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# ESTABILIDADE DE GENES DE REFERÊNCIA E INFLUÊNCIA DAS PROTEÍNAS MORFOGENÉTICAS ÓSSEAS 6 E 7 SOBRE O DESENVOLVIMENTO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS CAPRINOS

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia – Curso de Medicina, da Universidade Federal do Ceará como requisito parcial para obtenção do Título de Mestre em Biotecnologia.

Orientador: Prof. Dr. José Roberto Viana Silva.

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**Título do trabalho:** Estabilidade de genes de referência e influência das proteínas morfogenéticas ósseas 6 e 7 sobre o desenvolvimento *in vitro* de folículos pré-antrais caprinos.

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**"**O fracasso jamais o surpreenderá se sua decisão de vencer for suficientemente forte**".**

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## LISTA DE ABREVIATURAS E SÍMBOLOS

| | |
|---|---|
| ActR | : Receptor para ativina |
| ActR- IA | : Receptor para ativina tipo I-A |
| ActR-IB | : Receptor para ativina tipo I-B |
| ActR- IIA | : Receptor para ativina tipo II-A |
| ActR-IIB | : Receptor para ativina II-B |
| ALK-2 | : do ingles, activin receptor-like kinase type 2 |
| ALK-3 | : do ingles, activin receptor-like kinase type 3 |
| ALK-4 | : do ingles, activin receptor-like kinase type 4 |
| ALK-5 | : do ingles, activin receptor-like kinase type 5 |
| ALK-6 | : do ingles, activin receptor-like kinase type 6 |
| ANOVA | : Análise de variância |
| as | : Anti senso |
| Background | : Fluorescência de fundo |
| BMP | : Proteína morfogenética óssea |
| BMPR | : Receptor para proteína morfogenética óssea |
| BMPR- IA | : Receptor para proteína morfogenética óssea tipo I-A |
| BMPR- IB | : Receptor para proteína morfogenética óssea tipo I-B |
| BMPR-II | : Receptor para proteína morfogenética óssea tipo II |
| BMP-6 | : Proteína morfogenética óssea 6 |
| BMP-7 | : Proteína morfogenética óssea 7 |
| BMP-15 | : Proteína morfogenética óssea 15 |
| Br | : Bromo |
| °C | : Graus Celsius |
| Ca 2+ | : Íon cálcio |
| cAMP | : AMP cíclico |
| cDNA | : Ácido desoxiribonucléico complementar |
| CT ou CP | : Threshold cycle |
| COCs | : Complexos cúmulus-oócitos |
| $CO_2$ | : Dióxido de carbono |
| DAB | : Diaminobenzidina |
| DNA | : Ácido desoxirribonucléico |
| DNAse | : Desoxirribonuclease |
| dNTP | : Desoxinucleotídeos |
| EGF | : Fator de crescimento epidermal |
| FGF | : Fator de crescimento de fibroblasto |
| FGF-2 | : Fator de crescimento de fibroblastos-2 |
| FIV | : Fecundação *in vitro* |
| FOPA | : Folículo ovariano pré-antral |
| FSH | : Hormônio folículo estimulante |
| FSH-R | : Receptor para o hormônio folículo estimulante |
| GAPDH | : Gliceraldeído-3-fosfato-desidrogenase |
| GDF-9 | : Fator de crescimento e diferenciação-9 |
| GH | : Hormônio de crescimento |
| GH-R | : Receptor para o hormônio de crescimento |
| GT | : Granulosa + Teca |
| HEPES | : Àcido N-2-Hidroxietilpiperazina-N'-2'-Etanossulfônico |
| IGF-1 | : Fator de crescimento semelhante à insulina-1 |

| | |
|---|---|
| IHC | : Imunohistoquímica |
| KL | : Kit ligand |
| LH | : Hormônio luteinizante |
| LH-R | : Receptor para o hormônio luteinizante |
| MEM | : Meio essencial mínimo |
| $Mg^{+2}$ | : Magnésio |
| MOIFOPA | : Manipulação de oócitos inclusos em folículos pré-antrais |
| mRNA | : Ácido ribonucléico mensageiro |
| ng | : nanograma |
| PCR | : Reação em cadeia da polimerase |
| PGK | : Fosfoglicerato quinase |
| P450scc | : Enzima de clivagem da cadeia lateral do colesterol |
| P<0.05 | : Probabilidade menor do que 5% |
| P>0.05 | : Probabilidade maior do que 5% |
| RNA | : Ácido ribonucléico |
| RNAse | : Enzima ribonuclease |
| RPL | : Proteína ribossomal |
| rRNA | : RNAs ribossomais |
| RT | : Transcriptase reversa |
| -RT | : Menos transcriptase reversa |
| RT-PCR | : Transcriptase reversa da reação em cadeia da polimerase |
| s | : Senso |
| SMADS | : Mensageiros intracelulares |
| STAR | : Proteína reguladora da estereidogenese aguda |
| TGF-β | : Superfamília de fatores de crescimento transformante beta |
| TGF-ß2 | : Superfamília de fatores de crescimento transformante beta 2 |
| TGF-ßRIII | : Receptor de fatores de crescimento transformante beta - III |
| TßR-I | : Receptor de fatores de crescimento transformante beta - I |
| TßR-II | : Receptor de fatores de crescimento transformante beta - II |
| UBQ | : Ubiquitina |
| VEGF | : Fator de crescimento endotelial vascular |
| µg | : Micrograma |
| µL | : Microlitro |
| µm | : Micrômetro |
| + SD | : Mais ou menos o desvio padrão |
| % | : Percentagem |
| $X^2$ | : Qui-quadrado |

## RESUMO

Este trabalho tem como objetivo avaliar a estabilidade de genes de referência e a expressão de fatores de crescimento e de receptores de hormônios em folículos secundários de caprinos. Além disso, visa quantificar a expressão dos RNA mensageiros para BMP-6 e BMP-7 em folículos pré-antrais e antrais caprinos e avaliar os efeitos de FSH, BMP-6 e BMP-7 sobre o crescimento e expressão gênica em folículos secundários caprinos durante 6 dias de cultivo. Para avaliar a estabilidade dos genes de referência, folículos secundários (150-200 μm) foram isolados mecanicamente de ovários caprinos. Após a extração do RNA total e síntese de DNA complementar, realizou a quantificação dos mRNA, por PCR em tempo real, utilizando-se primers específicos para genes de referência (GAPDH, β-tubulina, β-actina, PGK, UBQ, RPL-19, rRNA18S), fatores de crescimento (GDF-9, BMP-15, BMP-6, FGF-2, VEGF, KL e IGF-1) e receptores de hormônio (FSH-R, LH-R e GH-R). Para avaliar a expressão de BMP-6 e BMP-7, folículos primordiais, primários e secundários, bem como pequenos e grandes folículos antrais foram obtidos e os níveis de mRNA de BMP-6 e 7 foram quantificados. Nos estudos *in vitro*, os efeitos da BMP-6 (50 ng/mL) e BMP-7 (50 ng/mL) na presença ou ausência de FSH (50 ng/mL) sobre o desenvolvimento *in vitro* de folículos secundários e sobre a expressão de mRNA para BMP-6 e 7 e FSH-R foi avaliada após 6 dias de cultivo. Os resultados mostraram que UBQ e β-actina são os genes de referência mais estáveis em folículos pré-antrais caprinos fresco cultivados por 12 dias. Os RNAs mensageiros para os fatores de crescimento (EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 e KL) e os receptores de FSH, LH e GH são expressos em diferentes níveis em folículos pré-antrais de caprinos, sendo que o IGF-1 e o EGF apresentaram, respectivamente, o maior e o menor nível de mRNA. O nível de mRNA para BMP-6 em folículos primários e secundários foi significativamente maior do que aqueles em primordial, enquanto que os níveis de mRNA para BMP-7 foi maior nas células da granulosa/teca de grandes do que nos pequenos folículos antrais. Após o cultivo de folículos secundários durante 6 dias, FSH aumentou o diâmetro folicular e FSH e BMP-7 aumentou significativamente os níveis de mRNA para BMP-7 e FSH-R. Já a BMP-6 na presença ou ausência de FSH aumentou o diâmetro dos folículos secundários. Além disso, FSH aumentou os níveis de mRNA para BMP-6, enquanto ambos BMP-6 e FSH e aumentaram os níveis de mRNA para FSH-R, após o período de cultivo. Em conclusão, UBQ e β-actina são os dois genes mais estáveis para folículos secundários caprinos e o FSH e as BMPs dos tipos 6 e 7 estimulam o crescimento de folículos pré-antrais *in vitro* durante 6 dias de cultivo.



**Palavras-chave:** BMP-6, BMP-7, receptores, genes de referência, folículos pré-antrais

## ABSTRACT

This study aims to evaluate the stability of reference genes and the expression of growth factors and hormone receptors in goat secondary follicles. It also seeks to quantify the expression of messenger RNA for BMP-6 and BMP-7 in goat preantral follicles and to evaluate the effects of FSH, BMP-6 and BMP-7 on growth and gene expression in secondary follicles goats after 6 days of culture. To evaluate the stability of reference genes, secondary follicles (150-200 μm) were mechanically isolated from goat ovaries. After extraction of total RNA and synthesis of complementary DNA, the quantification of mRNA was carried out by real-time PCR using specific primers for genes of reference (GAPDH, β-tubulin, β-actin, PGK, UBQ, RPL - 19, rRNA18S), growth factors (GDF-9, BMP-15, BMP-6, FGF-2, VEGF, KL and IGF-1) and hormone receptor (FSH-R, LH-R and GH-R). To evaluate expression of BMP-6 and BMP-7, primordial follicles, primary and secondary as well as small and large antral follicles were obtained and the mRNA levels of BMP-6 and 7 were measured. For *in vitro* studies, the effects of BMP-6 (50 ng / mL) and BMP-7 (50 ng / mL) in the presence or absence of FSH (50 ng / mL) on the development of secondary follicles and on the expression of mRNA for BMP-6 and 7 and FSH-R was evaluated after 6 days of culture. Results showed that mRNA for growth factors (EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 and KL) and the receptors for FSH, LH and GH are expressed in at different levels in preantral follicles of goats. Moreover, among the growth factors studied, IGF-1 and EGF had higher and lower levels or RNA, respectively. UBQ and β-actin genes were the mostr stable reference in fresh and cultured preantral follicles. The level of mRNA for BMP-6 in primary and secondary follicles was significantly higher than those in primordial follicles, while the levels of mRNA for BMP-7 was higher in granulosa cells / theca in large antral follicles than in small antral follicles. After culture of secondary follicles for 6 days, FSH increased follicular diameter and FSH and BMP-7 significantly increased the levels of mRNA for BMP-7 and FSH-R. In addition, BMP-6 in the presence or absence of FSH increased the diameter of secondary follicles after 6 days of culture. Real-time PCR shoed that FSH increased the levels of mRNA for BMP-6, while both BMP-6 and FSH and increased levels of mRNA for FSH-R, after a period of 6 days of culture. In conclusion, UBQ and β-actin are the two most stable genes for secondary follicles goats and FSH and BMPs of types 6 and 7 stimulate the growth of preantral follicles after 6 days of *in vitro* culture.



**Keywords:** BMP-6, BMP-7, hormone receptors, genes of reference, preantral follicle.

**SUMÁRIO**

## 1. **INTRODUÇÃO**

Os caprinos são considerados animais economicamente atrativos por serem importantes fontes de carne, leite e pele (SILVA, 2005). Além de estarem presentes em todos os continentes, no Brasil, os caprinos desempenham importante papel econômico e social, especialmente na Região Nordeste do país. Desta forma, o conhecimento da fisiologia ovariana, associado ao desenvolvimento de biotecnologias na área da reprodução animal, como por exemplo, o cultivo *in vitro* de folículos pré-antrais e a fecundação *in vitro*, abrem inúmeras possibilidades para proporcionar incremento genético e aumento na produtividade animal. Assim, é de grande importância o estudo dos fatores que controlam o desenvolvimento folicular ovariano nesta espécie.

As proteínas morfogenéticas ósseas (BMPs) constituem um subgrupo da superfamília de fatores de crescimento transformante beta (TGF-β), constituído por mais de 30 membros de proteínas. Diversos estudos têm demonstrado que as BMPs regulam o crescimento, diferenciação e apoptose em uma variedade de tecidos, incluindo o ovário (HUSSEIN *et al.*, 2005). Neste orgão, as BMPs exercem importantes funções na foliculogênese e na ovulação (SHIMASAKI *et al.*,1999; LEE *et al.*, 2001; OTSUKA e SHIMASAKI, 2002). A BMP-7 é produzida pelas células da teca de folículos secundários e antrais de ratas (SHIMASAKI *et al.*, 2004) e exerce suas funções biológicas interagindo com o receptor de ativina-IA ou receptor de BMP-IB que, após a ativação, recruta o receptor de ativina-IIA ou de BMP-II (SHIMASAKI *et al.*, 2004). A BMP-6 é produzida por oócitos, assim como por células da granulosa e da teca em bovinos (GLISTER *et al.*, 2004) e ovinos (JUENGEL *et al.*, 2005). Essa proteína atua no ovário estimulando a proliferação das células da granulosa, promovendo viabilidade celular e aumentando a produção de inibina-A, ativina-A e folistatina em células da granulosa de bovinos (GLISTER *et al.*, 2004).

Para facilitar a compreensão das funções dos diferentes fatores de crescimento em ovários caprinos é importante conhecer os locais de produção destes fatores. Para isto, a técnica de PCR em tempo real tem sido utilizada com bastante êxito para quantificação dos RNAs mensageiros que estão sendo produzidos pelos diferentes tipos de folículos ovarianos. Para a quantificação do mRNA por RT-PCR é necessário realizar uma adequada normalização utilizando genes de referência que podem apresentar níveis de expressão similar em vários tecidos (THELLIN *et al.*, 1999). Os genes de referência mais comumente usados para normalizar os padrões de expressão gênica são aqueles codificados pelos genes

gliceraldeído-3-fosfato-desidrogenase (GAPDH), β-actina, β-tubulina, fosfoglicerato quinase (PGK), ubiquitina (UBQ), proteína ribossomal (RPL) e RNA ribossômico (RNA 18S).

O ovário dos caprinos, ao nascimento, contém milhares de oócitos inclusos em folículos pré-antrais e já é bem conhecido que a grande maioria desses oócitos (99%) não ovula e morre por atresia durante as fases de crescimento e maturação. Assim, o isolamento de um grande número de folículos pré-antrais saudáveis, bem como o cultivo, a maturação e a fecundação *in vitro* dos seus oócitos inclusos abrem inúmeras possibilidades para a produção de milhares de embriões a partir de animais de alta produtividade, bem como para a compreensão das funções ovarianas. O estudo dos fatores de crescimento produzidos pelos folículos, associado com o isolamento e o cultivo *in vitro* de folículos pré-antrais justifica-se pelo fato do baixo rendimento do ovário mamífero no que concerne à produção e liberação de oócitos viáveis para a fecundação (FIGUEIREDO *et al.*, 2002).

Para uma melhor compreensão deste trabalho a revisão de literatura a seguir abordará aspectos relacionados ao ovário mamífero, foliculogênese e atresia folicular, fatores de crescimento importantes para a foliculogênese, cultivo folicular e reação em cadeia da polimerase (PCR) e as etapas para quantificação e expressão dos mRNA.

# 2. REVISÃO DE LITERATURA

## 2.1. Ovário mamífero

O ovário mamífero é um órgão dinâmico encarregado de proporcionar um ambiente ideal para a produção de hormônios e liberação dos gametas femininos. Em todas as espécies mamíferas, o ovário é composto de duas regiões distintas, uma medular e outra cortical, circundada pelo epitélio germinal. Na maioria das espécies, a medula ovariana está localizada mais internamente e consiste de um arranjo irregular de tecido conjuntivo fibroelástico e tecido nervoso e vascular que chega ao ovário através do hilo. O córtex ovariano, localizado mais externamente, consiste na região funcional do órgão, e é composto de tecido conectivo (fibroblastos, colágeno e fibras reticulares), folículos ovarianos e corpos lúteos em vários estágios de desenvolvimento ou de regressão (SILVA, 2005). Além dos nutrientes e hormônios provenientes da corrente sanguínea, outros fatores produzidos pelos diferentes tipos celulares descritos contribuem para a formação de um sistema bastante complexo que regula as funções do ovário, que incluem a produção de gametas e hormônios.

## 2.2. Foliculogênese e atresia folicular

Ao processo de formação, crescimento e maturação dos folículos ovarianos dá-se o nome de foliculogênese. Este processo inicia-se ainda na vida pré-natal, na maioria das espécies, com a formação do folículo primordial e culmina com o estágio de folículo pré-ovulatório (VAN DEN HURK e ZHAO, 2005).

Os folículos ovarianos são classificados de acordo com o grau de evolução em pré-antrais, compreendendo os primordiais, primários e secundários, e em antrais, caracterizados pela presença de uma área preenchida por fluido folicular, em que, a partir de então, passam a ser sub-classificados como terciários e pré-ovulatórios. Os folículos pré-antrais representam cerca de 90 a 95% de toda a população folicular (FIGUEIREDO *et al.*, 2008). As características morfológicas que marcam o início do crescimento de folículos primordiais são: aumento do diâmetro oocitário, proliferação das células da granulosa e transformação do formato destas células de achatado para cúbico. Quando o oócito é circundado por uma camada completa de células da granulosa de formato cúbico, os folículos são classificados

como primários. A multiplicação das células da granulosa dos folículos primários leva à formação de várias camadas destas células ao redor do oócito, formando os folículos secundários (Figura 1). Quando os folículos possuem duas a três camadas de células da granulosa, os precursores das células da teca são recrutados do estroma ovariano (PARROT e SKINNER, 2000). Com a intensa proliferação das células da granulosa, uma área preenchida por fluido folicular é identificada na camada granulosa e, a partir de então, os folículos passam a ser classificados como antrais. A partir desse estágio, o diâmetro folicular aumenta acentuadamente devido ao crescimento do oócito, multiplicação das células da granulosa, da teca e aumento da cavidade antral. No último estágio do desenvolvimento folicular, o folículo pré-ovulatório é caracterizado por um oócito circundado por células da granulosa especializadas que são denominadas de células do cúmulus (Figura 1).

A população folicular é estabelecida ainda na vida fetal (primatas e ruminantes – BETTERIDGE *et al.*, 1989) ou em um curto período de tempo após o nascimento (roedores – HIRSHFIELD, 1991). Entretanto, recentemente trabalhos têm demonstrado mecanismos envolvidos na formação, após o nascimento, de novas células germinativas e folículos na mulher (BUKOVSKI *et al.*, 2004) e em camundongas adultas (JOHNSON *et al.*, 2004). No entanto esses trabalhos ainda não são conclusivos havendo necessidade de estudos mais detalhados em outras espécies. O número de folículos primordiais em ovários caprinos ao nascimento tem sido estimado em 35.000 (LUCCI *et al.*, 1999). Já é bem conhecido que o desenvolvimento folicular é um processo complexo e dependente de vários hormônios e fatores de crescimento. Isto faz com que a grande maioria dos folículos primordiais presentes nos ovários, cerca de 99,9%, não cresça até a ovulação, mas morra por apoptose em um processo conhecido por atresia, fazendo com que o ovário seja um órgão de baixíssima produtividade (MARKSTROM *et al.*, 2002).

A atresia folicular e os fatores que influenciam este processo são extensivamente estudados e tem sido demonstrado que muitos hormônios e fatores de crescimento estão envolvidos. Durante o início do desenvolvimento folicular, estudos recentes têm demonstrado que fatores de crescimento, como por exemplo: ativina-A, BMP-4, GDF-9, FGF-2 e IGF-1 são extremamente importantes para a manutenção da viabilidade folicular (REYNAUD e DRIANCOURT, 2000). Em folículos antrais, as gonadotrofinas FSH e LH atuam como importantes inibidores da apoptose a partir do estágio em que eles estão aptos a serem recrutados (MARKSTROM *et al.*, 2002). Conforme observado, a progressão da apoptose em folículos ovarianos é dependente da regulação cooperativa de diferentes fatores endócrinos,

parácrinos e autócrinos. Provavelmente o balanço entre os fatores que promovem sobrevivência e aqueles que induzem a apoptose decidirá se um determinado folículo continuará o seu desenvolvimento ou sofrerá atresia (HSU e HSUEH, 2000). Como pode-se observar, os hormônios e os fatores de crescimento exercem importante papel no controle da folículogênese e da atresia folicular. Dentre estes fatores de crescimento podemos citar os pertencentes à subfamília das proteínas morfogenéticas ósseas (BMPs).

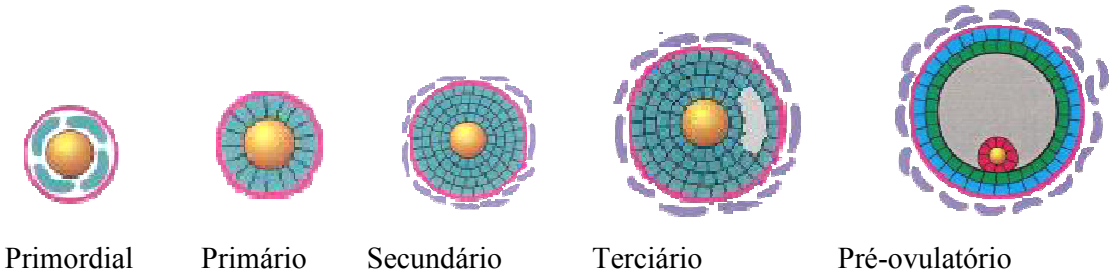


Figura 1: Caracterização morfológica de folículos pré-antrais e antrais.

**2.3. A subfamília de proteínas morfogenéticas ósseas**

A família de fatores de crescimento TGF-ß é composta por mais de 40 membros (CHANG *et al*., 2002), dentre os quais estão as BMPs que foram agrupadas na subfamília BMP, de acordo com a homologia estrutural de seus membros. Na subfamília BMP, os principais fatores que exercem funções no ovário são as BMP-2, BMP-4, BMP-6, BMP-7 e BMP-15, O nome proteína morfogenética óssea (BMP), foi citado pela primeira vez em 1965 por Urist (URIST, 1965) por ser um componente ativo no osso desmineralizado e em extratos de osso que são capazes de induzir a formação óssea em sítios ectópicos (SHIMASAKI *et al*., 2004).

A maioria dos membros da superfamília TGF-ß, incluindo as BMPs, exerce seus efeitos sobre as células-alvo através da formação de um complexo com dois tipos de receptores localizados na superfície das células, designados de Tipo-I e Tipo-II (CHANG *et al*., 2002). Em mamíferos, já foram identificados vários receptores do tipo-I [BMPR-IA (ALK3), BMPR-IB (ALK6), ActR-IA (ALK2), ActR-IB (ALK4), TßR-I (ALK5)], e do tipo-II [BMPR-II, ActR-IIA, ActR-IIB, TßR-II]. Geralmente, ligações entre membros da família TGF-ß e ativina ocorrem através de receptores tipo II, enquanto os membros da família BMP

têm alta afinidade por receptores tipo I (DE CAESTECKER, 2004). Também, um terceiro tipo de receptor, o TGF-ßRIII, tem sido demonstrado. Este pode atuar facilitando a ligação do TGF-ß2 ao receptor tipo II e, ainda, facilita a associação da inibina com o receptor tipo II de ativina, antagonizando, assim, as ações da ativina (GRAY *et al.*, 2002**)** e de uma série de BMPs (WIATER *et al.*, 2003).

A expressão de BMPR-II, BMPR-IA (ALK3) e BMPR-IB (ALK6) foi demonstrada em folículos primordiais, primários e secundários, bem como em oócitos, células da granulosa e da teca de folículos antrais caprinos. Os RNAs mensageiros para BMPR-IA (ALK3), BMPR-IB (ALK6) e BMPR-II foram detectados nos folículos primordiais, primários e secundários, bem como no oócito e células da granulosa de folículos antrais (SILVA *et al.*, 2004). Já os receptores para TGF-ß, TßRI ou ALK5 e TßRII, são aparentemente expressos em muitos tipos celulares.

### 2.3.1. Proteína morfogenética óssea -6 (BMP-6)

A BMP-6 é produzida por oócitos, células da granulosa e da teca em várias espécies (murinos: ERICKSON e SHIMASAKI, 2003; bovinos: GLISTER *et al.*, 2004; ovinos; JUENGEL e McNATTY, 2005). Para exercer suas funções biológicas, a BMP-6 interage com dois tipos (I e II) de receptores presentes na superfície celular (MASSAGUÉ e CHEN, 2000). Inicialmente, as BMPs ligam-se com receptores do tipo I, ou seja, com o receptor de ativina-IA ou com o receptor de BMP-IB. Após a ativação do receptor do tipo I, ocorre o recrutamento do receptor do tipo II, ou seja, receptores de ativina-IIA ou -IIB, ou receptores de BMP-II. A interação entre os receptores induz a fosforilação de mensageiros intracelulares (SMADS) que são deslocados para o núcleo, onde regulam a expressão de genes específicos (MASSAGUÉ, 2000). Silva *et al*. (2004a) demonstraram que os receptores das BMPs estão expressos em todos os tipos de folículos ovarianos na espécie caprina. Em camundongos, também já foi relatada a expressão destes receptores em folículos ovarianos (SHIMASAKI *et al.*, 2004).

As funções biológicas da BMP-6 foram identificadas no ovário utilizando culturas primárias de células da granulosa de ratas (OTSUKA *et al.*, 2001). A BMP-6 é eficaz na indução de FSH inibindo a síntese de progesterona. Contudo, considerando que a BMP-4 e -7 aumentam a produção de FSH induzida pelo estradiol, a BMP-6 não tem nenhum efeito sobre a produção de FSH induzida pelo estradiol. Além disso, BMP-6 inibe a síntese de

progesterona e da expressão correspondente da STAR e P450scc induzida por forskolina. Em contraste, BMP-6 tem efeito sobre estes parâmetros esteroidogênicos induzido por 8-bromo (Br)-cAMP (OTSUKA *et al.*, 2001). Em termos de fisiologia, deve-se notar que a expressão do mRNA de BMP-6 diminuiu rapidamente, no momento em que o folículo dominante foi selecionado durante o ciclo estral normal em ratos. Quando há perda da ação inibitória do FSH, a BMP-6, pode ser ligada ao mecanismo pelo qual os folículos dominantes são selecionados em ratas.

Estudos recentes mostraram que *in vitro*, a BMP-6 não influenciou a ativação de folículos primordiais caprinos após 7 dias de cultivo (ARAÚJO *et al.*, 2010). Além disso, outros estudos *in vitro* foram realizados para avaliar o efeito da BMP-6 sobre o crescimento folicular, principalmente na espécie murina (OTSUKA *et al.*, 2001). Durante o cultivo de células da granulosa de folículos antrais de camundongos foi demonstrado que 100ng/mL de BMP-6 inibe a síntese de progesterona, por meio da inibição de enzimas esteroidogênicas. Além disso, a BMP-6 também inibe a expressão de receptores de LH, mas não altera a produção de estradiol, em camundongos (OTSUKA *et al.*, 2001). Em bovinos, na concentração de 50 ng/mL, a BMP-6 estimula a proliferação das células da granulosa, promove a viabilidade celular e aumenta a produção de inibina-A, ativina-A e folistatina em células da granulosa de bovinos (GLISTER *et al.*, 2004). É importante ressaltar que a produção de BMP-6 diminui drasticamente durante a seleção do folículo dominante, sendo que esta redução pode estar relacionada com o mecanismo pelo qual os folículos dominantes são selecionados (SHIMASAKI *et al.*, 2004). Em resumo, a BMP-6 atua retardando o processo de diferenciação folicular, proporcionando o rápido crescimento do folículo por meio da multiplicação das células da granulosa.

### 2.3.2. Proteína morfogenética óssea -7 (BMP-7)

A presença de mRNA para BMP -7 foi identificada inicialmente em células da teca e os seus receptores (BMPR-II, ALK-3, e ALK-6) estavam localizados predominantemente nas células da granulosa de ovários de ratas (SHIMASAKI *et al.*, 1999). Posteriormente, foi demonstrado que a BMP-7 é produzida pelas células da teca de folículos secundários e antrais e exerce suas funções biológicas interagindo com o receptor de ativina-IA ou receptor de BMP-IB que, após a ativação, recruta o receptor de ativina-IIA ou de BMP-II (SHIMASAKI *et al.*, 2004). Com relação aos efeitos *in vivo* da BMP-7, Lee *et al.* (2001) injetaram BMP-7

no interior da bolsa ovariana de ratas e em seguida caracterizaram as alterações na foliculogênese, ovulação e esteroidogênese. Estes autores demonstraram que a BMP-7 reduz o número de folículos primordiais e aumenta o número de folículos primários, secundários e antrais, indicando que a BMP-7 promove a ativação e o crescimento dos folículos primordiais. Estudos recentes mostram que durante um cultivo de 7 dias, a concentração de 10ng/ml de BMP-7 aumenta o diâmetro folicular (ARAÚJO *et al*., 2010). Além disso, a administração da BMP-7 promoveu mitose nas células da granulosa e inibiu a produção de progesterona. Considerando que a progesterona é importante para o processo de ovulação (YOSHIMURA e WALLACH, 1987), a inibição da produção de progesterona pela BMP-7 pode estar relacionada com os mecanismos de inibição da ovulação. Estudos *in vitro*, também demonstraram que a BMP-7, na concentração de 100ng/mL, promove a ativação e o crescimento de folículos primordiais, bem como aumenta a expressão de receptores para FSH durante o cultivo de ovários de camundongas (LEE *et al.*, 2004). Durante o cultivo *in vitro* de células da granulosa de rata foi observado que a BMP-7 modula a ação do FSH, no sentido de aumentar a produção de estradiol e inibir a síntese de progesterona (SHIMASAKI *et al.*, 1999). É bem estabelecido que as células da granulosa de folículos em crescimento respondem ao estímulo do FSH *in vivo*, produzindo estradiol. Já a produção de progesterona é observada somente no estágio de folículo pré-ovulatório. Por outro lado, quando as células da granulosa são cultivadas *in vitro,* observa-se aumento na produção tanto de estradiol como de progesterona em resposta ao estímulo do FSH. Isto sugere que um inibidor da síntese de progesterona, ou seja, um inibidor da luteinização, deve estar atuando *in vivo* (SHIMASAKI *et al.*, 2004). A BMP-7 foi um dos primeiros fatores identificados com ação biológica que promove a inibição da luteinização em células da granulosa (SHIMASAKI *et al.*, 1999). Além disso, foi demonstrado que a BMP-7 aumenta a expressão da enzima P450 aromatase favorecendo a produção de estradiol (LEE *et al.*, 2001).

Usando cultivos primários de células da granulosa de rata em meio livre de soro, a BMP-7 recombinante foi identificada por modular a sinalização do FSH de uma forma que promove a produção de estradiol (aromatização), enquanto inibe a biossíntese de progesterona (SHIMASAKI *et al.*, 1999). Está bem estabelecido que as células da granulosa de folículos em crescimento respondem ao estímulo com FSH *in vivo* sintetizando estradiol, progesterona, mas não até o período pré-ovulatório. Por outro lado, quando as células da granulosa são cultivadas *in vitro*, sintetizam tanto estradiol como progesterona em resposta ao estímulo com FSH. Esses resultados sugerem que um seletivo inibidor da síntese de progesterona, ou seja,

um inibidor da luteinização, deve agir *in vivo* para modular a bioatividade do FSH de uma forma que reflete na esteroidogênese folicular normal durante o ciclo estral (FALCK *et al.*, 1959; NALBANDOV *et al.*, 1970). O desafio foi o de identificar a natureza deste inibidor de luteinização. Notavelmente, BMP-7 não afeta a esteroidogênese em células da granulosa na ausência de FSH em ratas (SHIMASAKI *et al.*, 1999). Os ratos com mutações nulas no gene BMP-7 morrem antes (WINNIER *et al.*, 1995) ou após o nascimento (DUDLEY *et al.*, 1995). Portanto, a tecnologia *knockout* em ratos não conseguiu fornecer informações diretas sobre o papel destas BMPs no ovário *in vivo*.

### 2.4. Outros fatores de crescimento importantes para a foliculogênese

Além das BMPs descritas anteriormente, vários outros fatores de crescimento participam diretamente do controle da folículogênese em várias espécies. Dentre estes fatores, inclui-se o fator de crescimento epidermal (EGF) que, em caprinos, estimula o aumento do diâmetro folicular, através da promoção do crescimento do oócito (SILVA *et al.*, 2004a) e do aumento do número de células foliculares (RAJARAJAN *et al.*, 2006). Além disso, o EGF pode atuar localmente no ovário controlando a ação de FSH e LH, pois alguns estudos mostraram que o EGF inibe a produção de receptores de LH (HATTORI *et al.*, 1995) e aumenta a expressão de receptores de FSH (LUCIANO *et al.*, 1994). Além do EGF, dois outros fatores de crescimento são conhecidos por serem essenciais para o desenvolvimento folicular normal, ou seja, o fator de crescimento de diferenciação 9 (GDF-9) e o GDF-9B, que também é conhecido como proteína morfogenética óssea 15 (BMP-15). O GDF-9 é um fator secretado pelo oócito (CHANG *et al.*, 2002) e células da granulosa (caprinos: SILVA *et al.*, 2006) e atua estimulando o crescimento de folículos primários e secundários, bem como a manutenção da viabilidade folicular e a proliferação de células da granulosa de humanos (HREINSSON *et al.*, 2002). A deleção do gene GDF-9 causou infertilidade com foliculogênese bloqueada no estágio primário em camundongos (DONG *et al*., 1996), enquanto que o tratamento com GDF-9 estimulou o crescimento de folículos pré-antrais em ratas (VITT *et al*., 2000), indicando participação fundamental do GDF-9 na regulação da foliculogênese pré-antral. O GDF-9B tem como primeiro alvo as células da granulosa e atua regulando sua proliferação e diferenciação (OTSUKA *et al.*, 2000). Estudos *in vitro* têm demonstrado que ele promove a proliferação das células da granulosa e estimula o desenvolvimento de folículos primordiais e primários em roedores, ou seja, é um importante

regulador da mitose das células da granulosa e do desenvolvimento folicular inicial (JUENGEL e McNATTY, 2005).

O Kit ligante (KL) é um fator de crescimento produzido localmente que desempenha importante papel no controle da função ovariana (YOSHIDA *et al.*, 1997). Em caprinos, a proteína e o mRNA para KL foram encontrados em folículos em todos os estágios de desenvolvimento, bem como no corpo lúteo, superfície do epitélio e tecido medular ovariano (SILVA *et al.*, 2006). A proteína KL foi demonstrada em células da granulosa de folículos a partir do estágio primordial. Já o mRNA foi detectado em células da granulosa isoladas de folículos antrais. O fator de crescimento de fibroblastos-2 (FGF-2) é outro fator que exerce influência sobre o desenvolvimento dos folículos ovarianos e é importante na regulação de várias funções ovarianas, tais como, mitose (ROBERTS e ELLIS, 1999), esteroidogênese (VERNON e SPICER, 1994), diferenciação (ANDERSON e LEE, 1993) e apoptose (TILLY *et al*., 1992) das células da granulosa. Ele atua promovendo o início do crescimento de folículos primordiais e primários (NILSSON *et al.*, 2001), bem como a esteroidogênese e a diferenciação em folículos antrais bovinos (VERNON e SPICER, 1994). Já o VEGF é um fator produzido pelas células da teca e células da granulosa (YAMAMOTO *et al*., 1997) em resposta a ação das gonadotrofinas (KOOS *et al*., 1995). O VEGF é um dos fatores responsáveis pela angiogênese folicular, pois ele atua estimulando a mitose de células endoteliais e aumenta a permeabilidade vascular (REDMER e REYNOLDS, 1996). Pesquisas mostraram que o VEGF promove o crescimento de folículos primários e secundários (DANFORTH *et al*., 2001). Alguns estudos *in vitro,* avaliando os efeitos de IGF-1 em bovinos, mostram sua importância sobre a proliferação das células da granulosa e da teca e na esteroidogênese (para revisão: SPICE e ECHTERNKAMP, 1995). Em bovinos, o IGF-1 aumenta os níveis de mRNA a partir do início da dominância (YUAN *et al.*, 1998). Spicer e Chamberlain (2000) mostraram que as mudanças intrafoliculares nos níveis de IGF-1 durante o desenvolvimento folicular em bovinos são devido ao estradiol e as gonadotrofinas induzidas por alterações na quantidade de IGF-1 produzidas por células da granulosa e da teca. Pesquisas demonstraram que a adição de IGF-1 ao meio de cultivo de grandes folículos secundários promove o aumento da incidência de dominância folicular e ovulação (GINTHER *et al.*, 2008). Para se estudar as funções biológicas de hormônios e fatores de crescimento, a técnica de cultivo *in vitro* tem sido utilizadas com bastante sucesso.

## 2.5. Cultivo folicular *in vitro*

Basicamente existem dois sitemas de cultivo folicular, no primeiro deles, o cultivo *in situ*, os folículos são cultivados inseridos no fragmento ovariano ou no ovário inteiro, enquanto que no segundo sistema os folículos são cultivados isoladamente sobre a placa ou um substrato, caracterizando o sistema bidimensional, ou inseridos no substrato, caracterizando o sistema tridimencional. Para o isolamento de grandes folículos pré-antrais (150-200μm) tem sido utilizado a técnica de microdissecação, que isola os folículos do estroma ovariano com o auxílio de agulhas. Esta técnica possibilita a manutenção da integridade das camadas tecais presentes em grandes folículos pré-antrais (TELFER, 1998).

O cultivo de folículos pré-antrais está inserido na biotécnica de manipulação de oócitos inclusos em folículos ovarianos pré-antrais (MOIFOPA - FIGUEIREDO *et al*., 2007). Tal biotécnica tem como principal objetivo resgatar oócitos oriundos de folículos pré-antrais, a partir do ambiente ovariano, e posteriormente cultivá-los *in vitro* até a maturação, prevenindo-os da atresia e possibilitando sua utilização em outras biotécnicas como FIV, transgenia e clonagem. Para alcançar esse objetivo é necessário o desenvolvimento de um sistema de cultivo *in vitro* ideal para cada etapa do desenvolvimento folicular.

O cultivo *in vitro* de folículos pré-antrais é uma técnica que vem sendo largamente empregada com o intuito de avaliar o efeito de diferentes substâncias, em diferentes concentrações e em diferentes fases do desenvolvimento folicular, a fim de mimetizar *in vitro* os eventos que ocorrem *in vivo* no ovário durante a foliculogênese. Nas últimas duas décadas, vários sistemas de cultivo foram desenvolvidos e os resultados são dependentes do tipo de meio, sistema de cultivo utilizado e da espécie animal estudada (EPPIG e SCHOEDER, 1989; BOLAND *et al.*, 1994; FORTUNE, 2003). Recentemente, o grupo de pesquisa liderado pelo prof. José Ricardo de Figueiredo obteve grande avanço com o cultivo *in vitro* de folículos pré-antrais na espécie caprina, tendo sido relatado à produção do primeiro embrião *in vitro* a partir do cultivo de folículos pré-antrais caprinos (Diário do Nordeste*,* 2009). No entanto, esta técnica ainda precisa ser aperfeiçoada e umas das estratégias é avaliar os níveis de mRNA e de proteínas para hormônios e fatores de crescimento que são produzidos por cada categoria folicular. Isto poderia fornecer informações importantes para o desenvolvimento de meios de cultivo eficientes. Para avaliar os níveis de mRNA e localização das protéinas, as técnicas de PCR em tempo real e imunohistoquímica (IHC) são bastante utilizadas para esta finalidade.

## 2.6. Reação em Cadeia da Polimerase (PCR)

A Reação em Cadeia da Polimerase (PCR, do inglês Polymerase Chain Reaction), é um método de amplificação de ácido desoxirribonucléico (DNA) sem o uso de um organismo vivo, por exemplo, a *Escherichia coli* ou leveduras (BRUCE *et al.*, 1999). Esta técnica foi desenvolvida por Kary Mullis no final da década de 1980, tendo-lhe sido posteriormente, em 1993, atribuído o Prêmio Nobel de Química pelo seu trabalho.

A técnica de PCR baseia-se no processo de replicação de DNA que ocorre *in vivo*. Durante a PCR são usadas elevadas temperaturas de forma a separar as moléculas de DNA em duas cadeias simples, permitindo então a ligação de oligonucleotídeos iniciadores (*primers*), também em cadeia simples e geralmente constituídos por 15 a 30 nucleotídeos obtidos por síntese química. Para amplificar determinada região são necessários dois iniciadores complementares das seqüências que flanqueiam o fragmento de DNA a amplificar, nos seus segmentos terminais 3´, de modo a permitir a atuação da DNA polimerase durante a síntese da cadeia complementar, usando como molde cada uma das duas cadeias simples constituintes do DNA a amplificar (BROWN, 2003).

Para realizar PCR são necessárias pequenas quantidades do DNA alvo, um tampão salino contendo a polimerase, oligonucleotídeos iniciadores, os quatros desoxiribonucleotídeos constituintes do DNA e o co-fator $Mg^{2+}$. Esta mistura é submetida a vários ciclos de amplificação que consistem em: (1) desnaturação do DNA alvo pelo calor (tipicamente 1 minuto a 94-96ºC), de modo a separar as duas cadeias; (2) associação dos iniciadores por ligações de hidrogênio ao DNA alvo em cadeia simples e (3) extensão dos iniciadores através da síntese da cadeia complementar de cada cadeia molde, catalisada pela DNA polimerase (tipicamente 1 minuto a 72ºC) (BROWN, 2003).

O processo envolvendo estes três passos pode ser repetido várias vezes (25 a 40 ciclos), sendo possível aumentar, em cada ciclo, duas vezes a concentração de DNA pré-existente (Figura 2). Com a realização de 25 ciclos de amplificação seguidos, a concentração de DNA aumenta $2^{25}$ vezes embora, na prática, possa haver alguma ineficiência no processo de amplificação (BUSTIN, 2002).

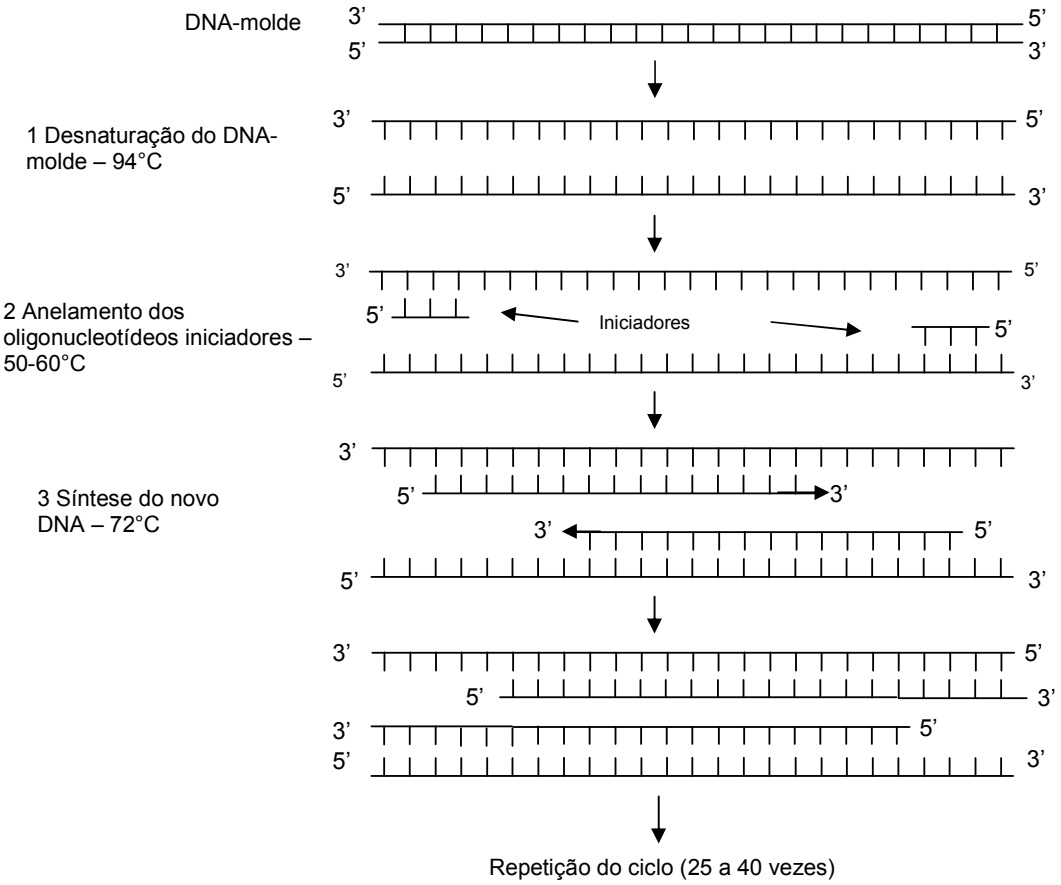


Figura 2: Modelo do processo de amplificação exponencial de DNA através da técnica de PCR (BROWN, 2003).

A PCR é um método muito sensível de análise e por isso é realizado com muito cuidado para evitar contaminações que possam inviabilizar ou tornar errôneo o resultado. Esta é uma das técnicas mais comuns utilizadas em laboratórios de pesquisas médicas e biológicas para diversas tarefas, como o sequenciamento de genes e diagnóstico de doenças hereditárias, usado em testes de paternidade e medicina forense, detecção de diagnóstico de doenças infecciosas e criação de organismos transgênicos (NOVAIS e ALVES, 2004).

No campo da genômica funcional, a introdução da PCR e dos microarranjos de DNA permite o conhecimento e a descrição dos perfis transcriptômicos de vários mecanismos fisiológicos e patológicos observados em todos os tipos celulares. Na PCR por transcriptase reversa, o mRNA é extraído da célula e, a partir dele, é sintetizado o DNA complementar (cDNA) pela enzima transcriptase reversa. Esta enzima tem atividade endonucleásica, degradando o mRNA original e permitindo que seja formada a fita de DNA complementar, o

cDNA. Ao cDNA obtido são adicionados DNA polimerase, desoxiribonucleotídeos e as seqüências de DNA iniciadoras específicas do gene de interesse (*primers*). Da mesma maneira com que é executada a PCR, em um termociclador, são realizados ciclos de desnaturação, anelamento e amplificação, e os resultados são quantificados em relação a uma curva padrão. A introdução da tecnologia de PCR em tempo real (RT-PCR em tempo real) permite a quantificação de um determinado mRNA com elevada sensibilidade (OVERBERGH *et al.*, 1999). O RT-PCR fornece um panorama geral sobre as seqüências ativas ou inativas de DNA, não fornecendo informações mais aprofundadas sobre a regulação da expressão gênica, nem sobre as modificações epigenéticas sofridas pela molécula protéica após a tradução do mRNA para se tornar funcional.

### 2.6.1. PCR em tempo real: amplificação e quantificação

A reação de amplificação em tempo real, uma variante da reação de PCR convencional, representa grande avanço nos métodos moleculares de auxílio diagnóstico, particularmente por facilitar as tarefas de quantificação da expressão gênica em determinado tecido ou amostra biológica. Esta técnica utiliza marcadores fluorescentes para controlar a produção da amplificação de produtos durante cada ciclo da reação de PCR, combinando a amplificação e detecção de ácidos nucléicos em um ensaio homogêneo evitando a necessidade de gel de eletroforese para detectar amplificação dos produtos. Sua especificidade e sensibilidade têm feito a RT-PCR em tempo real, a técnica de referência para detecção e/ou comparação dos níveis de RNA (BUSTIN *et al.,* 2005). Uma maneira comum de revelação fluorescente do produto amplificado consiste na utilização da sonda *TaqMan* ou do agente *SybrGreen*.

O *SybrGreen* é um fluoróforo que se liga de maneira inespecífica ao DNA em fita dupla (NOVAIS e ALVES, 2004). O princípio do método está baseado na detecção de fluorescência no tubo de reação à medida que a dupla fita de DNA é gerada, em virtude da concentração do corante *SybrGreen* entre as cadeias de DNA geradas. A presença de DNA em fita dupla na solução é capaz de aumentar essa emissão de luz em cerca de 100 vezes para uma mesma concentração de *SybrGreen*. Este fluoróforo é um agente inespecífico e revela qualquer dupla fita gerada na reação de amplificação. Em decorrência desse fato, os ensaios que utilizam esse agente devem ter desenho cauteloso para evitar possíveis resultados errôneos (GIULIETTI *et al.*, 2001).

O outro sistema de emissão de luz, *a TaqMan*, é bastante eficiente uma vez que aumenta a especificidade da reação, mas apresenta um maior custo pela utilização de um oligonucleotídeo modificado além dos *primers* habituais para PCR. A figura 3 apresenta, de forma esquemática, a utilização da atividade 5' exonuclease da Taq DNA polimerase em sondas marcadas com corantes fluorescentes (TaqMan). Essas sondas, específicas para o segmento gênico cuja expressão se deseja estudar, apresentam um fluoróforo na posição 5' da sonda, capaz de absorver a energia luminosa emitida pelo equipamento e dissipá-la na forma de luz e calor, em comprimento de onda diferente do original (Figura 3). Entretanto, na sua posição nativa, toda a luz emitida por esse fluoróforo é absorvida pela substância quelante presente na extremidade 3' da sonda. Dessa forma, o sistema óptico do equipamento não é capaz de detectar fluorescência no tubo de reação. Por outro lado, se a reação for capaz de gerar produtos (amplicons), ou mais precisamente, se for detectado e amplificado, a sonda irá hibridizar-se com o alvo gerado e ficará exposta à atividade de exonuclease da polimerase. Como conseqüência, essa sonda será degradada e o fluoróforo ficará distante da substância quelante que agora não mais será capaz de absorver a luz emitida (BUSTIN, 2002).

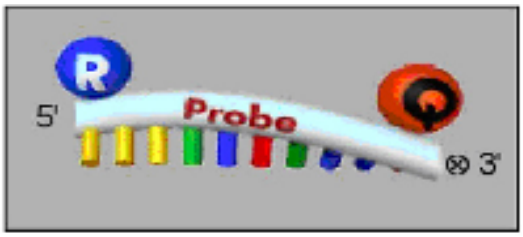


Figura 3: Representação esquemática da sonda TaqMan. R: repórter; Q: quelante (NOVAIS e ALVES, 2004).

O equipamento de PCR em tempo real utiliza uma fonte de luz capaz de excitar o fluoróforo envolvido na reação. A fluorescência eventualmente produzida pela amostra é detectada pelo sistema. O momento da reação de PCR em que a fluorescência de determinada amostra é detectada inequivocamente acima da fluorescência de fundo (background) é comumente denominado de CT ou CP (threshold cycle, crossing point), como ilustra a figura 5. Nessa nova situação, a cada estímulo luminoso gerado pelo equipamento corresponderá uma segunda emissão de luz pelo fluoróforo. Portanto, a emissão de luz será proporcional à

quantidade de produto gerado no tubo de reação. Esse é, por sua vez, proporcional à quantidade inicial de alvos da reação de amplificação (BUSTIN *et al.*, 2005).

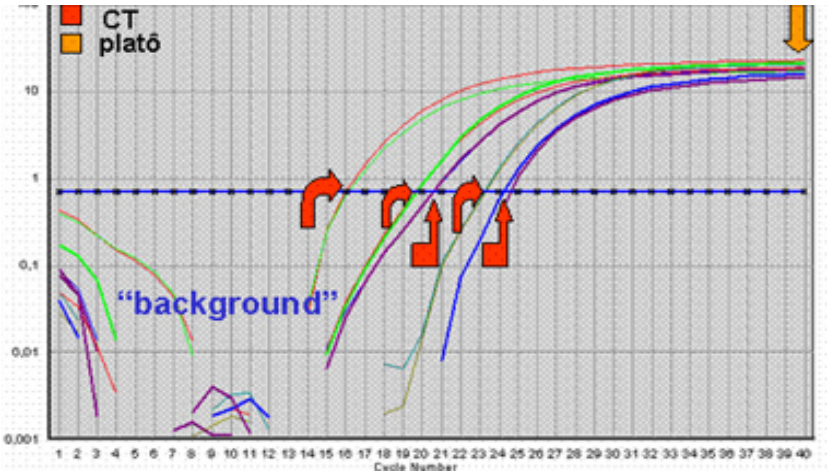


Figura 5: Curvas de monitoramento de fluorescência que ilustram a amplificação em tempo real mostrando os CTs de cinco conjuntos de *primers* em uma única amostra (NOVAIS e ALVES, 2004).

Para normalização da expressão dos genes alvos, genes de referência têm sido utilizados com bastante sucesso, pois eles normalmente apresentam pequenas diferenças em seus perfis de expressão sob diversas condições experimentais (CZECHOWSKI *et al.*, 2005). Como exemplos de genes de referência podemos citar GAPDH, β-tubulina, β-actina, PGK, UBQ, RPL-19 e rRNA18S.

As β-actinas são proteínas altamente conservadas que estão envolvidas em processos que são cruciais para o desenvolvimento dos tecidos e órgãos, como por exemplo, na motilidade, estrutura e integridade celular. A β-actina codifica uma proteína do citoesqueleto e foi um dos primeiros RNAs a ser utilizado como um padrão interno, sendo ainda defendida como uma referência quantitativa para ensaios de RT-PCR (KREUZER *et al.*, 1999). A β-actina é uma das seis formas diferentes de actina que foram até agora identificadas. Como o GAPDH, a β-actina é expressa em altos níveis em quase todos os tecidos e linhagens celulares, fazendo com que ela seja ideal como marcador controle.

O GAPDH é freqüentemente utilizado como um controle endógeno de análise quantitativa de RT-PCR, uma vez que, em alguns sistemas experimentais, a sua expressão é muito constante (EDWARDS e DENHARDT 1985; WINER *et al.*, 1999). No entanto, as

concentrações de GAPDH podem variar entre diferentes indivíduos (BUSTIN e MCKAY, 1999), como por exemplo, durante a gravidez (CALE *et al.*, 1997), com a etapa de desenvolvimento (PUISSANT *et al.*, 1994; CALVO *et al.*, 1997) e durante o ciclo celular (MANSUR *et al.*, 1993).

A Ubiquitina (UBQ) é uma pequena proteína altamente conservada de 8 kilodaltons que se torna covalentemente ligada a resíduos de lisina (Lys) de proteínas do alvo (HERSHKO e CIECHANOVER, 1992). Esta proteína é composta por 76 aminoácidos e desempenha um papel fundamental nos processos de degradação de proteínas, reparo do DNA, transdução de sinal, regulação transcricional e controle de receptores por endocitose (CHRISTENSEN *et al.*, 1992; CHAN *et al.*, 1995; OKAZAKI *et al.*, 2000). Van Tol *et al.* (2007) utilizaram com sucesso a UBQ para normalizar a expressão de vários genes em oócitos bovinos antes e após cultivo *in vitro.*

A β-tubulina é uma proteína encontrada nos microtúbulos. Os isotipos β-tubulina diferem principalmente na sua região C-terminal. Esta região tem sido demonstrada ser a região de ligação para um número de proteínas associadas aos microtubulos (MAPs) (LITTAUER *et al.*, 1986; PASCHAL *et al.*, 1989; SERRANO *et al.*,1985). A β-tubulina tem sido utilizada para normalizar a expressão gênica em vários tecidos, como por exemplo: Nijhof *et al.*, 2009 e Silveira *et al.*, 2009.

Como exemplos de outros genes de referência podemos citar PGK, RPL-19 e rRNA18S, que já foram utilizados para normalizar dados de PCR em tempo real em diferentes tipos de tecidos (SCHMIDT e DELANEY, 2010; KOK *et al.*, 2005).

## 2.7. Imunohistoquímica

Após a localização dos mRNAs, a imunohistoquímica é um método de diagnóstico complementar, que tem sido utilizada com bastante eficiência para localizar as respectivas proteínas. Esta técnica tem permitido a identificação de diferentes tipos de proteínas (enzimas, receptores e produtos de genes), que estão relacionados ao comportamento biológico das células, sendo utilizada com freqüência para avaliar a expressão de receptores de fatores de crescimento e hormônios presentes nos ovários, além de ser utilizada também como técnica de avaliação de proliferação das células da granulosa (ABIR *et al.*, 2006).

Vários estudos demonstraram que a técnica de imunohistoquimica pode ser utilizada com sucesso para localizar proteínas de fatores de crescimento em ovários caprinos. Dentre os fatotres localizados por esta técnica, podemo citar o VEGF (BRUNO *et al.*, 2009), o IGF-1 (SILVA *et al.*, 2009), o GH (SILVA *et al.*, 2009), o EGF (SILVA *et al.*, 2006a), o KL (SILVA *et al.*, 2006a), BMP-15 e GDF-9 (SILVA *et al.*, 2004).

## 3. PROBLEMA

O ovário desempenha duas funções prioritárias para o sistema reprodutivo de fêmeas mamíferas, sendo responsável pela: 1) diferenciação e liberação de um oócito maturo para fertilização (McGEE e HSUEH, 2000) e 2) síntese e secreção de hormônios que são essenciais para o desenvolvimento folicular, ciclicidade estral/menstrual e manutenção do trato reprodutivo e suas funções (HIRSHFIELD, 1991). Para que este órgão funcione perfeitamente, faz-se necessário a participação dos fatores de crescimento presentes no ovário mamífero que podem ser regulado por gonadotrofinas, somatotrofinas e por fatores intra-ovarianos. Inúmeros fatores de crescimento sintetizados pelas células foliculares atuam modulando o efeito dos hormônios e regulando o desenvolvimento dos folículos ovarianos (MARTINS *et al.,* 2008). Alterações de expressão gênicas são detectadas por meio da avaliação dos níveis de mRNA das amostras que se deseja estudar. Os genes de referência, que são utilizados para normalizar os dados, podem variar entre os diferentes grupos de amostras e por isso, vários estudos (POHJANVIRTA *et al.*, 2006; BANDA *et al.*, 2007; VAN ZEVEREN *et al.*, 2007; LIN *et al.*, 2009) tem sido realizados buscando uma adequada normalização dos dados para se distinguir entre o papel real (biológico) e os desvios resultantes de processos. Apesar destes estudos em outras espécies, levantou-se a seguinte problemática: qual(is) será(ão) o(s) genes de referências mais estável(is) em folículos pré-antrais caprinos?

Outros estudos também têm sido feito para se avaliar a expressão de receptores e fatores de crescimento em folículos ovarianos caprinos (XU *et al*., 1995; MCNATTY *et al*.,1999; GUTIERREZ *et al*., 2000; WEBB *et al.*, 2003), bem como para entender as interações dos fatores de crescimento com as gonadotrofinas (OTSUKA *et al.*, 2001; FORTUNE *et al.*, 2004; BURATINI *et al.*, 2005). No entanto, ainda não se sabe se existe diferença entre os níveis de expressão de receptores de FSH, LH e GH, bem como dos fatores de crescimento EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 e KL em folículos secundários caprinos. Vários estudos *in vitro* demonstrarm que as BMPs-6 e 7 são importantes reguladores da foliculogênese ovariana, mas em caprinos, ainda não se sabe se estes fatores promovem o crescimento folicular e controlam a expressão de R-FSH, BMP-6 e BMP-7 em ovários caprinos.

## 4. HIPÓTESES

1) Os genes de referência são expressos em diferentes níveis em folículos pré-antrais caprinos.

2) Os RNA mensageiros para as BMPs dos tipos 6 e 7 são expressos em diferentes níveis em folículos primordiais, primários e secundário, bem como em COCs e células da granulosa / teca de pequenos e grandes folículos antrais em caprinos.

3) O FSH e as BMPs dos tipos 6 e 7 estimulam o crescimento de oócitos inclusos em folículos pré-antrais *in vitro* durante 6 dias de cultivo e controlam a expressão de RNA para BMP-6, BMP-7 e R-FSH.

## 5. JUSTIFICATIVA

A compreensão da fisiologia ovariana em caprinos é de grande importância para o desenvolvimento do Nordeste do Brasil, pois esta região concentra cerca de 88% do rebanho caprino do país (10,4 milhões de animais). Considerando a dimensão territorial brasileira, bem como as condições climáticas favoráveis ao desenvolvimento da caprinocultura no Nordeste, este rebanho é numericamente inexpressivo, principalmente quando comparado com os bovinos, cujo efetivo nacional é da ordem de 150 milhões de animais.

No córtex do ovário mamífero contém uma grande população de folículos e a maior parte desses (cerca de 90%) estão no estágio inicial de desenvolvimento. Considerando que cerca de 99,9% desses folículos são eliminados pelo processo de atresia, faz-se necessário o estudo dos fatores de crescimento produzidos pelos folículos, juntamente com o isolamento e o cultivo *in vitro* de folículos pré-antrais para promover a maturação dos seus oócitos, prevenindo-os da atresia. Compreender a fisiologia ovariana em caprinos, através do desenvolvimento de biotecnologias na área da reprodução animal, como por exemplo, o estudo da expressão gênica através da técnica de PCR em tempo real é de grande importância para a compreensão da foliculogênese ovariana, fornecendo informações importantes que podem contribuir para acelerar a multiplicação e o melhoramento genético dos rebanhos. A quantificação do mRNA para BMP-6 e BMP-7 em diferentes categorias foliculares, bem como a avaliação dos níveis de mRNA para receptores de hormônios e outros fatores contribuirá de para elucidar a foliculogênese na espécie caprina. Além disso, a determinação de qual(is) gene(s) de referência(s) são mais estáveis em folículos secundários caprinos antes e após cultivo in vitro é essencial para avaliar o efeito do cultivo, bem como de hormônio e fatores de crescimento sobre a expressão de genes alvo. Todos estes estudos poderão contribuir de forma importante para o desenvolvimento de meios de cultivo eficientes para promover o desenvolvimento *in vitro* de folículos secundários. Além disso, para o estudo da foliculogênese ovariana, a quantificação de mRNA pode fornecer subsídeos para a identificação dos fatores de crescimento que devem ser adicionados ao meio de cultivo para determinar as interações ideais e promover a maturação oocitária.

O estudo da expressão dos fatores de crescimento e o cultivo de folículos pré-antrais *in vitro* é de grande importância para a reprodução animal. Os FOPA isolados do ambiente ovariano, bem como da influência endócrina e nutricional comum ao organismo animal, poderão ser cultivados *in vitro* em presença da proteína morfogenética óssea 6 e 7, cujo efeito individual ou associado poderá ser avaliado e controlado. Nos estágios finais de

desenvolvimento folicular, os folículos tornam-se dependentes de gonadotrofinas (KUMAR *et al.*, 1997) e as BMPs 6 e 7 tem sido descritas com ações modulatórias sobre os níveis de expressão de receptores de FSH (BMP-6: ARAÚJO, 2010; BMP-7: SHI *et al.*, 2009). Isto sugere um papel desses fatores de crescimento na preparação dos folículos para a ovulação.

Portanto, a estratégia para obter, com êxito, o completo desenvolvimento *in vitro* de um grande número de folículos pré-antrais se baseia num estudo minucioso da biologia molecular do ovário no que se refere à identificação dos mRNA para os fatores de crescimento que estão sendo produzidos pelos folículos. Em seguida, os fatores de crescimento seriam adicionados ao meio de cultivo para determinar as interações ideais para promover a maturação oocitária.

## 6. OBJETIVOS

### 6.1. Gerais

- Definir qual (is) genes de referência são mais estáveis em folículos secundários caprinos, antes e após cultivo *in vitro*.
- Avaliar os efeitos de um meio de cultivo, que contenha FSH e BMP-6 e 7, sob o crescimento e expressão gênica em folículos secundários caprinos durante 6 dias de cultivo.

### 6.2. Específicos

- Avaliar a estabilidade de genes de referência para quantificação de mRNA em folículos secundários caprinos.
- Quantificar a expressão dos RNA mensageiros para as BMPs dos tipos 6 e 7 em folículos primordiais, primários, secundários e antrais na espécie caprina.
- Avaliar o efeito do FSH sozinho ou em associação com fatores de crescimento (BMP- 6 e 7) sobre o crescimento de oócitos inclusos em folículos pré-antrais *in vitro* durante 6 dias de cultivo.
- Avaliar o efeito do FSH sozinho ou em associação com fatores de crescimento (BMP- 6 e 7) sobre a expressão de mRNA para BMP-6, -7, FSH-R.

7. ARTIGO 1

# Stability of housekeeping genes and expression of locally produced growth factors and hormone receptors in goat preantral follicles

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**Resumo**

O objetivo do presente estudo foi investigar a estabilidade dos sete genes de referência, e a expressão relativa de fatores de crescimento (EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 e KL) e receptores hormonais (FSH, LH e GH) em folículos pré-antrais. Para avaliar a estabilidade dos genes de referência, folículos frescos foram microdissecados (150-200 μm) e cultivados *in vitro* por 12 dias. Além disso, os folículos isolados frescos foram usados para comparar a expressão de vários fatores de crescimento e receptores hormonais antes do cultivo. Ambos os folículos, frescos e cultivados, foram submetidos à extração do RNA total e síntese do cDNA. Após a amplificação do cDNA por PCR em tempo real, o programa de software geNorm foi utilizado para avaliar a estabilidade do gliceraldeído-2-fosfato desidrogenase (GAPDH), β-tubulina, β-actina, fosfogliceroquinase (PGK), rRNA 18S, ubiquitina (UBQ ) e proteínas ribossomais 19 (RPL-19). Além disso, os níveis de mRNA folicular dos vários fatores de crescimento em estudo foram comparados. Os resultados demonstraram que, em folículos pré-antrais, UBQ e β-actina foram os genes de referência mais adequados e, portanto, podem ser utilizados como parâmetros para normalizar dados *in vitro*. Em contraste, o RNA 18S foi o gene menos estável entre os genes de referência testados. Na análise de mRNA para vários receptores de hormônios hipofisários, folículos frescos apresentaram níveis significativamente mais elevados de mRNA para R-FSH do que para R-LH e R-GH, e não houve nenhuma diferença entre os níveis de mRNA para R-GH e R-LH. Em relação a expressão do mRNA para os fatores de crescimento em folículos pré-antrais, os níveis de mRNA para EGF foram significativamente menores do que dos outros fatores de crescimento estudados. Sucessivamente, os maiores níveis de mRNA em folículos pré-antrais, foram observados para GDF-9, BMP-15, BMP-6, FGF-2, VEGF, KL e IGF-1,. Em conclusão, UBQ e β-actina são os genes de referência mais estáveis em folículos pré-antrais caprinos frescos e cultivados por 12 dias. Além disso, nos folículos frescos, altos níveis de mRNA para R-FSH foram detectados, enquanto entre os oito fatores de crescimento, IGF-1 é o mais expresso e EGF o menos expresso.

**Palavras-chave:** folículos ovarianos, fatores de crescimento, genes de referência, cabra, mRNA, receptores de hormônios hipofisários

**Stability of housekeeping genes and expression of locally produced growth factors and hormone receptors in goat preantral follicles**

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**Abstract**

The aim of the present study was to investigate the stability of six housekeeping genes, and the relative expression of growth factors (EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 and KL) and hormone receptors (FSH, LH and GH) in goat preantral follicles. To evaluate to stability of housekeeping genes micro-dissected fresh follicles (150-200 μm) as well as follicles that have been *in-vitro* cultured for 12 days were used. In addition, isolated fresh follicles were used to compare expression of various growth factors and hormone receptors before culture. Both fresh and cultured follicles were subjected to total RNA extraction and synthesis of cDNA. After amplification of cDNA by real-time PCR, the geNorm software programme was used to evaluate the stability of glyceraldehydes-2-phophate dehydrogenase (GAPDH), β-tubulin, β-actin, phosphoglycerokinase (PGK), 18S rRNA, ubiquitin (UBQ) and ribosomal protein 19 (RPL-19). In addition, follicular steady-state levels of mRNA from the various growth factors under study were compared. Results demonstrated that, in goat preantral follicles, UBQ and β-actin were the most suitable reference genes and thus could be used as parameters to normalise data from future in-vitro studies. In contrast, 18S RNA appeared the least stable gene among the tested housekeeping genes. Analysis of mRNA for several hypophyseal hormone receptors in fresh preantral

follicles showed higher FSH-R mRNA levels than those of LH-R and GH-R, and no difference between GH-R and LH-R mRNA levels. In regard growth factor mRNA expression in goat preantral follicles, EGF mRNA levels appeared significantly lower than those of the other studied growth factors. Increasingly higher relative mRNA levels were observed for GDF-9, BMP-15, BMP-6, FGF-2, VEGF, KL and IGF-1, successively. In conclusion, UBQ and β-actin are the most stable housekeeping genes in fresh and 12-days cultured caprine preantral follicles. Furthermore, in fresh follicles, high levels of FSH-R mRNA are detected while among eight growth factors, IGF-1 is the most highly expressed and EGF the weakest expressed compound.

**Key-words:** ovarian follicles, growth factors, housekeeping genes, goat, mRNA, hypophyseal hormone receptors

## 1. Introduction

The mammalian ovary contains thousands of follicles at birth and preantral (i.e., primordial, primary and secondary) follicles represent 90% of this follicular population. However, the vast majority (99.9%) of these follicles become atretic during their growth and maturation (MARKSTRÖM *et al.*, 2002). Although many preantral follicles degenerate (VAN DEN HURK *et al*., 1998), numerous healthy ones still can be used in assisted reproductive programmes after *in vitro* growth and maturation (SILVA *et al.,* 2002). Because of this, different isolation techniques and culture systems for preantral follicles have been developed in the past years. In various species (bovine: GUTIERREZ *et al.,* 2000; ovine: Cecconi *et al.,* 1999**;** murine: Cortvrindt *et al.,* 1996; caprine: ZHOU and ZANG, 2005; Huanmin and Young, 2000), preantral follicles have been cultured up to antral stages of development. Systemically delivered pituitary hormones and locally produced growth factors have an important role in maintaining follicular viability and to promote follicle growth and differentiation (for reviews, see FORTUNE, 2003; VAN DEN HURK and ZHAO, 2005). These compounds act on follicles through their receptors. Such binding sites for FSH (FSH-R) have been demonstrated in granulosa cells of preantral follicles in hamsters (Roy and Treacy, 1993), cows (WANDJI *et al.*, 1992), sheep (ECKERY *et al*., 1997) and rats (MONNIAUX and RENVIERS, 1989), suggesting that FSH is involved in early follicle growth. Indeed, FSH has been demonstrated to stimulate *in-vitro* growth of primary and

secondary follicles from various mammalian species (e.g., human: ROY and TREACY, 1993; ABIR *et al*., 1997; WRIGHT *et al*., 1999; cow: HULSHOF *et al*., 1995; WANDJI *et al*., 1996; sheep: NEWTON *et al*., 1999, and mouse: SPEARS *et al*., 1998; CORTVRINDT *et al*., 1997). In rat secondary follicles, LH receptors (LH-R) have been demonstrated in the theca layer (KISHI and GREENWALD, 1999a) which was stimulated to produce progesterone and androstendione after addition of equine chorionic gonadotrophin to the follicular culture medium (KISHI and GREENWALD, 1999b). As in rodents, bovine secondary follicles also expressed LH receptors in theca cells (BRAW-TAL and ROTH, 2005). In rat ovaries, growth hormone receptor (GH-R) mRNA was abundantly expressed in granulosa cells (CARLSSON *et al*., 1993; ZHAO *et al*., 2002), while positive immunostaining for GHR was detected in both oocytes and granulosa cells (ZHAO *et al*., 2002). *In-vivo* experiments showed that GH promotes ovarian follicle development (GONG *et al*., 1991; GONG *et al*., 1997) and reduces regression of mouse follicles (DANILOVICH *et al*., 2000). In ovine (ECKERY *et al*., 1997) and human (ABIR *et al*., 2008) preantral follicles, expression of GH-R mRNA was demonstrated in both oocytes and granulosa cells. Thus far, however, there is little information on the relative expression of hypophyseal hormone receptors and growth factors in goat preantral follicles.

Pituitary hormones may influence ovarian folliculogenesis through the control of follicular growth factor production, and locally produced growth factors on their turn may regulate hormone actions on follicles through up-regulation or down-regulation of the receptors of these endocrine compounds (VAN DEN HURK and ZHAO, 2005). Among the ovarian growth factors, epidermal growth factor (EGF) is expressed in human (REEKA *et al*., 1998; QU *et al*., 2000) and hamster (GARNETT *et al*., 2002) primordial and primary follicles, and in caprine primordial, primary and secondary follicles (SILVA *et al.,* 2006a). In goats, EGF promotes primordial follicle growth (SILVA *et al.,* 2004a), while in rats, EGF locally controls the action of FSH and LH by inhibiting LH-R synthesis (HATTORI *et al.,* 1995) and increases that of FSH-R (LUCIANO *et al.,* 1994).

Fibroblastic growth factor-2 (FGF-2) mRNA is localised in granulosa cells and theca cells of developing rat follicles (GUTHRIDGE *et al*., 1992; ORTEGA *et al.,* 2007) and in preantral human follicles (QUENNEL *et al*., 2004; BEN-HAROUSH *et al*., 2005). FGF-2 has been found to activate mouse (NILSSON *et al,* 2001) and caprine (MATOS *et al*., 2006) primordial follicles and to stimulate subsequent growth to primary stages and beyond. Transcripts for IGF-1 mRNA were detected in rat granulosa cells of developing secondary,

early antral and preovulatory follicles (OLIVER *et al*., 1989). In goat follicles IGF-1 mRNA is expressed in all stages of development (SILVA *et al.,* 2007) and the IGF-1 protein stimulates preantral follicle growth in goats (ZHOU and ZANG, 2005) and rats (ZHAO *et al.*, 2001). IGF-1 binds with higher affinity for IGFR-1 and low affinity for IGFR-2 (SILVA *et al*., 2009), which are present in granulosa cells of primary follicles, secondary and antral (MONGET *et al*., 1989). Like IGF-1 mRNA, Kit ligand (KL) expression was detected at all stages of caprine follicular development (SILVA *et al*., 2006b). In rabbit and murine follicles, KL is produced by granulosa cells and has an important role in primordial follicle activation, recruitment of theca cells, antrum formation and oocyte maturation (HUTT *et al.*, 2006**)**. The receptor for KL (c-kit) is expressed by oocytes and thecal-interstitial cells, enabling them to respond to this growth factor (KNIGHT and GLISTER, 2006).

During human folliculogenesis, vascular endothelial growth factor (VEGF) is produced by theca cells (YAMAMOTO *et al*., 1997) and, late in follicle development, also in granulosa cells (KAMAT *et al*., 1995). In response to gonadotrophins, the ovarian VEGF level is increased in rats (KOOS, 1995), wherby it promotes growth of preantral follicles (DANFORTH *et al*., 2001), the number of healthy follicles and follicular angiogenesis (TAJIMA *et al*., 2005). In the goat, VEGF receptor-2 is expressed in oocytes and granulosa cells of all follicular stages, while VEGF maintains follicular ultrastructural integrity and promotes primordial follicle growth (BRUNO *et al*., 2009).

TGF-β family members, like the bone morphogenetic proteins 6 (BMP-6) and 15 (BMP-15) and growth differentiation factor-9 (GDF-9) are also implicated in the growth of preantral follicles. BMP-6 is expressed in goat preantral follicles (SILVA *et al.,* 2007) and promotes granulosa cell viability in cows (GLISTER *et al.,* 2004). This factor plays its biological activity by binding with the receptors bone morphogenetic protein receptor II (BMPRII) and BMPRIB (also termed ALK6) (SHIMASAKI *et al*., 2004). BMP-15 is expressed in the oocytes of all types of goat follicles (SILVA *et al.,* 2004b), and stimulates both granulosa cell proliferation and development of primordial and primary follicles (JUENGEL and MCNATTY, 2005). GDF-9 is secreted by oocytes (mouse: CHANG *et al.,* 2002) and granulosa cells (goat: SILVA *et al.,* 2004b) of preantral follicles. In humans, it promotes follicular viability and growth of preantral follicles by granulosa cell proliferation (HREINSSON *et al.,* 2002). It has recently been established that GDF-9 signaling involves interaction with TGF-βRI (also known as ALK5) and BMPRII on the target cell surface, while BMP-15 signaling involves BMPRIB and BMPRII (JUENGEL and MCNATTY,

2005). Expression of each of these receptor types has been detected in granulosa cells from the primordial/primary follicle stage onwards (JUENGEL and MCNATTY, 2005).

To optimise preantral follicle development *in vitro*, it is important to study follicular mRNA levels of hormone receptors and growth factors. Hereby, the choice of a correct reference gene to normalise gene expression in quantitative real-time PCR is essential to truly reflect biological processes (GARCIA-VALLEJO *et al.,* 2004). Housekeeping genes are used as endogenous controls for normalising expression levels evaluated with RT-PCR. Ideal housekeeping genes are constitutively expressed, do not respond to external stimuli and exhibit little or no sample-to-sample or run-to-run variation (BANDA *et al*., 2008). Most of the housekeeping genes are involved in basic cell metabolism [for example: glyceraldehyde-3-phophate dehydrogenase (GAPDH), phosphoglycerokinase (PGK), 18S rRNA, ubiquitin (UBQ) and ribosomal protein 19 (RPL-19)] or are cytoskeletal structural proteins [β-tubulin and β-actin]. However, after several reports of variation in putative housekeeping genes (SCHMITTGEN and ZAKRAJSEK, 2000; SCHMID *et al.,* 2003; RADONIC *et al.,* 2004; HUGGETT *et al.,* 2005) it has become clear that no reference gene can be assumed to be suitable for any given set of condition. Vandesompele *et al.* (2002) demonstrated that errors in expression data up to 20-fold can be generated by use of only a single reference gene. Various studies showed the stability of housekeeping genes in tissues from mice (KOUADJO *et al.,* 2007), pig (NYGARD *et al.,* 2007) and in embryos of bovine (GOOSSENS *et al.,* 2005), swine (KUYK *et al.,* 2007) and murine (MAMO *et al.,* 2007) species. Currently, however, there are no data about both the stability of housekeeping genes and the levels of mRNA for hormone receptors, like FSH, LH and GH, and growth factors, like EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 and KL, in caprine preantral follicles.

The aim of the present study is to investigate the stability of GAPDH, β-tubulin, β-actin, PGK, UBQ, RNA 18S, RPL19 expression in fresh and in-vitro cultured goat preantral follicles, and to evaluate the relative expression of growth factors (EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 and KL) and hormone receptors (FSH, LH and GH) in fresh follicles.

## 2. Material and Methods

### Ovaries, follicle isolation and *in vitro* culture

Ovaries of adult goats (n=10) were collected in local abattoir immediately after slaughter. After collection, the ovaries were washed once in alcohol 70% for about ten seconds, and then twice in 0.9 % saline solution and transported to the laboratory at 4°C for up to 1h. Then, the ovaries were carefully dissected and placed immediately in warmed culture medium, consisting of α-MEM. Briefly, ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface and large preantral follicles were visualized under the stereomicroscope, manually isolated using 26-gauge needles attached to a syringe and washed two times in α-MEM. After isolation, follicles were transferred to 100 µL drops containing fresh medium at room temperature for evaluation. For this study, only follicles (150-200 µm in diameter) with a centrally-located oocyte surrounded by compact layers of granulosa cells, an intact basal membrane, and no antral cavity were selected. Isolated follicles from five ovaries were randomly distributed into three groups of ten preantral follicles that were stored at -80$^{o}$C until RNA extraction.

For *in-vitro* culture, another group of isolated follicles (n=30) from five ovaries were randomly transferred to 100 µL drops containing fresh medium under mineral oil to further evaluate the follicular quality. Then, health preantral follicles were individually cultured in 25 µL drops of culture medium in petri-dishes (60 x 15 mm, Corning, USA). The culture medium was called α-MEM$^{+}$ and consisted of α-MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/mL bovine serum albumin (BSA), insulin 6.25 µg/mL, transferrin 6.25 µg/mL and selenium 6.25 ng/mL (ITS), 2 mM glutamine, 2 mM hypoxantine and 50 µg/mL of ascorbic acid under mineral oil. Incubation of follicles was conducted at 39 °C, for 12 days. Fresh media were prepared and immediately incubated for 1h at 39°C prior to use. Preantral follicles were randomly distributed in α-MEM$^{+}$ that was supplemented with 100 ng/mL FSH (from day 0 to day 6) and 500 ng/mL FSH (from day 6 to day 12). Every other day, 5 µL of the culture media was added because of evaporation and at least 30 follicles were cultured. After culture, three groups of ten cultured follicles were collected and stored at - 80$^{o}$C until RNA extraction.

**RNA Extraction and cDNA Synthesis**

Total RNA was extracted using the Trizol® reagent (Invitrogen, São Paulo, Brazil). The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (Amersham, Biosciences. Cambridge, England). For each sample, the RNA concentrations were adjusted and 44 ng/mL was used to synthesise cDNA. Before the reverse transcription reaction, samples of RNA are incubated for 5 minutes at 70°C and then cooled in ice. The reverse transcription was performed in a total volume of 20 µL composed of 10 µL of sample RNA, 4 µL reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNAsin, 150 units of reverse transcriptase Superscript III, 0036 U random primers, 10 mM DTT and 0.5 mM of each dNTP (Invitrogen, São Paulo, Brazil). The mixture was incubated at 42°C for 1h, subsequently at 80°C for 5 min, and finally stored at -20°C. The negative control is prepared under the same conditions, but without addition of reverse transcriptase.

**PCR amplification and determination of gene stability**

To identify the most stable housekeeping gene for its use in studies with fresh and cultured preantral follicles, quantification of mRNA for glyceraldehydes-2-phophate dehydrogenase (GAPDH), β-tubulin, β-actin, phosphoglycerokinase (PGK), 18S rRNA, ubiquitin (UBQ) and ribosomal protein 19 (RPL-19) was performed with the use of Syber green. Each reaction in real time (20 µL) containing 10 µL of Syber green Master Mix® (Applied Biosystems, Warrington, UK), 7.3 µL of ultra pure water, 1 µL of cDNA and 0.85 M of each primer real-time PCR is performed in a thermocycler (Master Cycler, Eppendorf, Germany). The primers, chosen to carry out amplification of different housekeeping genes, are shown in Table 1. The reactions of the cDNA by PCR amplification consist of initial denaturation and polymerase activation for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C and 30 sec at 60°C. The extension will be held for 20 min at 72°C.

Gene stability was evaluated using the geNorm software programme (VANDERSOMPELLE *et al.,* 2002). Briefly, this approach relies on the principle that the expression ratio of two perfect reference genes would be identical in all samples in all experimental conditions or cell types. Variation in the expression ratios between different samples reflects expression instability of one or both of the genes. Therefore, increasing

variation in this ratio corresponds to decreasing expression stability. The geNorm software can be used to calculate the gene expression stability measure (M), which is the mean pair-wise variation for a gene compared with all other tested control genes. Genes with higher M-values have greater variation in expression. The stepwise exclusion of the gene with the highest M-value allows the ranking of the tested genes according to their expression stability.

**Quantification of mRNA for hormone receptors and growth factors**

Quantification of mRNA for different growth factors and hormone receptors were performed to compare their expression in fresh preantral follicles. The primer chosen to carry out amplification of different growth factors (EGF, FGF-2, BMP-6, BMP-15, KL, VEGF, GDF-9 and IGF-1) as well as the receptor for LH, FSH and GH are shown in Tables 2 and 3, respectively.

**Statistical analysis**

Data of mRNA expression of different growth factors (EGF, FGF-2, BMP-6, BMP-15, KL, VEGF, GDF-9 and IGF-1) in large preantral follicles were analyzed by the paired t-test ($P<0.05$). Comparison among R-LH, R-FSH and R-GH were performed using the non parametric Kruskal-Wallis test ($P<0.05$).

**3. Results**

**Stability of housekeeping genes in caprine preantral follicles**

Analysis of starting cDNA determined gene expression stability in fresh and 12-days cultured goat preantral follicles and resulted in gene expression stability values M for each gene. Therefore, stepwise exclusion of unstable genes and subsequent recalculation of the average M-values resulted in a ranking of the genes based on their M-values with the two most stable genes leading the ranking. After stepwise elimination of the least stable gene (18S RNA) it was revealed that the genes with the highest expression stability in goat preantral follicles before and after *in vitro* culture were β-actin and ubiquitin (Figure 1A, B).

**Expression of FSH-R, LH-R, GH-R and growth factors in caprine preantral follicles**

Real time-PCR demonstrated that the steady-state levels of FSH-R mRNA in fresh preantral follicles are higher than those of LH-R mRNA. However, neither difference was observed between FSH-R and GH-R no between LH-R mRNA and GH-R mRNA in caprine preantral follicles (Figure 2). Expression profiles for eight genes (EGF, GDF-9, BMP-15, VEGF, FGF-2, BMP-6, IGF-1 and KL) were determined relative to the least expressed gene. As shown in Table 1, EGF had the lowest levels of mRNA (1.0) in goat preantral follicles and was used as calibrator. When compared to EGF, all growth factors studied had higher relative mRNA expression rates, i.e., in sequence of increasing expression, GDF-9, BMP-15, BMP-6, FGF-2, VEGF, KL and IGF-1.

Relative to GDF-9, the steady-state levels of BMP-6 and IGF-1 mRNA were significantly higher (Figure 3A), whereas, compared to BMP-15 mRNA, IGF-1 and KL mRNA had higher levels (Figure 3B). No significant differences were observed between the mRNA levels of VEGF, BMP-6, FGF-2, KL and IGF-1 (Figure 3C), but the mRNA levels of KL and IGF-1 were significantly higher than that of FGF-2 (Figure 3D). Furthermore, the mRNA level of IGF-1 was significantly higher than that of FGF-2 (Figure 3E), but not different from that of KL (Figure 3F).

## 4. Discussion

The current study demonstrated that, among the tested housekeeping genes, β-actin and ubiquitin are the most stable genes in goat preantral follicles, while 18sRNA is the least stable gene. In comparison, ubiquitin, β-actin and GAPDH were the three most stable housekeeping genes in bovine oocytes, while ubiquitin and PGK were the most stable genes in fresh and cultured bovine cumulus cells (VAN TOL *et al.,* 2007). Different from bovine oocytes, GAPDH was considered the least stable gene in zebrafish embryos (LIN *et al.*, 2009). The data confirm the opinion of Garcia-Vallejo *et al.* (2004) that assessment of the most suitable housekeeping gene(s) for different types of animal tissues and cells is inevitably, and that no reference gene can at forehand be assumed to be suitable for them.

In caprine preantral follicles, derived from the same animals that have been used for the housekeeping gene studies, the level of FSH-R mRNA was higher compared to that of

LH-R. Thus far, little is known about the relative expression of hypophyseal hormone receptors in ovarian preantral follicles. Previous studies showed binding sites/receptors for FSH in granulosa cells of preantral and antral follicles and that FSH may act on the development of (WANDJI *et al.*, 1992; HULSHOF *et al*., 1995; GUTIERREZ *et al.,* 2000) and growth factor expression in (JOYCE *et al*., 1999; WANG e ROY, 2004; THOMAS *et al.,* 2005) early and more advanced follicles of various mammalian species. Braw-Tal e Roth (2005) demonstrated the presence of LH-R in theca cells of bovine preantral follicles and that this is accompanied by reduced follicular atresia. FSH and LH appeared both able to support murine preantral follicle development *in vitro* (CORTVRINDT *et al.*, 1998, WU *et al.,* 2000).

In caprine preantral follicles, the level of GH-R mRNA was not significantly aberrant from those of FSH-R and LH-R. To our knowledge there is nothing known in literature about the relative expression levels of these hormones in preantral follicles of other animals. Eckery *et al*. (1997) previously demonstrated abundant expression of GH-R mRNA in oocytes and granulosa cells of ovine preantral and antral follicles. In the bovine ovary, GH-R mRNA was detected in oocytes of primordial and primary follicles and in follicular cells from the primary follicle stage onward (cow: KOLLE *et al*., 1998; rat: ZHAO *et al*., 2002). In cows, GH was found to promote granulosa cell proliferation and to increase progesterone synthesis (LANGHOUT *et al.,* 1991), and in rodents, to promote preantral follicle development by positively affecting the proliferation and ultrastucture of both granulosa and theca cells (LIU *et al.,* 1998; KIKUCHI *et al.,* 2001; ZHAO *et al*., 2000; KOBAYASHI *et al.,* 2000).

Among the studied growth factor mRNAs in caprine preantral follicles, IGF-1 appeared most highly expressed, followed by KL, VEGF, FGF-2, BMP-6, BMP-15, GDF-9 and EGF, in decreasing extent of expression successively. Involvement of IGF-1 in early stages of folliculogenesis was shown in knock-out experiments with mice, since the development of preantral and antral follicles was impaired in animals lacking the IGF-1 gene (ELVIN and MATZUK, 1998). *In-vitro* studies demonstrated that IGF-1 stimulates the development of bovine (GUTIERREZ *et al*., 2000; FORTUNE *et al*., 2004) and rat (ZHAO *et al*., 2001) preantral follicles. KL did increase the number of developing follicles during culture of mouse ovaries, suggesting a role in primordial follicle activation (PARROT and SKINNER, 1999). The expression of KL was stimulated by BPM-15, while KL inhibited BMP-15 expression. In contrast, GDF-9 inhibited KL expression in cultured granulosa cells (JOYCE *et al*., 2000).

VEGF production was brought about by theca cells of human developing follicles (YAMAMOTO *et al*., 1997) and in granulosa cells of human maturing follicles (KAMAT *et al*., 1995), and was found to promote follicular angiogenesis in ruminants and primates (REDMER and REYNOLDS, 1996). Injection of VEGF in mouse ovaries not only increased vascularisation, but also promoted follicle development and reduced follicular apoptosis (QUINTANA *et al*., 2004). Alterations in the levels of mRNA for VEGF were observed during primordial to primary follicle transition in rat ovaries (KEZELE *et al*., 2005). In preantral follicles, FGF-2 expression was previously shown in bovine oocytes and granulosa cells (VAN WEZEL *et al*., 1995) and in human oocytes (BEN-HAROUSH *et al*., 2005) and granulosa cells (QUENNELL *et al.,* 2004). FGF-2 appeared an important regulator of early folliculogenesis, promoting primordial follicle activation (mouse: NILSSON *et al*., 2001, goat: MATOS *et al.,* 2006), growth of activated follicles (goat: MATOS *et al*., 2006), and granulosa cell proliferation (chicken: ROBERTS and ELLIS, 1999). FGF-2 also plays a major role in ovarian angiogenesis (REYNOLDS and REDMER, 1998; BERISHA *et al.*, 2004).

In ovine ovaries, BMP-6 mRNA was previously detected in oocytes of all follicular stages, but not in granulosa and theca cells (JUENGEL *et al.,* 2006). However, in rat (ERICKSON and SHIMASAKI, 2003) ovaries BMP-6 was expressed in both oocytes and granulosa cells. In mouse granulosa cells, BMP-6 was found to inhibit FSH-R expression and to increase FSH-stimulated progesterone production (OTSUKA *et al.,* 2001). Expression of BMP-15 mRNA was previously detected in all preantral follicle stages (SILVA *et al*., 2004b). *In-vitro* studies furthermore showed that BMP-15 decreases expression of FSH-R (OTSUKA *et al*., 2001) and increases that of KL in rat granulosa cells (OTSUKA and SHIMASAKI, 2002). Thomas *et al.* (2005) also demonstrated that BMP-15 increases expression of KL in cultured oocytes and granulosa cells, and that the level of BMP-15 transcripts was reduced in the presence of FSH. BMP15 induced granulosa cell proliferation was inhibited after blocking KL and c-kit signaling during co-culture of rat granulosa cells and oocytes, which suggests that BMP-15 acts via KL (OTSUKA and SHIMASAKI, 2002; SHIMASAKI *et al*., 2003). Shimasaki *et al*. (2003) hypothesised that an interaction between BMP-15 and KL promotes the development of primordial follicles, while GDF-9 was secreted by oocytes from growing follicles to stimulate granulosa cell proliferation and to modulate KL action. In the current experiments, expression of BMP-15 may have caused the relative high level of KL in caprine preantral follicles.

Despite being one of the least expressed growth factor in caprine preantral follicles, GDF-9 was 91.0 times more expressed than the most weakly expressed EGF gene. Hreinsson *et al*. (2002) showed that GDF-9 promotes survival and growth of cultured human preantral follicles, while it inhibits KL expression in granulosa cells (mouse: JOYCE *et al.,* 2000, hamster: WANG and ROY, 2004) and preantral follicle development (hamster: WANG and ROY, 2004). Consequently, relatively low expression of GDF-9 may have contributed for high levels of KL in caprine preantral follicles. In addition, GDF-9 synergises with FSH to promote growth and differentiation of murine preantral follicles (HAYASHI *et al*., 1999). In bovine, GDF-9 reduced the production of progesterone and oestradiol by granulosa cells (SPICER *et al.,* 2006) and that of progesterone and androstenedione by theca cells (SPICER *et al.,* 2008), respectively.

In caprine preantral follicles, EGF came up as the least expressed gene among the growth factor mRNAs tested. In mice, the follicular EGF mRNA level is regulated by LH (HSIEH *et al.*, 2009), while EGF, amphiregulin, epiregulin, and betacellulin are potent stimulators of oocyte maturation and cumulus expansion. Several papers reported that EGF inhibts the expression of LH-R (e.g., HATTORI *et al*., 1995) and increases the expression of FSH-R (e.g., LUCIANO *et al*., 1994). *In-vitro* studies furthermore showed that EGF stimulates growth of bovine preantral follicles (GUTIERREZ *et al.,* 2000), and promotes granulosa cell proliferation in porcine preantral follicles (MORBECK *et al.,* 1993), as well as growth of these follicles up to the antral stage, while reducing degeneration of the granulosa cells (MAO *et al*., 2004).

In conclusion, ubiquitin and β-actin are the most stable genes among several tested putative housekeeping genes in fresh and cultured caprine preantral follicles, and thus are most useful in normalising starting quantities of cDNA during RT-PCR analysis. Among eight tested growth factors, IGF-1 was most abundantly expressed in goat follicles, whereas EGF showed the weakest expression. Knowing the levels of mRNA for hormone receptors and growth factors in fresh preantral follicles is very important for *in vitro* studies, since will provide helpful information to choose which growth factor or hormone will be used as a supplement for culture medium.

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Table 1: Primer pairs used in real-time PCR for quantification of growth factors mRNAs in fresh and 12-days cultured caprine preantral follicles.

| Target gene | Primer sequence (5′→3′) | Sense (s) Anti-sense (as) | Position | Genbank accession n°. |
|---|---|---|---|---|
| GDF-9 | ACAACACTGTTCGGCTCTTCACCC | s | 332 – 356 | GI:51702523 |
| | CCACAACAGTAACACGATCCAGGTT | as | 426-451 | |
| IGF-1 | ATGCTCTCCAGTTCGTGTGT | s | 182-202 | GI:217666 |
| | TTGAGAGGCGCACAGTACATCT | as | 319-341 | |
| BMP-6 | ACACATGAACGCCACCAACCAT | s | 141-163 | GI:76262832 |
| | AGGATGACGTTGGAGTTGTCGT | as | 262-284 | |
| BMP-15 | AAGTGGACACCCTAGGGAAA | s | 237-257 | GI:8925958 |
| | TTGGTATGCTACCCGGTTTGGT | as | 362-384 | |
| VEGF | ATGCGGATCAAACCTCACCAAAGC | s | 74-98 | GI:21747875 |
| | TGCAGGAACATTTACACGTCTGCG | as | 251-275 | |
| FGF-2 | AGTGTGTGCAAACCGTTACCTTGC | s | 228-252 | GI:536865 |
| | ATACTGCCCAGTTCGTTTCAGTGC | as | 375-399 | |
| KL | AGCGAGATGGTGGAACAACTGTCA | s | 211-235 | GI:16580734 |
| | GTTCTTCCATGCACTCCACAAGGT | as | 328-352 | |
| EGF | TCCCAGGTTCTCTTAAGTGCCT | s | 46-68 | GI:6031163 |
| | AACAGCCGCTTATCAAGCACATCC | as | 218-242 | |

Table 2: Primer pairs used in real-time PCR for quantification of housekeeping genes in fresh and 12-days cultured caprine preantral follicles.

| Target gene | Primer sequence (5′→3′) | Sense (s) Anti-sense (as) | Position | Genbank accession n°. |
|---|---|---|---|---|
| GAPDH | TGTTTGTGATGGGCGTGAACCA | s | 288- 309 | Gi:27525390 |
| | ATGGCGTGGACAGTGGTCATAA | as | 419-440 | |
| β- actin | ACCACTGGCATTGTCATGGACTCT | s | 188-211 | GI:28628620 |
| | TCCTTGATGTCACGGACGATTTCC | as | 363-386 | |
| RNA-18S | TTTGGTGACTCTAGATAACCTCGGGC | s | 175-201 | GI:58760943 |
| | TCCTTGGATGTGGTAGCCGTTTCT | as | 334-358 | |
| PGK | AGCCTTCCGAGCTTCACTTT | s | 444-466 | GI:77735550 |
| | AAACCTCCAGCCTTCTTTGGCA | as | 541-563 | |
| RPL-19 | ATGAAATCGCCAATGCCAACTCCC | s | 89-113 | GI:94966830 |
| | TTGGCAGTACCCTTTCGCTTACCT | as | 233-257 | |
| UBQ | GAAGATGGCCGCACTCTTCTGAT | s | 607-631 | GI:57163956 |
| | ATCCTGGATCTTGGCCTTCACGTT | as | 756-780 | |
| β-tubulin | TTCATTGGCAACAGCACAGCCA | s | 1100-1121 | GI: 114052730 |
| | TCGTTCATGTTGCTCTCAGCCT | as | 1229-1250 | |

Table 3: Primer pairs used for mRNA quantification of RNA for FSH-R, GH-R and LH-R in fresh and 12-days cultured caprine preantal follicles.

| Target gene | Primer sequence (5´→3´) | Sense (s) Anti-sense (as) | Position | Genbank accession n°. |
|---|---|---|---|---|
| LH- R | CGATTTCACCTGCATGGCACCAAT | a | 14-38 | GI: 20977242 |
| | GATTGGCGCATGAATTGACGGGAT | as | 117-141 | |
| GH-R | TGGAATACTTGGGCTAACAGTGAC | a | 35-59 | GI: 9968297 |
| | TCCTTCCTTGAGGAGATCTGGA | as | 145-167 | |
| FSH-R | AGGCAAATGTGTTCTCCAACCTGC | a | 250-274 | GI:95768228 |
| | TGGAAGGCATCAGGGTCGATGTAT | as | 316-340 | |

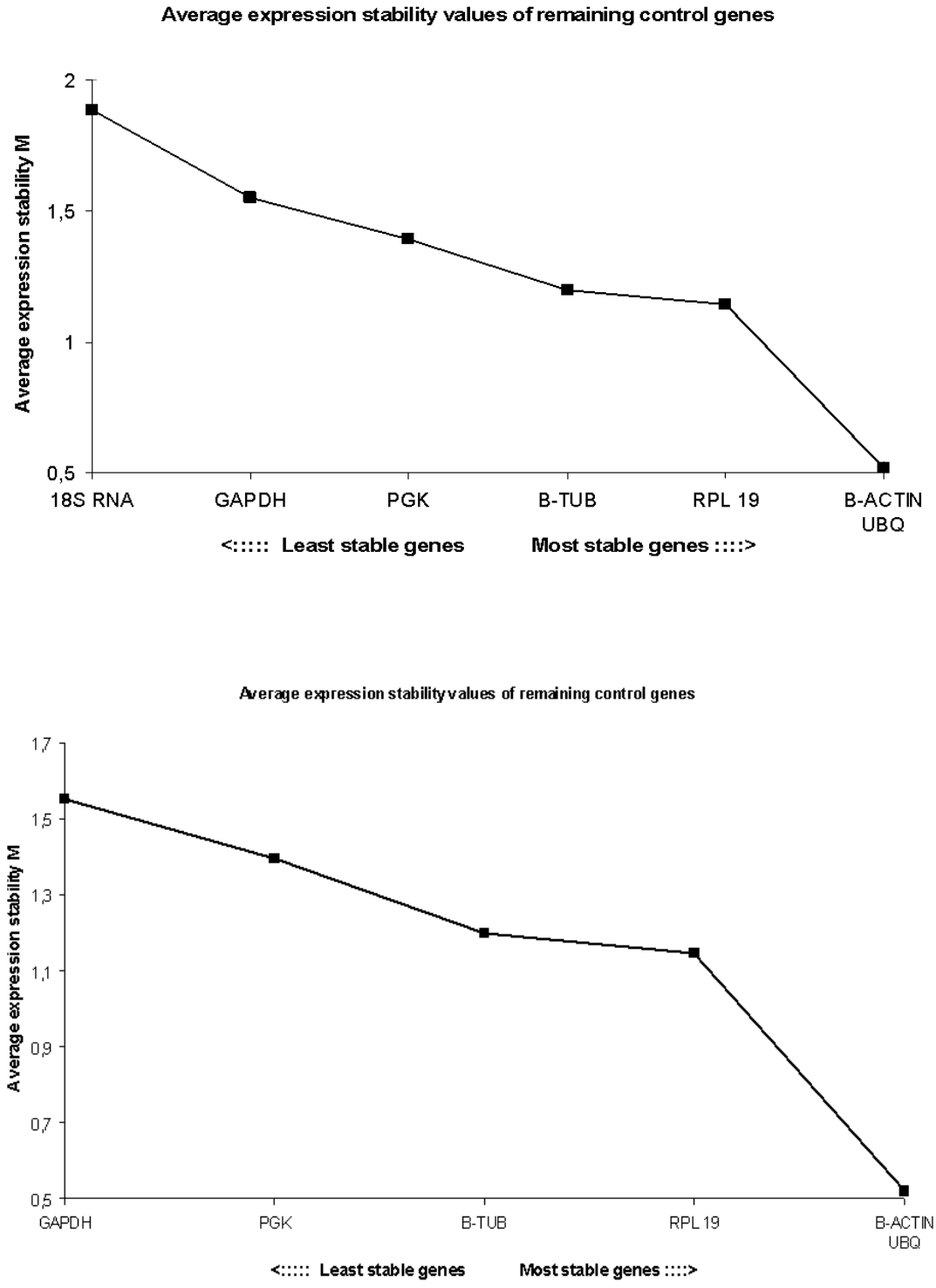



Figure 1: Stability of housekeeping genes in goat preantral follicles before (A) and after (B) elimination of the least stable housekeeping gene (18S RNA).

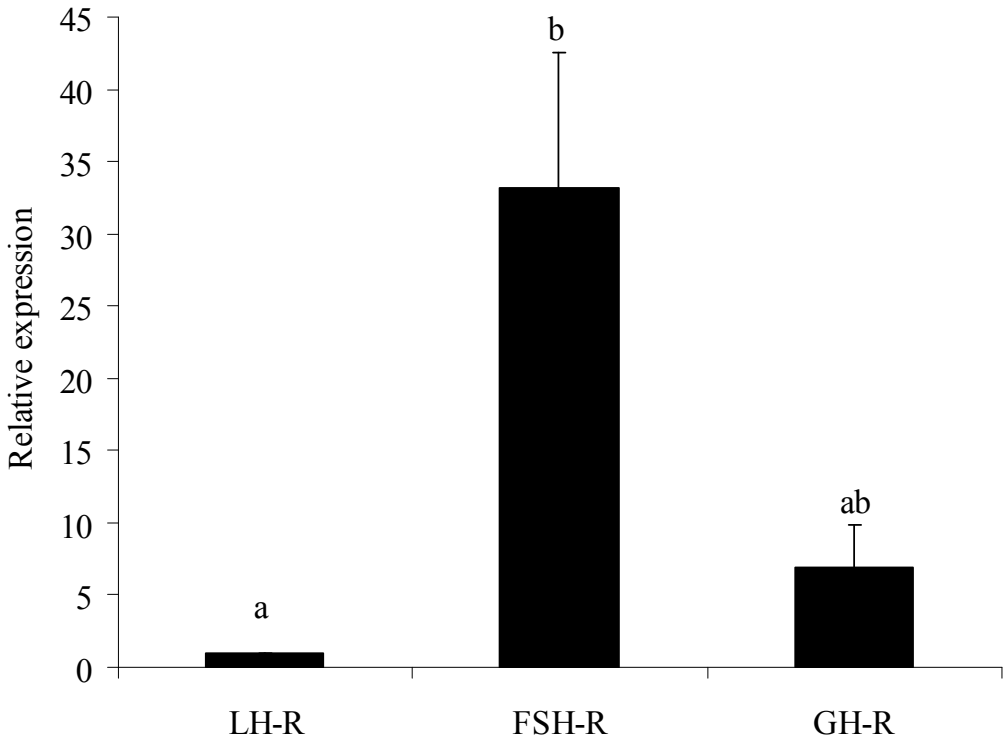
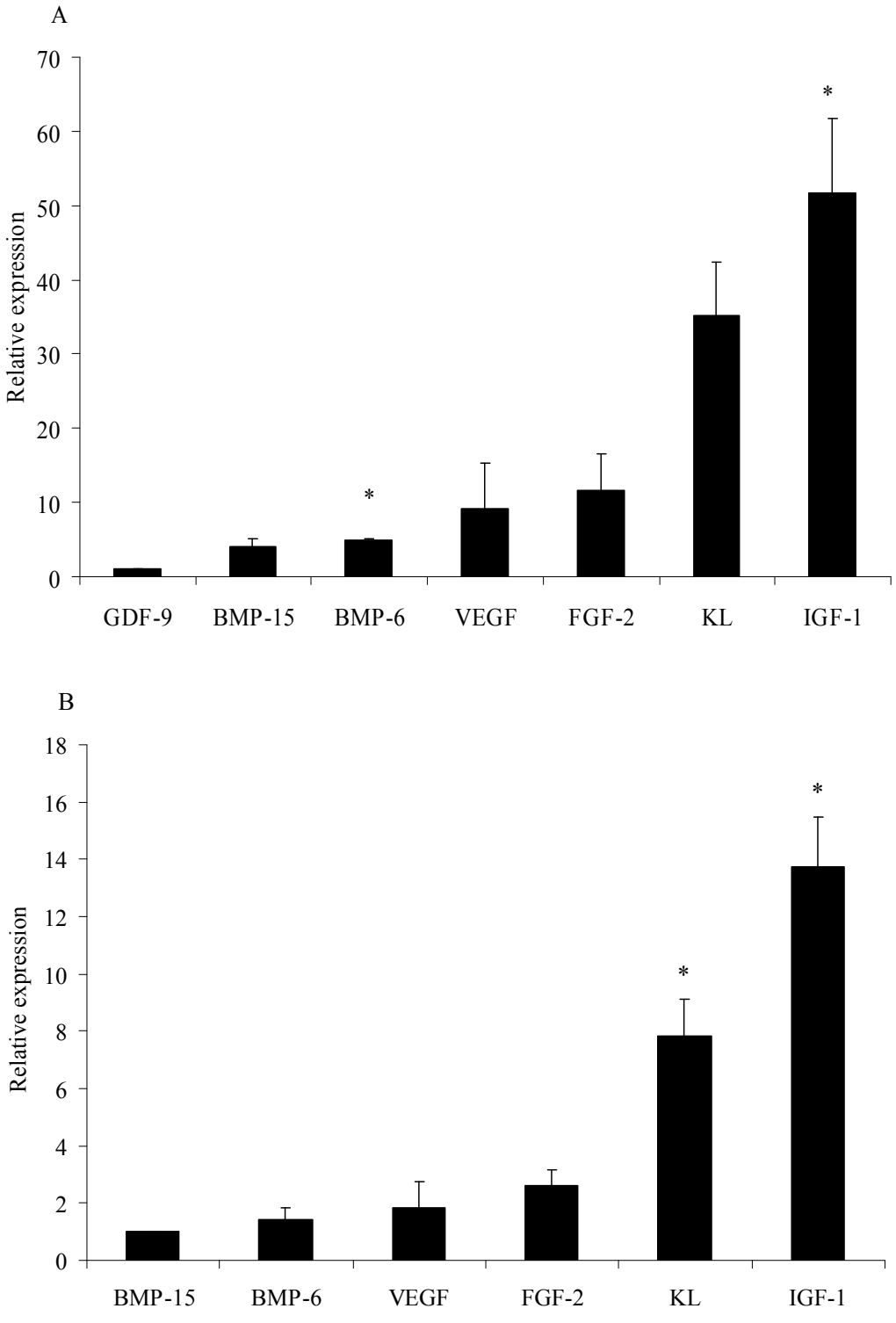



Figure 2: Steady-state levels of FSH-R, LH-R, and GH-R mRNA in caprine preantral follicles.

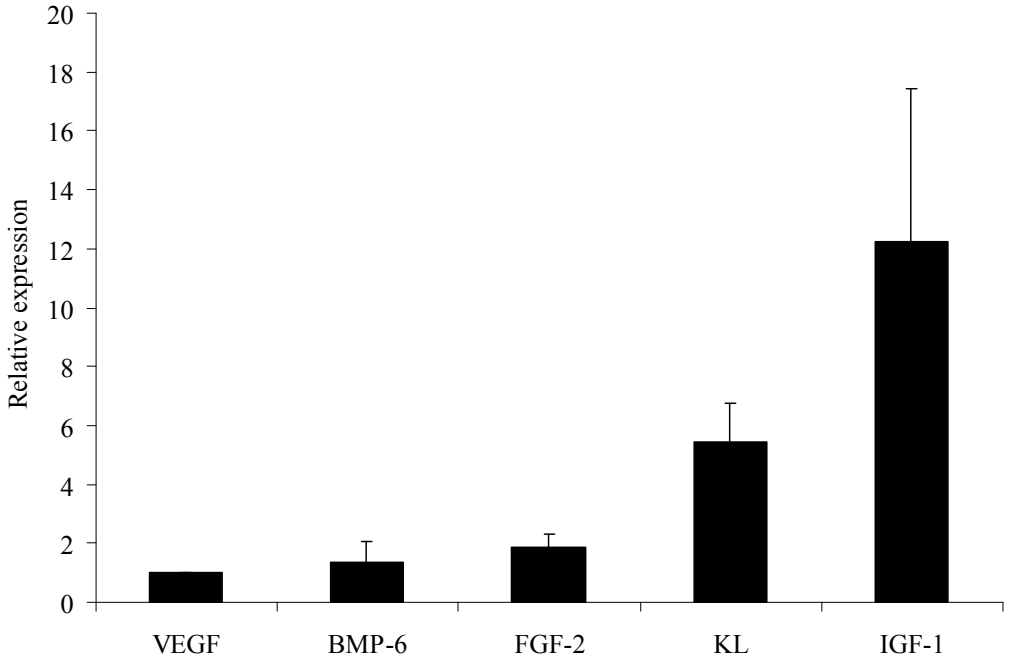
a, b: significantly different ($P<0.05$).

Table 4: Steady-state levels (mean ± SEM) of mRNA for various growth factors (GDF-9, BMP-15, BMP-6, FGF-2, VEGF, KL and IGF-1) compared to that of EGF in caprine preantral follicles. * significantly different ($P<0.05$) from growth factor compared with.

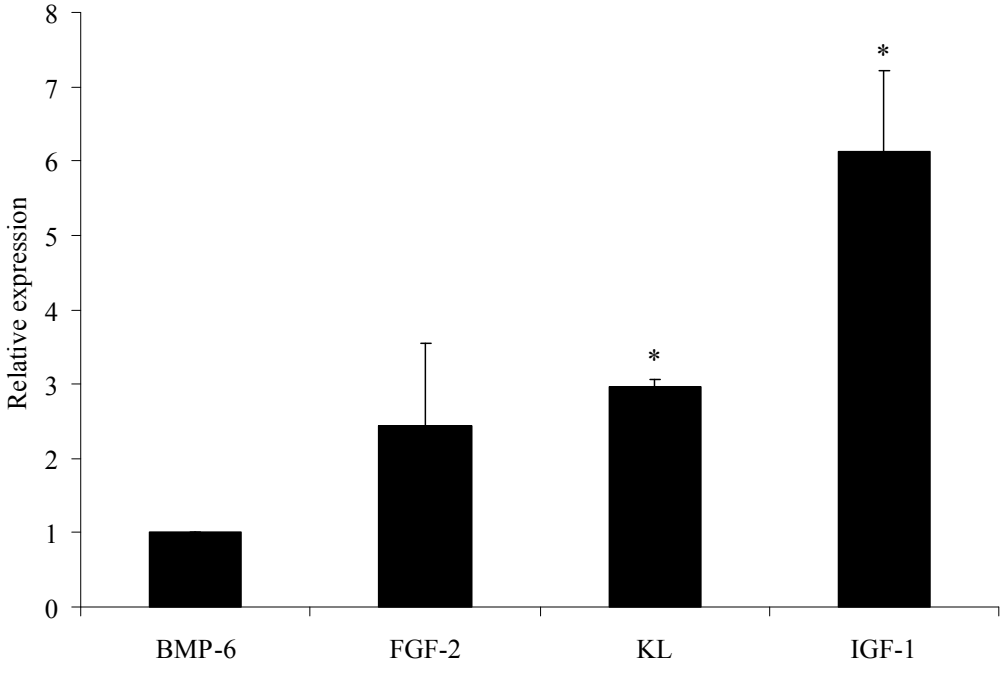
| GROWTH FACTORS | RELATIVE mRNA LEVELS |
|---|---|
| EGF | 1.0 ± 0 |
| GDF-9 | 91.0 ± 14.6 * |
| BMP-15 | 335.8 ± 32.6 * |
| BMP-6 | 453.4 ± 81.6 * |
| FGF-2 | 962.6 ± 100.2 * |
| VEGF | 1323.7 ± 58.2 * |
| KL | 2371.8 ± 201.1 * |
| IGF-1 | 4502.5 ± 123.0 * |


A
Relative expression
70
60
50
40
30
20
10
0
*
*
GDF-9
BMP-15
BMP-6
VEGF
FGF-2
KL
IGF-1
B
Relative expression
18
16
14
12
10
8
6
4
2
0
*
*
BMP-15
BMP-6
VEGF
FGF-2
KL
IGF-1

C


Relative expression
20
18
16
14
12
10
8
6
4
2
0
VEGF
BMP-6
FGF-2
KL
IGF-1

D


Relative expression
8
7
6
5
4
3
2
1
0
*
*
BMP-6
FGF-2
KL
IGF-1

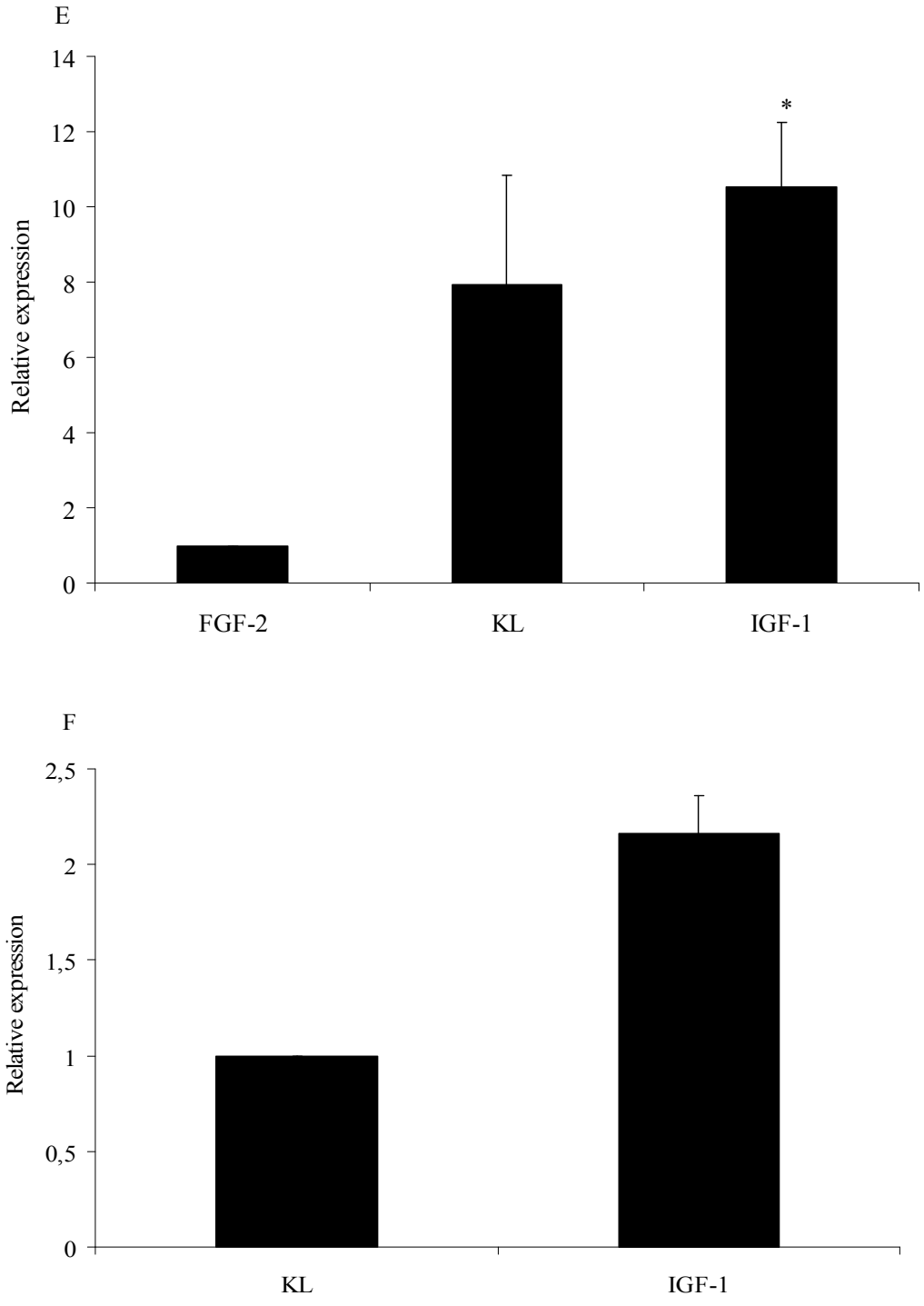



Figure 3A-F: Steady-state level (mean ± *SEM)* of mRNA for the growth factors BMP-15, VEGF, FGF-2, BMP-6, IGF-1, and KL compared to that of GDF-9 (A), BMP-15 (B), VEGF (C), FGF-2 (D), IGF-1 (E) and KL (F) in goat preantral follicles.

* significantly different ($P<0.05$) from growth factor compared with.

8. ARTIGO 2

# Levels of BMP-6 mRNA in goat ovarian follicles and *in vitro* effects of BMP-6 on secondary follicle development

A ser submetido para publicação no periódico Theriogenology

**Resumo**

O objetivo do presente estudo foi verificar o nível de mRNA para BMP-6 em diferentes fases de desenvolvimento folicular e investigar a influência da BMP-6 sobre a sobrevivência e o crescimento de folículos secundários cultivados por 6 dias. Para tanto, folículos primordial, primário e secundário, bem como pequenos e grandes folículos antrais de cabra, foram obtidos e os níveis de mRNA para BMP-6 foram quantificados por PCR em tempo real. A expressão da proteína BMP-6 em folículos caprinos foi demonstrada por imunohistoquímica. A influência da BMP-6 na presença ou ausência de FSH tanto no desenvolvimento de folículos secundários quanto na expressão de mRNA para BMP-6 e R-FSH, além do diâmetro folicular e a formação de antro foram avaliados após 6 dias de cultivo. Os resultados mostraram que o nível de mRNA para BMP-6 em folículos primários e secundários foi significativamente maior do que em primordial. Nos pequenos e grandes folículos antrais, níveis similares de mRNA para BMP-6 foram observados em oócitos e células da granulosa. A proteína BMP-6 foi expressa em oócitos de todas as categorias foliculares e nas células da granulosa de folículos secundários. Após 6 dias de cultivo, BMP-6 na presença ou ausência de FSH aumentou o diâmetro dos folículos secundários cultivados. Além disso, FSH aumentou os níveis de mRNA para BMP-6, enquanto BMP-6 e FSH interagiram e aumentaram os níveis de mRNA para RFSH, após o cultivo de folículos durante 6 dias. Em conclusão, ocorre um aumento nos níveis de mRNA para BMP-6 durante a transição de folículos primordial para primário / secundário e FSH e BMP-6 promovem o crescimento de folículos secundários caprinos. Além disso, FSH aumenta o nível de mRNA para BMP-6 e FSH e BMP-6 aumentam os níveis de mRNA para R-FSH no cultivo de folículos secundários.

**Palavras-chave:** BMP-6, folículos secundários, RFSH

**Levels of BMP-6 mRNA in goat ovarian follicles and *in vitro* effects of BMP-6 on secondary follicle development**

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**Abstract**

The aim of the present study was to verify the level of BMP-6 mRNA in follicles of different stages of development and to investigate the influence of BMP-6 on the survival and growth of secondary follicles cultured for 6 days. Therefore, goat primordial, primary and secondary follicles, as well as small and large antral follicles were obtained and the mRNA levels of BMP-6 were quantified by PCR in real time. Expression of BMP-6 protein in goat follicles was demonstrated by immunohistochemistry. The influence of BMP-6 in the presence or absence of FSH on both the development of secondary follicles and the expression of mRNA for BMP-6 and FSH-R was evaluated after 6 days of culture. Furthermore, the follicular diameter and the formation of the antrum were evaluated before and after 6 days of culture. The results showed that the level of mRNA for BMP-6 in primary and secondary follicles was significantly higher than those in primordial follicles. In small and large antral follicles, similar levels of BMP-6 mRNA were observed in oocytes and granulosa cells. BMP-6 protein was expressed in oocytes of all categories of follicles and in granulosa cells from secondary follicles. After 6 days of culture, BMP-6 in the presence or absence of FSH increased diameter of cultured secondary follicles. In addition, FSH increased the levels of mRNA for BMP-6 while both BMP-6 and FSH interacted and increased the levels of mRNA for FSH-R

after culture of follicles for 6 days. In conclusion, the levels of mRNA for BMP-6 is increased during transition from primordial to primary / secondary follicles and both FSH and BMP-6 promote growth of goat secondary follicle. Furthermore, FSH increases the level of mRNA for BMP-6 and both FSH and BMP-6 increases the levels of mRNA for FSH-R in cultured secondary follicles.

**Key words:** BMP-6**,** secondary follicles, FSH-R

## 1. Introduction

Ovarian folliculogenesis is a lengthy process marked by the proliferation and differentiation of both somatic and germ cells. During the growth of primordial follicles toward the stage of pre-ovulatory follicles, several growth factors produced locally in the ovary act by modulating the effects of FSH. Although the latter stages of follicular development are primarily regulated by pituitary gonadotropins (FSH and LH), there is evidence that this process is based on complex actions and interactions between locally produced hormones and growth factors (KNIGHT *et al*., 2003; WEBB *et al.*, 2007).

The bone morphogenetic protein (BMP) family is the largest within the TGF-ß superfamily of growth factors and some studies have shown that BMPs regulate growth, differentiation and apoptosis in a variety of tissues including the ovary (SHIMASAKI *et al.*, 2004). In the ovary, the presence of mRNA for BMP-6 was observed in oocytes, granulosa and theca cells in several species (murine: ERICKSON and SHIMASAKI, 2003; cattle: GLISTER *et al.*, 2004; sheep; JUENGEL and McNATTY, 2005) and the protein was detected in both somatic cells of sheep ovarian follicles (JUENGEL *et al.*, 2004; CAMPBELL *et al.*, 2006). However, in caprine species, neither the levels of mRNA for BMP-6 at different stages of follicles development nor the presence of BMP-6 protein had been described. To perform its biological functions, BMP-6 initially bind to type I receptors, i.e. with the activin receptor-IA (ALK-2) or BMPR-IB (ALK-6). Upon activation of the type I receptor, type II receptor is recruited, i.e. activin receptor-IIA or -IIB, or BMPR-II. The interaction between the receptors induces phosphorylation of intracellular messengers (SMADS) that are recruited to the nucleus, where they regulate the expression of specific genes (MASSAGÉ and CHEN, 2000). In sheep, the expression of both BMP receptors, BMPR-IA and BMPR-IB, were observed in somatic cells of the ovary at all stages of folliculogenesis (SOUZA *et al.*, 2002).

*In vitro* studies to evaluate the effect of BMP-6 on the follicular growth were mainly carried out in murine species (OTSUKA *et al.*, 2001). Culture of granulosa cells from mice antral follicles shows that BMP-6 inhibits the synthesis of progesterone, through the inhibition of steroidogenic enzymes. In cultured granulosa cells from rodents, BMP-6 lack mitogenic activity and inhibits the action of FSH on the production of progesterone, but has no effect on estradiol production (OTSUKA *et al.*, 2001; MIYOSHI, 2006). During the selection of the dominant follicle the production of BMP-6 is decreased, and this may be related to the mechanism by which dominant follicles are selected (SHIMASAKI *et al.*, 2004). In granulosa cells, BMP-6 inhibits FSH stimulation of StAR and P450scc mRNA expression without affecting the expression of P450arom mRNA. Furthermore, BMP-6 inhibits progesterone synthesis and the corresponding expression of StAR and P450scc induced by forskolin. In contrast, BMP-6 has no effect on these steroidogenic parameters induced by 8-bromo (Br)-cAMP (OTSUKA *et al.*, 2001). However, the *in vitro* effects of BMP-6 in the presence or absence of FSH on both development of secondary follicles and on expression of BMP-6 and FSH-R are still unknown.

The present study aimed (1) to verify the steady-state level of BMP-6 mRNA during different follicular stages in goat ovaries, (2) to demonstrate the presence of BMP-6 protein in goat follicles, (3) to investigate a possible influence of BMP-6 on the survival and growth of secondary follicles after culture for 6 days, and (4) to evaluate the effects of BMP-6 and FSH on the levels of mRNA for FSH-R and BMP-6 after 6 days culture.

## 2. Materials and Methods

### Messenger RNA quantification for BMP-6 in goat ovarian follicles

To evaluate mRNA expression, ovaries (n=30) of goats (*Capra hircus*) were collected and rinsed in saline (0.9% NaCl) containing antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). After this preparation, 10 ovaries were utilized for isolation of primordial, primary, and secondary follicles. The remaining ovaries were used for collection of cumulus–oocyte complexes (COCs), mural granulosa cells, and thecal cells from small and large antral follicles. Primordial, primary, and secondary follicles were isolated using a mechanical procedure, as previously described (LUCCI *et al.*, 1999). After isolation, these follicles were washed several times to completely remove the stromal cells and were then placed by

category into separate Eppendorf tubes in groups of 10. This procedure was completed within 2 h, and all samples were stored at –80°C until the RNA was extracted. From a second group of ovaries (n=20), COCs aspirated from small (1–3 mm) and large (3–6 mm) antral follicles were recovered. Compact COCs were selected from the follicle content as described by Van Tol and Bevers (1998). Thereafter, groups of 10 COCs were stored at -80°C until RNA extraction. To collect mural granulosa and theca cell complex, small (n=10) and large antral follicles (n=10) were isolated from ovaries (n=5) and dissected free from stromal tissue with forceps as previously described (VAN TOL AND BEVERS, 1998). The follicles were then bisected and mural granulosa / theca were collected and stored at -80°C.

Isolation of total RNA was performed using Trizol plus purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 ml of Trizol® solution was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 g for 3 minutes at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz units/mL) for 15 minutes 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 minutes at 70°C, and chilled on ice. Reverse transcription was then performed in a total volume of 20µl, which was comprised of 10 µl of sample RNA, 4 µl 5X reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNAseout, 150 units Superscript III reverse transcriptase, 0.036 U random primers (Invitrogen, São Paulo, Brazil), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 hour at 42°C, for 5 minutes at 80°C, and then stored at -20°C. Negative controls were prepared under the same conditions but without the inclusion of the reverse transcriptase.

Quantification of the mRNA for BMP-6 was performed using SYBR Green. PCR reactions were composed of 1 µl cDNA as a template in 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µl of ultra-pure water, and 0.5 µM of each primer. The primers were designed to perform amplification of mRNA for BMP-6 and housekeeping genes are shown in Table 1. The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 minutes 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 minutes 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 expression levels.

**Expression of BMP-6 protein in goat ovarian follicles**

Ovaries (n=10) of adult goats were collected in local slaughterhouse, washed with saline (0.9% NaCl) containing antibiotics (1000 µg/mL penicillin and 100 µg/mL streptomycin) and transported to the laboratory at 32°C. Ovaries were fixed in paraformaldehyde (4%) for 18h, dehydrated and embedded in paraffin. Then sections of 5µm were obtained and mounted on glass slides. Immunohistochemistry was performed according to the protocol described previously (SILVA *et al.*, 2004). Briefly, the epitopes were activated through incubation in citric acid to 98-100°C for 7 minutes, whereas nonspecific binding was blocked through incubation in 5% normal goat serum diluted in PBS. Then, the sections were incubated for 18h at 4°C with monoclonal anti-BMP-6 produced in mice (1:50, Santa Cruz Biotechnology, Santa Cruz, USA). Subsequently, the sections were incubated for 45 minutes with secondary antibody biotinylated anti-mouse IgG (Vector laboratories, Burlingame, CA, USA), diluted 200 times in PBS containing 5% normal goat serum. The sections were incubated for 45 minutes with avidin-biotin (1:600, Vectastain Elite ABC kits, Vector Laboratories, Burlingame, CA, USA). The location of the protein was demonstrated with diaminobenzidine DAB (0.05% DAB in Tris / HCl pH 7.6, 0.03% $H_2O_2$ - Sigma Chemicals, Poole, Dorset, UK). Finally, the sections were counterstained with hematoxylin, dehydrated and mounted in Canada balsam. The negative controls were carried out by replacing the primary antibody for IgG of the same species as the primary antibody was produced.

The ovarian follicles were classified as primordial, primary, secondary, small antrall and large antral follicles according to the diameter and morphology of the oocyte and granulosa cells. In the different follicular compartments (oocyte and granulosa cells), the immunostaining was classified as absent (-), weak (+), moderate (+ +) or strong (+++).

**Effect of BMP-6 on growth of goat secondary follicles and expression of FSH-R and BMP-6**

Ovaries of goats (n = 10) were collected from a slaughterhouse and transported to the laboratory in MEM containing antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin) at 32°C in maximum of 1 hour.

In the laboratory, surrounding fat tissue and ligaments were stripped off from the ovaries. Ovarian cortical slices (1 to 2 mm in diameter) were cut from the ovarian surface using a surgical blade under sterile conditions. The ovarian cortex was subsequently placed in fragmentation medium, consisting of MEM plus HEPES. Secondary follicles of approximately 200 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of ovarian cortex using 25 gauge (25 G) needles. After isolation, follicles were transferred to 100 µL drops containing fresh medium under mineral oil to further evaluation of the follicular quality. Follicles with a visible oocyte, surrounded by granulosa cells, an intact basement membrane and no antral cavity were selected for culture.

For *in vitro* studies, after selection, follicles were individually cultured in 25 µL drops of culture medium in petri dishes (60 x 15 mm, Corning, USA). Control culture medium consisted of α- MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/mL bovine serum albumin (BSA), ITS (insulin 6.25 ng/mL, transferrin 6.25 ng/mL and selenium 6.25 ng/mL), 2 mM glutamine, 2 mM hypoxantine and 50 µg/mL of ascorbic acid under mineral oil. Incubation was conducted at 39° C, for 6 days. For treatments, control culture medium was supplemented with 50 ng/mL of FSH, 50 ng/mL of BMP-6 (Sigma, St. Louis, USA) or both. These concentrations of FSH and BMP-6 were those that promoted the highest growth rates *in vitro* preantral follicles in previous studies (GLISTER *et al.*, 2004; MATOS *et al.*, 2007). For culture, the follicles were randomly chosen and incubated for 6 days in the incubator with 5% $CO_2$ in air at 39 °C. Every other day, 5 µL of the culture media were added to the drops. The culture was replicated four times, and a mean number of 29 follicles were used per treatment. The morphology and follicular diameter were assessed at the beginning and end of cultivation with the aid of an inverted microscope. In addition, the percentages of secondary follicles that reached the antrum formation *in vitro* was determined.

To evaluate the effect of BMP-6 on expression of mRNA for FSH-R and BMP-6 itself after a six-day culture period, for each treatment, groups of six follicles were collected at the

end of culture period and stored at -80°C until extraction of total RNA. Quantification of mRNA was performed as described previously and the primers for BMP-6 and FSH-R are shown in Table 1. Glyceraldehyde-2-phophate dehydrogenase (GAPDH), ubiquitin (UBQ) and β-actin were used as endogenous controls for normalization of gene expression (Table 1).

**Statistical analysis**

Data of mRNA expression in primordial, primary and secondary follicles were compared by nonparametric Kruskal-Walis test, while *t* test was used for paired comparisons of mRNA expression in small and large antral follicles ($P<0.05$). Follicle diameters before and after culture were also compared by nonparametric Kruskal-Walis test, while the percentages of follicles that reached antrum formation were compared by chi-square test ($P<0.05$).

**3. Results**

Quantification of mRNA demonstrated that primary and secondary had significantly higher the levels of BMP-6 than primordial follicle ($P<0.05$). However, no difference in the levels of BMP-6 mRNA was observed between primary and secondary follicles. In antral follicles, the levels of mRNA for BMP-6 in both COCs and granulosa / theca cells did not differ between small and large follicles ($P>0.05$) (Figure 1). When the expression of BMP-6 mRNA was compared between COCs and granulosa / theca cells of small or large antral follicles, no significant difference was observed ($P>0.05$) (Figure 1).

Immunohistochemical study showed a moderate reaction for BMP-6 in oocytes of most categories of follicles, except in oocytes of small antral follicles that had a strong reaction. In addition, a weak immunoreaction for BMP-6 was also observed in granulosa cells from secondary follicles. On the other hand, cumulus, mural granulosa, and theca cells of small and large antral follicles did not show a positive reaction for BMP-6 (Figure 2).

After culture of secondary follicles for six days, a significant increase in their diameters was observed in all treatments, when compared to day 0 (Table 2). When the increase of follicular diameter was compared among treatments, the results showed that the presence of BMP-6, FSH or both BMP-6 and FSH significantly increase follicular growth when compared to MEM alone ($P<0.05$). However, no significant differences among

treatmens were observed in the percentages of follicles that reached antrum formation (Table 2).

After evaluation of level of mRNA for FSH-R and BMP-6 in cultured follicles, figure 3 shows that FSH increased significantly the levels of RNAm for BMP-6 when compared to MEM alone or supplemented with BMP-6 ($P<0,05$). In addition, BMP-6 and FSH interacted and increased the levels of mRNA for FSH-R after 6 days of culture (Figure 4).

**4. Discussion**

This study shows for the first time that during transition from primordial to primary / secondary follicles occur an increase in the level of mRNA for BMP-6 and that both FSH and BMP-6 increase secondary follicle diameter after 6 days of culture. Furthermore, FSH increased the level of mRNA for BMP-6 and both FSH and BMP-6 increased the levels of mRNA for FSH-R in cultured secondary follicles.

In goats early follicles, during transition from primordial to primary follicles occurs an increase in the level of mRNA for BMP-6. Recent *in vitro* studies showed that BMP-6 did not influence goat primordial follicles activation after 7 days of culture (ARAÚJO *et al*., 2010). Probably, BMP-6 is important only after primary follicle stage since high levels of mRNA were found in this type of follicle. This confirms results of previous studies showing that BMP-6 acts on development follicles, by promoting oocyte growth and granulosa cell proliferation and differentiation (KRYSKO *et al.*, 2008). In studies with *in situ* hybridization, Erickson and Shimasaki (2003) showed that the expression of BMP-6 mRNA in rat oocytes was developmentally regulated and there was no detectable BMP-6 mRNA in primordial and primary follicles, however a weak hybridization signal was detected in oocytes of early secondary follicles when the second layer of GCs was forming. Differences between species or in sensibility of techniques can explain the expression of mRNA for BMP-6 in goat primordial and primary follicles, but not in rat. The mRNAs for BMP receptors (BMPR-IA, -IB, and II) are expressed in oocytes and granulosa cells of goat ovarian follicles (SILVA *et al.*, 2004) as well as in follicles of other mammalian species (murine: [ELVIN *et al.*, 2000]; ovine: [SOUZA *et al.*, 2002]; [MCNATTY *et al.*, 2005]; bovine: [GLISTER *et al.*, 2002]). In COCs and granulosa/theca cell from goat small and large antral follicles the mRNA for BMP-6 was expressed at similar levels. The expression of BMP-6 mRNA has been reported in mouse oocytes from antral follicles (ELVIN *et al.*, 2000). Correspondingly, studies in animals

have shown that BMP-6 expression is abundant in both oocytes and granulosa cells of bovine antral follicle (GLISTER *et al.*, 2004). Shi et al. (2009) have shown that BMP-6 is strongly expressed in in human granulosa cells and oocytes in tertiary follicles. This distribution of BMP-6 mRNA in granulosa and oocyte compartments highlights possible diverse roles in cell–cell communication within the follicle. Immunoreaction for BMP-6 protein demonstrated a moderate reaction in oocytes of most categories of follicles, except in oocytes of small antral follicles that had a strong reaction. In addition, a weak immunoreaction for BMP-6 was also observed in granulosa cells from secondary follicles. Juengel *et al.*, (2004) has determined mRNA expression for BMPs 2, 4, 6 and 7 in sheep ovaries and has reported that although mRNA is detectable by PCR for all these BMPs, only BMP-6 protein was detectable by immunohistochemistry with expression being restricted to the oocyte. On the other hand, Glister *et al.* (2004) has reported specific protein expression of BMP-4 and 7 in isolated bovine theca cells whilst BMP-6 was mainly expressed by granulosa cells and the oocyte.

The effects of BMP-6 on secondary follicles and their interactions with FSH co-treatment have not been explored previously in caprine. This study showed an increase in the goat oocyte diameter of secondary follicles cultured with BMP-6 in the presence or absence of FSH. Since BMP-6 is not a mitogenic factor for granulosa cells of murine species (OTSUKA *et al.,* 2001), probably this effect was due to the combination of BMP-6 and some compounds in the culture medium, like ascorbic acid. In a recent study, in which BMP-6 was tested during culture of primordial in medium without ascorbic acid, BMP-6 reduced viability of primordial follicles was observed (ARAÚJO *et al*., 2010). Rossetto *et al.* (2009) have shown that combination of ascorbic acid and FSH maintained follicular integrity and promoted follicular activation and growth after long-term *in vitro* culture of caprine preantral follicles. At physiological concentrations, ascorbic acid, or vitamin C, is a potent free radical scavenger, protecting cells against the damage caused by reactive oxygen species (CARR *et al.*, 1999). Interesting, in the control medium containing ascorbic acid, but not FSH or BMP-6, reduced follicular growth was observed in this study. Another possibility is that BMP-6 is promoting goat granulosa cell proliferation since a significant increase in follicle diameter was observed in goat primordial follicles cultured in the presence of BMP-6 (ARAÚJO *et al.*, 2010). In addition, the BMP-6 has been suggested as an important local factor able to inhibit the stimulatory actions of FSH in granulosa cells of murine species (SHIMSAKI *et al.,* 2004), but in goat secondary follicle BMP-6 did modulate the action of FSH in promoting follicle growth.

After culturing secondary follicles for 6 days, FSH increased the level of mRNA for BMP-6 and both FSH and BMP-6 increased the level of mRNA for FSH-R. Receptors for FSH have been shown to be expressed from primary follicle onward in ovine species (TISDALL *et al*., 1995) and the stimulatory effect of FSH on BMP-6 expression can explain the high level of BMP-6 in goat primary and secondary follicles shown in this study. Autocrine BMP-6 can interact with FSH and increase expression of FSH-R in goat developing follicles, being important for the development of primary (ARAÚJO *et al.*, 2010) and secondary follicles. In cultured granulosa cells from mice antral follicle, it was shown that BMP-6 inhibits the synthesis of progesterone induced by FSH through the inhibition of steroidogenic enzymes (OTSUKA *et al.,* 2001). Miyoshi *et al.* (2007) showed BMP-6 inhibited FSH induced progesterone production in a dose-dependent manner and BMP-6 had no effect on FSH-induced P450arom expression regardless of the presence of oocytes. StAR mRNA levels induced by FSH were significantly suppressed by both BMP-6 regardless of oocyte actions. Also cAMP production induced by FSH was inhibited by BMP-6 regardless of the presence or absence of oocytes. Thus, the inhibitory effects of BMP-6 on cAMP synthesis were in parallel with the reduction in progesterone production (MIYOSHI *et al.,* 2007).

In conclusion, the present study provides evidence that the levels of mRNA for BMP-6 is increased during transition from primordial to primary / secondary follicles and that both FSH and BMP-6 promote growth of goat secondary follicle. Furthermore, FSH increases the level of mRNA for BMP-6 and both FSH and BMP-6 increases the levels of mRNA for FSH-R in cultured secondary follicles.

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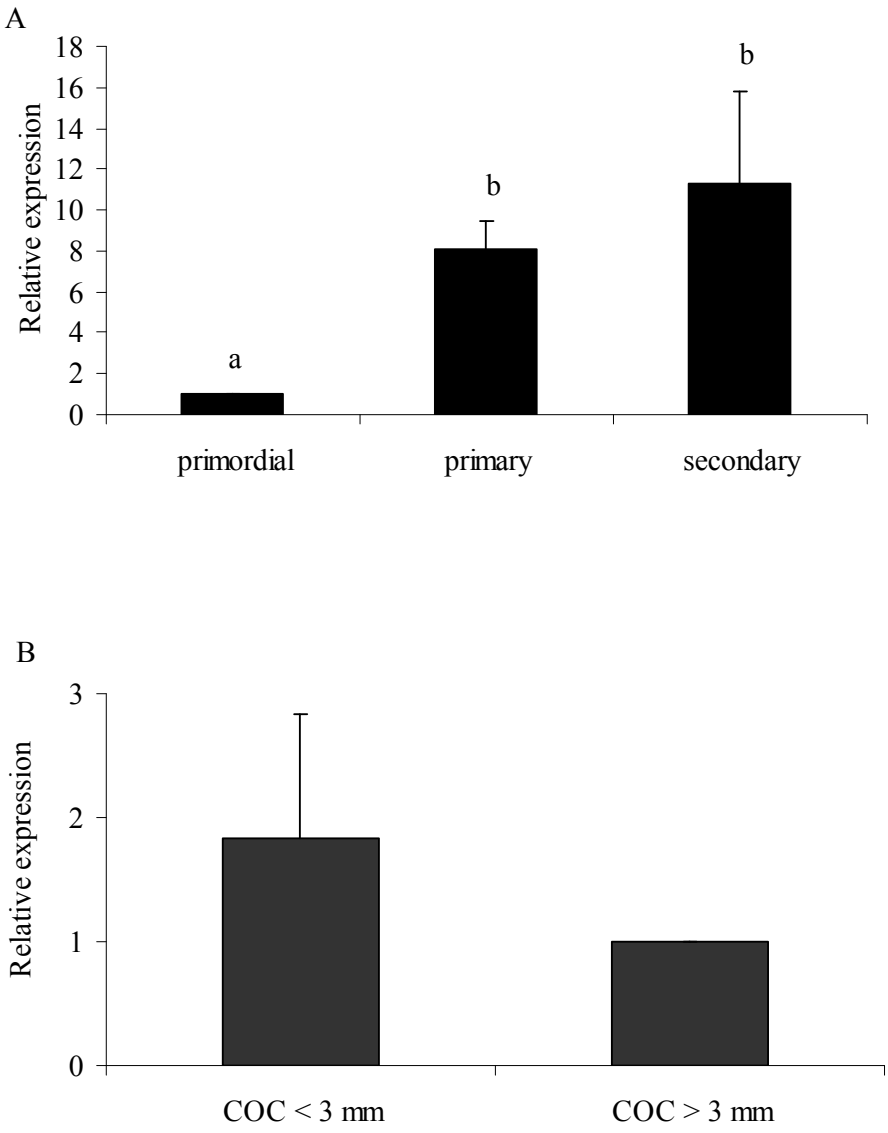
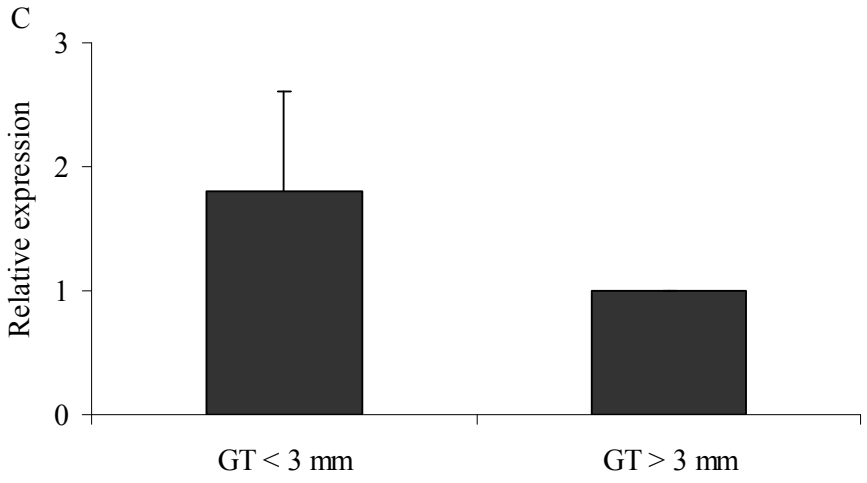
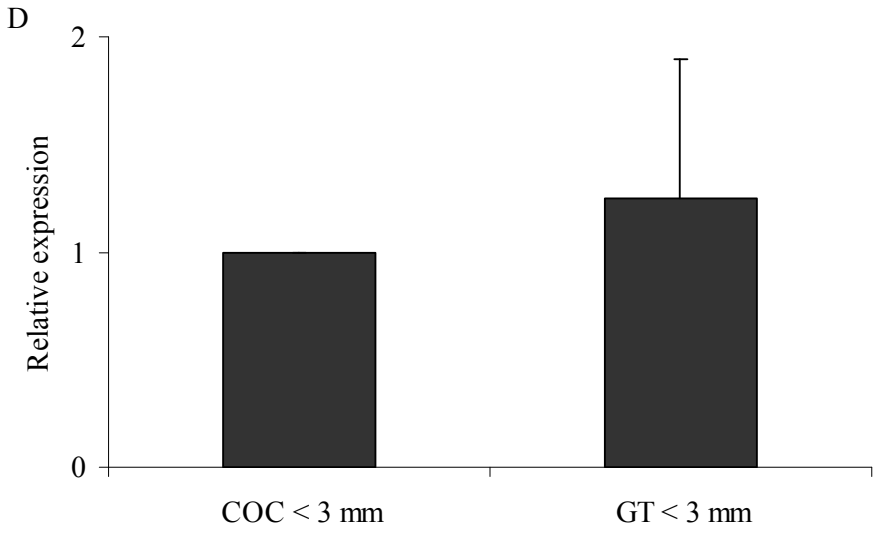
Table 1: Primer pairs used for as endogenous controls for normalization of gene expression in real-time PCR.

| Target gene | Primer sequence (5´→3´) | Sense (s) Anti-sense (as) | Position | Genbank accession n°. |
|---|---|---|---|---|
| GAPDH | TGTTTGTGATGGGCGTGAACCA | s | 288- 309 | Gi:27525390 |
| | ATGGCGTGGACAGTGGTCATAA | as | 419-440 | |
| β-ACTIN | ACCACTGGCATTGTCATGGACTCT | s | 188-211 | GI:28628620 |
| | TCCTTGATGTCACGGACGATTTCC | as | 363-386 | |
| UBQ | GAAGATGGCCGCACTCTTCTGAT | s | 607-631 | GI:57163956 |
| | ATCCTGGATCTTGGCCTTCACGTT | as | 756-780 | |
| BMP-6 | ACACATGAACGCCACCAACCAT | s | 141-163 | GI:76262832 |
| | AGGATGACGTTGGAGTTGTCGT | as | 262-284 | |
| FSH-R | AGGCAAATGTGTTCTCCAACCTGC | a | 250-274 | GI:95768228 |
| | TGGAAGGCATCAGGGTCGATGTAT | as | 316-340 | |


A
Relative expression
18
16
14
12
10
8
6
4
2
0
a
b
b
primordial
primary
secondary
B
Relative expression
3
2
1
0
COC < 3 mm
COC > 3 mm


C
Relative expression
3
2
1
0
GT < 3 mm
GT > 3 mm


D
Relative expression
2
1
0
COC < 3 mm
GT < 3 mm

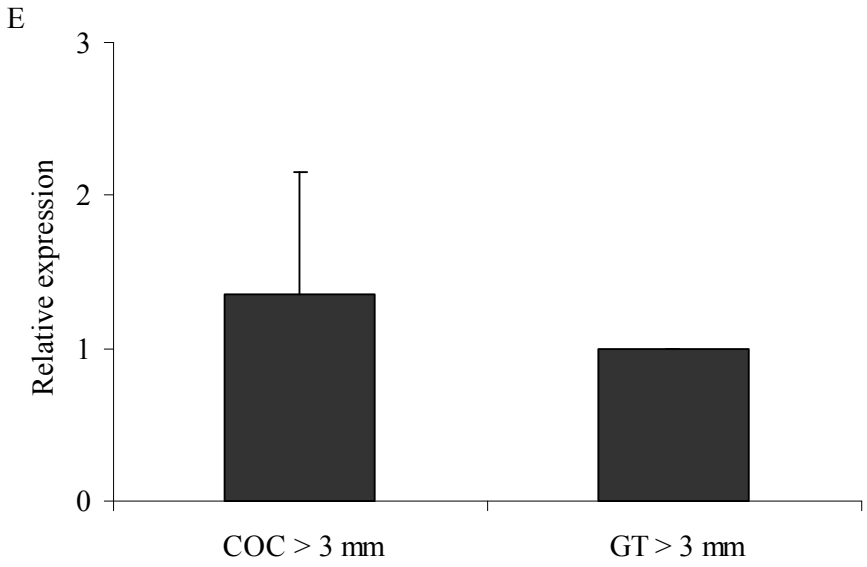



**Figure 1**. Expression of BMP-6 mRNA in goat ovarian follicles (means ± SD). **A**) primordial, primary and secondary follicles, **B**) COCs from small and large antral follicles, **C**) granulosa / theca cells from small and large antral follicles, D) COCs and granulosa / theca cells from small antral follicles, and E) COCs and granulosa / theca cells from large antral follicles.

* ($P<0.05$)

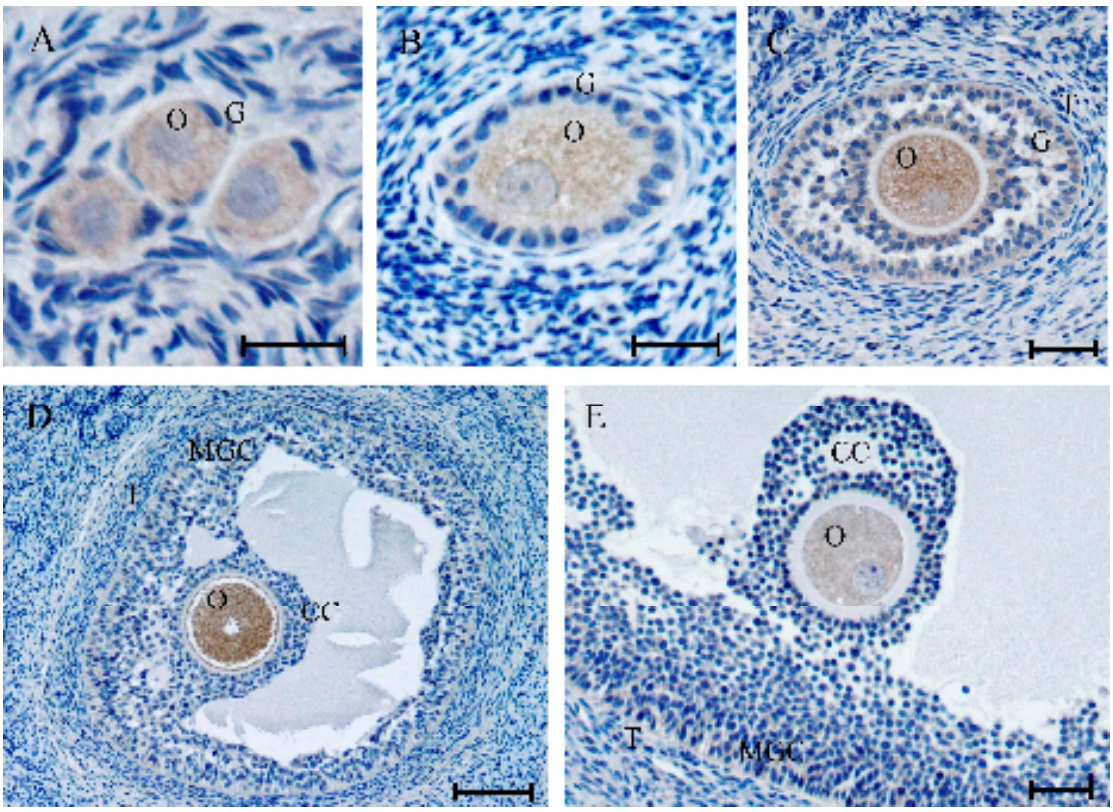



**Figure 2**. BMP-6 immunoreactivity in goat ovarian follicles. **(A)** Primordial follicle, **(B)** primary follicle, **(C)** secondary follicle, **(D)** small antral follicle, **(E)** large antral follicle. O: oocyte, G: granulosa cells, CC: cumulus cells, MGC: mural granulosa cells, T: theca cells. Scale bars represent 30µm (A, B, C) and 50µm (D, E).

**Table 2**: Follicle diameter and antrum formation of secondary follicles cultured with goat BMP-6 in the presence or absence of FSH.

| Treatments | Day 0<br>Diameter± SEM | Day 6<br>Diameter± SEM | Growth<br>Diameter ± SEM | Antrum formation |
|---|---|---|---|---|
| MEM | 204.0 ± 8.2 | 275.3 ± 15.1* | 71.2 ± 8.7 [a] | 44,8% (13/29) |
| MEM + FSH | 226.0 ± 6.0 | 353.9 ± 12.9* | 127.8 ± 8.8 [b] | 69.4% (25/36) |
| BMP-6 | 214.5 ± 11.0 | 340.7 ± 18.2* | 126.1 ± 13.0 [b] | 58.0% (18/31) |
| BMP-6 + FSH | 228.7 ± 14.7 | 344.5 ± 17.0* | 115.8 ± 8.8 [b] | 60% (12/20) |

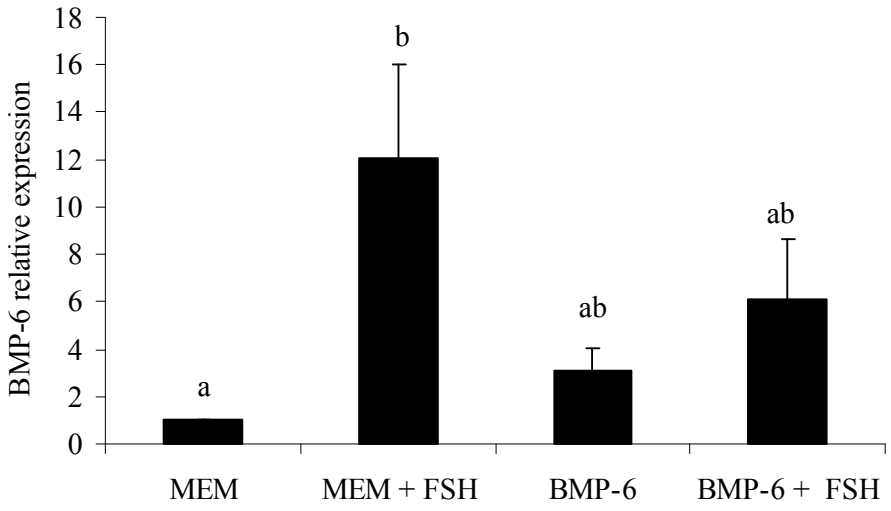



**Figure 3:** Steady state level of mRNA for BMP-6 in goat secondary follicles cultured for 6 days in MEM supplemented with FSH, BMP-6 or both.

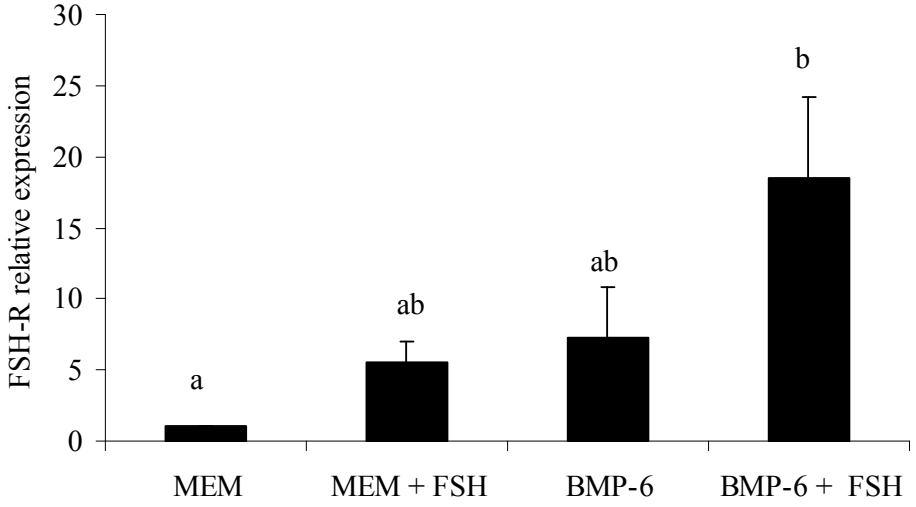



**Figure 4:** Steady state level of mRNA for FSH-R in goat secondary follicles cultured for 6 days in MEM supplemented with FSH, BMP-6 or both.

9. ARTIGO 3

# Levels of mRNA for BMP-7 in goat ovarian follicles and *in vitro* effects of BMP-7 on secondary follicle development

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**Resumo**

Este estudo teve como objetivo quantificar os níveis de mRNA para BMP-7 em folículos ovarianos caprinos em diferentes estágios de desenvolvimento, bem como avaliar os efeitos de FSH e BMP-7 no crescimento e na expressão de R-FSH e BMP-7 após cultivo de folículos secundários. Para este fim, folículos primordial, primário e secundário, bem como pequenos e grandes folículos antrais caprinos foram obtidos e os níveis de mRNA para BMP-7 foram quantificados por PCR em tempo real. Os efeitos da BMP-7 na presença ou ausência de FSH tanto no desenvolvimento de folículos secundários quanto na expressão de mRNA para BMP-7 e R-FSH, além disso, o diâmetro folicular e a formação de antro foram avaliados antes e após 6 dias de cultivo. Os níveis de mRNA para BMP-7 foram maiores em células da granulosa mural / teca de grandes folículos antrais do que em pequenos folículos antrais, mas não foi detectado mRNA para BMP-6 em folículos primordiais, primários e secundários, bem como em COCs de folículos antrais. Após o cultivo de folículos secundários durante 6 dias, FSH aumentou o diâmetro folicular e ambos FSH e BMP-7 aumentaram significativamente os níveis de mRNA para BMP-7 e R-FSH. Em conclusão, os níveis de mRNA para BMP-7 foram expressos em células da granulosa mural / teca de pequenos e grandes folículos antrais e BMP-7 aumentou o diâmetro folicular de folículos secundários de cabra. Além disso, BMP-7 interagiu com FSH e promoveu um aumento nos níveis de mRNA para BMP-7 e R-FSH, após 6 dias de cultivo.

**Palavras-chave:** BMP-7, foliculogênese, R-FSH, cultivo, caprino

**Levels of mRNA for BMP-7 in goat ovarian follicles and *in vitro* effects of BMP-7 on secondary follicle development**

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**Abstract**

This study aimed to quantify the mRNA levels for BMP-7 in goat ovarian follicles at different stages of developments as well as to evaluate the effects of FSH and BMP-7 on growth and on expression of FSH-R and BMP-7 in cultured secondary follicles. To this end, goat primordial, primary and secondary follicles, as well as small and large antral follicles were obtained and the mRNA levels of BMP-7 were quantified by PCR in real time. The effects of BMP-7 in the presence or absence of FSH on both the development of secondary follicles and expression of mRNA for BMP-7 and FSH-R was evaluated after 6 days of culture. Furthermore, the follicular diameter and the formation of the antrum were evaluated before and after 6 days of culture. The levels of mRNA for BMP-7 was higher in mural granulosa / theca cells from large antral follicles than in small antral follicles, but was neither found in primordial, primary and secondary follicles nor in COCs from antral follicles. After culture of secondary follicles for 6 days, FSH increased follicular diameter and both FSH and BMP-7 increased significantly the levels of mRNA for BMP-7 and FSH-R. In conclusion the levels of mRNA for BMP-7 was expressed in mural granulosa / theca cells from small and large antral follicles and BMP-7 increased the follicular diameter of goat secondary follicle. Furthermore,

the BMP-7 interacted with FSH and promoted an increase in levels of mRNA for BMP-7 and FSH-R after 6 days of cultivation.

**Keywords:** BMP-7, folliculogenesis**,** FSH-R, culture, caprine

## 1. Introduction

Folliculogenesis is the process by which primordial follicles grow and develop into the ovulatory follicle. Through this process, a healthy follicle is usually selected for maturation. Evidence indicates that bone morphogenetic proteins (BMPs), members of the superfamily of transforming growth factor-β (TGF-β), play a key role in ovarian folliculogenesis in mammals (SHIMASAKI *et al.*, 2004 e KNIGHT *et al.*, 2006).

With respect to BMP-7, Lee *et al.* (2004) showed that this growth factor is produced by thecal cells of secondary follicles and antral follicles and promotes growth and activation of primordial follicles and increases the expression of receptors for FSH during the culture of ovaries of mice. Miyoshi *et al.* (2006) elucidated the effects of BMP-7 on steroidogenesis, showing that this protein increases the production of estradiol-induced FSH and that the co-culture with oocytes regulates the effects of BMP-7 in the production of estradiol-induced FSH. Moreover, during *in vitro* culture of granulosa cells of rats, it was observed that BMP-7 modulates the action of FSH to increase estradiol and inhibit the synthesis of progesterone (SHIMASAKI *et al.*, 1999). It is well established that BMP-7 increases the mRNA levels of FSH in culture of ovaries of female rats (LEE *et al.*, 2004) and that BMP-7 and their receptors are expressed in human ovarian follicles (ABIR *et al.*, 2008). Studies with laboratory animals have shown that factors produced in the ovary, such as BMP-7 are involved in controlling the growth of oocyte and proliferation of granulosa cells, but in other animals the role of this factor has not been studied. However, the effects of BMP-7 in secondary follicles and their interactions with FSH have not been explored previously in caprine.

The present study aimed (1) to verify the steady-state level of BMP-7 mRNA at different follicular stages in goat ovaries, (2) to investigate a possible influence of BMP-7 on the survival and growth of secondary follicles after culture for 6 days, and (4) to evaluate the effects of BMP-7 and FSH on the levels of mRNA for FSH-R and BMP-7 after culture of secondary follicles for 6 days.

## 2. Materials and Methods

### Messenger RNA quantification for BMP-7 in goat ovarian follicles

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

Isolation of total RNA was performed using Trizol® plus purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 mL of Trizol solution was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 g for 3 minutes at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz units/mL) for 15 minutes 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 minutes at 70°C, and chilled on ice. Reverse transcription was then performed in a total volume of 20 μL, which was comprised of 10 μL of sample RNA, 4 μL 5X reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNAseout, 150 units Superscript III reverse

transcriptase, 0.036 U random primers (Invitrogen, São Paulo, Brazil), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 hour at 42°C, for 5 minutes at 80°C, and then stored at -20°C. Negative controls were prepared under the same conditions but without the inclusion of the reverse transcriptase.

Quantification of the mRNA for BMP-7 was performed using Syber green. PCR reactions were composed of 1 µl cDNA as a template in 7.5 µl of Syber Green Master Mix® (PE Applied Biosystems, Foster City, CA), 5.5 µl of ultra-pure water, and 0.5 µM of each primer. The primers were designed to perform amplification of mRNA for BMP-7 (Table 1). The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 minutes 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 minutes 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 expression levels.

**Effect of BMP-7 on growth of goat secondary follicles and expression of FSH-R and BMP-7**

Ovaries of goats (n=10) were collected from a slaughterhouse and transported to the laboratory in MEM containing antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin) at 32 °C in maximum of 1 hour.

In the laboratory, surrounding fat tissue and ligaments were stripped off from the ovaries. Ovarian cortical slices (1 to 2 mm in diameter) were cut from the ovarian surface using a surgical blade under sterile conditions. The ovarian cortex was subsequently placed in fragmentation medium, consisting of MEM plus HEPES. Secondary follicles of approximately 200 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of ovarian cortex using 25 gauge (25 G) needles. After isolation, follicles were transferred to 100 µL drops containing fresh medium under mineral oil to further evaluation of the follicular quality. Follicles with a visible oocyte, surrounded by granulosa cells, an intact basement membrane and no antral cavity were selected for culture.

For *in vitro* studies, after selection, follicles were individually cultured in 25 µL drops of culture medium in petri dishes (60 x 15 mm, Corning, USA). Control culture medium consisted of α- MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/mL bovine serum albumin

(BSA), insulin 6.25 μg/mL, transferrin 6.25 μg/mL and selenium 6.25 ng/mL (ITS), 2 mM glutamine, 2 mM hypoxantine and 50 μg/mL of ascorbic acid under mineral oil. Incubation was conducted at 39° C, for 6 days. For treatments, control culture medium was supplemented with 50 ng/mL of FSH, 50 ng/mL of BMP-7 (R & D Systems Europe, Abingdon, Oxon, UK) or both. These concentrations of FSH and BMP-7 were those that promoted the highest growth rates *in vitro* preantral follicles in previous studies (GLISTER *et al*., 2004; MATOS *et al.*, 2007). For culture, the follicles were randomly chosen and incubated for 6 days in the incubator with 5% $CO_2$ in air at 39 °C. Every other day, 5 μL of the culture media were added to the drops. The culture was replicated four times, and a mean number of 25 follicles were used per treatment. The morphology and follicular diameter were assessed at the beginning and end of cultivation with the aid of an inverted microscope. In addition, the percentages of secondary follicles that reached the antrum formation *in vitro* was determined.

To evaluate the effect of FSH and BMP-7 on expression of mRNA for FSH-R and BMP-7 after a six-day culture period, for each treatment, groups of six follicles were collected at the end of culture period and stored at -80°C until extraction of total RNA. Primers for BMP-7, FSH-R and housekeeping genes (glyceraldehyde-2-phophate dehydrogenase (GAPDH), ubiquitin (UBQ) and β-actin) are shown in table 1.

**Statistical analysis**

Data of BMP-7 mRNA levels in small and large antral follicles were compared by *t* test ($P<0.05$). Nonparametric Kruskal-Walis test was used to compare follicle diameters and the levels of mRNA for BMP-7 and FSH-R after 6 days of culture, while the percentages of follicles that reached antrum formation were compared by chi-square test ($P<0.05$).

**3. Results**

Amplification of cDNA from primordial, primary and secondary follicles, as well as from COCs from small and large antral follicles resulted in no PCR product, showing that mRNA for BMP-7 is not expressed in these type of cells. However, mRNA for BMP-7 was detected in samples of mural granulosa / theca cells from small and large antral follicles. As shown in figure 1, the levels of mRNA for BMP-7 was significantly higher in mural granulosa / theca cells from large antral follicles than in small antral follicles.

After culture of secondary follicles for six days, a significant increase in their diameters was observed in all treatments, when compare to day 0 (Table 2). When the increase of follicular diameter was compared among treatments, the results showed that the presence of FSH or both BMP-7 and FSH significantly increase follicular growth, when compared to MEM alone ($P<0.05$). On the other hand, BMP-7 alone did not increase follicular diameter, when compared to MEM. In addition, no significant differences among treatments were observed in the percentages of follicles that reached antrum formation (Table 2).

As shown in Figure 2, BMP-7 interacted with FSH and promoted an increase in levels of mRNA for BMP-7 when compared with secondary follicles cultured with MEM only, but there was no significant difference between follicles cultured im medium supplemented with either BMP-7 or FSH. When FSH was added to culture medium, no significant difference in mRNA levels of FSH-R ws observed when compared to cultivation in MEM alone. In addition, after culturing secondary follicles with MEM or MEM plus BMP-7, no significant difference in levels of mRNA for R-FSH was detected. However, BMP-7 and FSH interacted and promoted a significant increase in the levels of mRNA for FSH-R, when compared with control medium.

**4. Discussion**

This is study shows for the first time that the mRNA for BMP-7 was detected in samples of mural granulosa / theca cells from small and large antral follicles and that BMP-7 increase secondary follicle diameter after 6 days of culture. Furthermore, both FSH and BMP-7 increased the levels of mRNA for BMP-7 and FSH-R in cultured secondary follicles.

The results of this study suggest that as small antral follicles develop to more advanced stages, the expression of BMP-7 is increased and provide the first evidence for a regulatory role for BMP-7 in ovarian follicle development in goats. However, in sheep ovaries, no expression of BMP-7 mRNA was observed in granulosa cells, theca, cumulus, oocyte or antral healthy follicles (1-2 mm) by using the technique of in situ hybridization. However, using the technique of RT-PCR, there was weak expression of BMP-7 in granulosa cells from 1-2 mm follicles from ovine ovaries (JUENGEL *et al.*, 2006). Previous studies showed that mRNA expression for BMP-7 was increased in the theca cells from secondary to

early tertiary and dominant follicles of rodents, as well as the corpus luteum and stromal cells (ERICKSON and SHIMAZAKI, 2003).

The effects of BMP-7 in secondary follicles and their interactions with FSH have not been explored previously in caprine. This study showed that the presence of FSH or both BMP-7 and FSH increase secondary follicle growth in caprine species. Araújo et al. (2010) showed that BMP-7 facilitates the transition of primordial follicles into goat primary follicles. Previous studies shoed that the mRNAs for BMP receptors (BMPR-IA, -IB, and II) are expressed in oocytes and granulosa cells of goat ovarian follicles (SILVA *et al*, 2004) as well as in follicles of other mammalian species (murine: [ELVIN *et al.*, 2000]; ovine: [SOUZA *et al.*, 2002]; [MCNATTY *et al.*, 2005]; bovine: [GLISTER *et al.*, 2002]). Lee et al. (2001) showed that BMP-7 is one of the factors that is involved in the control of granulosa cells proliferation, since this protein is secreted by the theca cells of large antral follicles and can stimulate growth of adjacent follicles. Other studies have shown that administration of BMP-7 promoted mitosis in granulosa cells and inhibited the production of progesterone. Considering that progesterone is important for the process of ovulation (YOSHIMURA and WALLACH, 1987), inhibition of progesterone production by BMP-7 may be related to mechanisms of inhibition of ovulation. During *in vitro* culture of granulosa cells of rat was observed that BMP-7 modulates the action of FSH, to increase the production of estradiol and inhibit the synthesis of progesterone (SHIMASAKI *et al.*, 1999). It is well established that granulosa cells of growing follicles respond to FSH stimulation *in vivo*, producing estradiol. The production of progesterone is observed mainly in the stage of preovulatory follicle. Moreover, when granulosa cells are cultured *in vitro* there is an increase in production of both estradiol and progesterone in response to the stimulus of FSH. This suggests that an inhibitor of the synthesis of progesterone, ie, an inhibitor of luteinization should be acting *in vivo* (SHIMASAKI *et al.*, 2004). Other results also demonstrate the importance of BMP-7 in the control of steroidogenesis *in vitro* after culture of granulosa cells. When levels of estradiol and progesterone were measured after 48 h of culture, an increase and a reduction in the production of estrogen and progesterone was observed, respectively (SHIMASAKI *et al.*, 1999). After *in vivo* administration of BMP-7 in the bursa rat ovarian there was a proliferation of granulosa cells, reduction of primordial follicles and growth of developing follicles, and antral follicles (LEE *et al.*, 2001).

In the present study, it was found that FSH and BMP-7 interacted and induced FSH-R mRNA expression in goat secondary follicles, suggesting that this may contribute to

increasing FSH sensitivity and thus promoting folliculogenesis. Notably, BMP-7 significantly increased FSH-R mRNA levels in human granulosa cells (SHI *et al.*, 2009). Moreover, the culture of neonatal mouse ovary in medium supplemented with BMP-7 increased FSH-R mRNA (LEE *et al.*, 2004). Previous studies demonstrated that early follicular development is not dependent on gonadotropins, but subsequently receptor expression of FSH increases as follicles grow (KUMAR *et al*., 1997). Thus, the results of this study suggests that BMP-7 acts by modulating the expression of mRNA for FSH-R is preparing follicles to further development.

In conclusion, the present study provides evidence that the levels of mRNA for BMP-7 are increased as the antral follicles grow and that both FSH and BMP-7 promote growth of goat secondary follicle. Furthermore, both FSH and BMP-7 increases the levels of mRNA for BMP-7 and FSH-R in cultured secondary follicles.

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Table 1: Primer pairs used for housekeeping genes, BMP-7 and FSH-R.

| Target gene | Primer sequence (5´→3´) | Sense (s) Anti-sense (as) | Position | Genbank accession n°. |
|---|---|---|---|---|
| GAPDH | TGTTTGTGATGGGCGTGAACCA | s | 288- 309 | Gi:27525390 |
| | ATGGCGTGGACAGTGGTCATAA | as | 419-440 | |
| β-ACTIN | ACCACTGGCATTGTCATGGACTCT | s | 188-211 | GI:28628620 |
| | TCCTTGATGTCACGGACGATTTCC | as | 363-386 | |
| UBQ | GAAGATGGCCGCACTCTTCTGAT | s | 607-631 | GI:57163956 |
| | ATCCTGGATCTTGGCCTTCACGTT | as | 756-780 | |
| BMP-7 | AGGCAGGCATGTAAGAAGCA | s | 78-98 | AF:508311 |
| | TTGGTGGCGTTCATGTAGGA | as | 203-223 | |
| FSH-R | AGGCAAATGTGTTCTCCAACCTGC | a | 250-274 | GI:95768228 |
| | TGGAAGGCATCAGGGTCGATGTAT | as | 316-340 | |

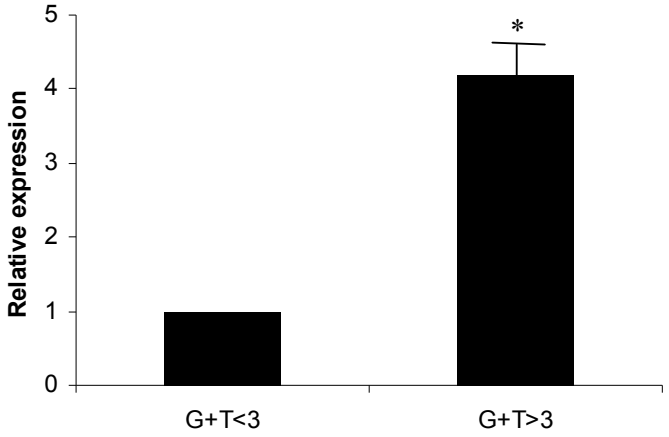


**Figure 1**: Level of messenger RNA for BMP-7 in mural granulosa / theca cells from small and large antral follicles. **G + T**: mural granulosa / theca cells.
* $P < 0.05$

**Table 2**: Follicle diameter and antrum formation in goat secondary follicles cultured in media supplemented with BMP-7 in the presence or absence of FSH.

| **CULTURE** | **Day 0** | **Day 6** | **Growth** | **Antrum formation (%)** |
|---|---|---|---|---|
| | Diameter ± SEM | Diameter ± SEM | Diameter ± SEM | |
| **MEM** | 206.0 ± 8.7 | 281.7 ± 15.6* | 75.7 ± 8.8 [a] | 40.7% (11/27) |
| **MEM +FSH** | 226.0 ± 6.0 | 353.9 ± 12.9* | 127.8 ± 8.8 [b] | 69.4% (25/36) |
| **BMP-7** | 256.4 ± 14.7 | 317.7 ± 18.0* | 61.2 ± 13.6 [a] | 47.0% (8/17) |
| **BMP-7 + FSH** | 250.0 ± 14.8 | 358.0 ± 17.1* | 108.4 ± 13.3 [b] | 65.0% (13/20) |

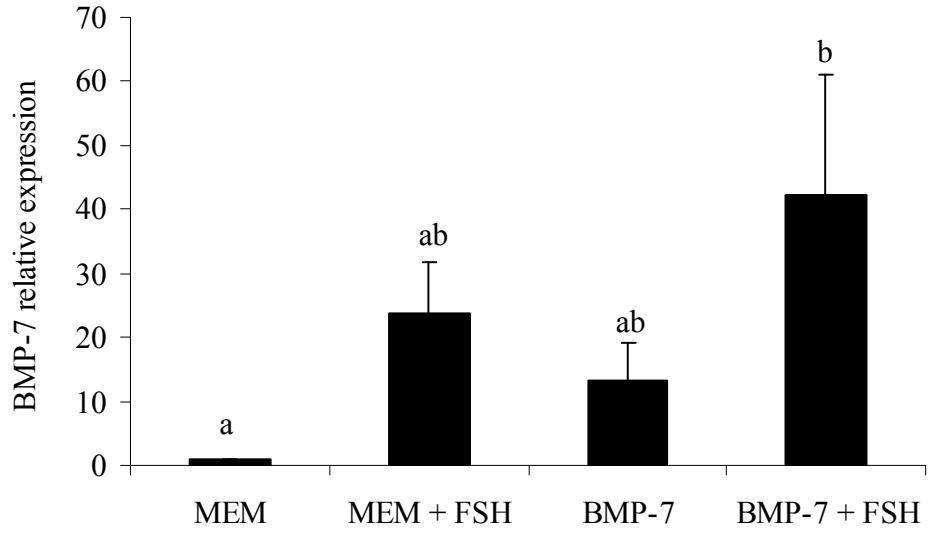


Figure 2. Steady state level of mRNA for BMP-7 in goat secondary follicles cultured for 6 days in MEM supplemented with FSH, BMP-7 or both.

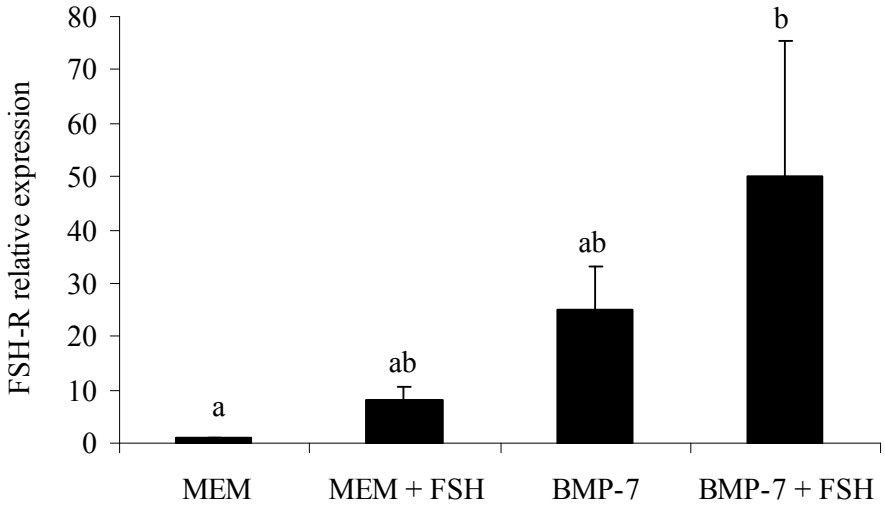


Figure 3. Steady state level of mRNA for FSH-R in goat secondary follicles cultured for 6 days in MEM supplemented with FSH, BMP-7 or both.

## 10. Conclusões e Perspectivas

- UBQ e β-actina são os genes de referência mais estáveis em folículos secundários caprinos;
- IGF-1 é um fator de crescimento que apresenta maior níveis de RNAm dentre os fatores estudados nesta categoria folicular;
- Folículos primários e secundários apresentaram maiores níveis de RNAm para BMP-6 do que folículos primordiais;
- Os níveis de RNAm para BMP-7 foram maiores nas células da granulosa / teca de grandes folículos antrais;
- O FSH e as BMPs dos tipos 6 e 7 estimulam o crescimento de folículos secundários *in vitro* durante 6 dias de cultivo;
- FSH aumentou os níveis de RNAm para BMP-6, enquanto BMP-6 e 7 em adição com o FSH aumentaram os níveis de RNAm para R-FSH, após o cultivo de folículos secundários caprinos.

A quantificação do RNAm para fatores de crescimento e receptores de hormônio podem contribuir para o desenvolvimento de um meio de cultivo eficiente que promova o crescimento de folículos pré antrais até a maturação. Desta forma, é necessário, a realização de estudos posteriores para cultivar folículos secundários em longo prazo, visando obter completa maturação oocitária.

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