A.; BOHLEN, P.; SAUER, M. V.; KITAJEWSKI, J. Vascular endothelial growth factor receptor 2-mediated angiogenesis is essential for gonadotropin-dependent follicle development. *The Journal of Clinical Investigation*, v. 112, p. 659–669, 2003.

ZIMMERMANN, R. C.; XIAO, E.; BOHLEN, P.; FERIN, M. Administration of anti-vascular endothelial growth factor receptor 2 antibody in the early follicular phase delays follicular selection and development in the rhesus monkey. *Endocrinology*, v. 143, p. 2496–2502, 2002.