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ELLEN DE VASCONCELOS DA CUNHA

INFLUÊNCIA DAS PROTEÍNAS MORFOGENÉTICAS ÓSSEAS 2 (BMP-2) E 4 (BMP-4) SOBRE O CRESCIMENTO, EXPRESSÃO GÊNICA E ULTRAESTRUTURA DE FOLÍCULOS PRÉ-ANTRAIS BOVINOS CULTIVADOS *IN VITRO*

FORTALEZA
2017

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Orientador: Prof. Dr. José Roberto Viana Silva

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Aprovada em: 27/07/2017

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“Aprender é a única coisa que a mente nunca se cansa, nunca tem medo e nunca se arrepende”.

Leonardo da Vinci

RESUMO

Os objetivos deste estudo foram: 1) avaliar o efeito de diferentes concentrações de BMP-2 (10, 50 ou 100 ng/mL) e BMP-4 (10, 50 ou 100 ng/mL) sobre a ativação, sobrevivência e crescimento de folículos pré-antrais bovinos inclusos em tecido ovariano cultivado *in vitro* por 6 dias; 2) avaliar a expressão de RNA mensageiros para *GDF-9*, *BMP-15*, *PCNA*, *Bax* e *Bcl2* em folículos pré-antrais bovinos após 6 dias de cultivo *in vitro* na presença de BMP-4; 3) avaliar o efeito de diferentes concentrações de BMP-2 (10, 50 ou 100 ng/mL) sobre o desenvolvimento *in vitro* de folículos secundários isolados durante 18 dias de cultivo; 4) avaliar o efeito da BMP-2 (10 ng/mL) associada ao FSH, bem como a expressão de RNAs mensageiros para *GDF-9*, *NLRP-5* e *NPM-2* em folículos secundários cultivados por 18 dias e 5) avaliar o efeito da interação de ambas, BMP-2 e 4, sobre o crescimento/viabilidade de folículos secundários bovinos isolados após 18 dias de cultivo *in vitro*, 6) bem como quantificar os níveis de RNAs mensageiros para *GDF-9, Ciclina B1*, *BMPR1A*, *BMPR1B*, *BMPRII*, *FSHR* e *SMAD1* antes e após o cultivo destes folículos. Para o cultivo *in situ*, fragmentos de córtex ovariano foram cultivados *in vitro* por 6 dias em α-MEM^{+} adicionado de diferentes concentrações de BMP-2 ou BMP-4. Antes e após o cultivo, os fragmentos de tecido ovariano foram fixados, analisados por histologia clássica e microscopia eletrônica de transmissão. Os folículos foram classificados em primordiais, primários e secundários, bem como em normais ou atrésicos. Além disso, os diâmetros oocitário e folicular foram avaliados. Para o cultivo de folículos isolados, folículos secundários foram microdissecados e cultivados por: a) 12 dias em α-MEM^{+} com diferentes concentrações de BMP-2 (10, 50 ou 100 ng/mL); b) 18 dias em α-MEM^{+} suplementado com BMP-2 (10 ng/mL) associada ou não ao FSH sequencial (50, 100 ou 200 ng/mL) e c) 18 dias em TCM199^{+} suplementado com BMP-2 (10 ng/mL), BMP-4 (100 ng/mL) ou a com a interação de ambas. Após o cultivo, os folículos foram avaliados morfologicamente e seus diâmetros foram mensurados. A técnica de RT-PCR foi utilizada para quantificar os RNAs mensageiros. Para o cultivo *in situ*, os resultados mostraram que BMP-2 e BMP-4 não influenciam a ativação de folículos primordiais *in vitro*. Entretanto, a adição de BMP-4 na concentração de 100 ng/mL aumentou os diâmetros oocitário e folicular de folículos primários e secundários inclusos em tecido ovariano cultivado *in vitro* por 6 dias. A BMP-4 não influenciou a expressão de RNAs mensageiros para *GDF-9, BMP-15, PCNA, Bax e Bcl2* após o cultivo. Em relação ao cultivo de folículos secundários isolados, observou-se que a BMP-2 na concentração de 10 ng/mL, sozinha ou associada ao FSH, manteve a ultraestrutura folicular após 12 dias de cultivo. Além

disso, a BMP-2 (10 ng/mL) promoveu crescimento folicular, formação de antro, manteve a viabilidade e modulou a expressão de *GDF-9*, *NLRP-5* e *NMP-2* dos folículos após 18 dias de cultivo *in vitro*. A BMP-4 (100 ng/mL) promoveu crescimento folicular, formação de antro e manteve a viabilidade folicular após o cultivo *in vitro*. Entretanto, as BMPs 2 e 4 juntas não influenciaram os níveis de expressão de RNA mensageiro para *GDF-9*, *Ciclina B1*, *BMPR1A*, *BMPR1B*, *BMPRII*, *FSHR* e *SMAD1*. Em conclusão, a adição de BMP-2 (10 ng/mL) e BMP-4 (100 ng/mL) ao meio de cultivo promove crescimento e contribui para a manutenção da ultraestrutura de folículos pré-antrais bovinos após cultivo *in vitro*.

Palavras-chave: Vaca. Cultivo. Ativação folicular. Crescimento

ABSTRACT

The objectives of this study were: 1) to evaluate the effect of different concentrations of BMP-2 (10, 50 or 100 ng/mL) and BMP-4 (10, 50 or 100 ng/mL) on the activation, survival and growth of bovine preantral follicles included in ovarian tissue cultured *in vitro* for 6 days; 2) to evaluate the expression of messenger RNA for *GDF-9*, *BMP-15*, *PCNA*, *Bax* and *Bcl2* in bovine preantral follicles after 6 days of *in vitro* culture in presence of BMP-4; 3) to evaluate the effect of different concentrations of BMP-2 (10, 50 or 100 ng/ml) on the *in vitro* development of isolated secondary follicles during 18 days of culture; 4) to evaluate the effect of BMP-2 (10 ng/mL) associated with FSH, as well as the expression of messenger RNAs for *GDF-9*, *NLRP-5* and *NPM-2* in secondary follicles cultured for 18 days and 5) to evaluate the effect of the interaction of both BMP-2 and 4, on the growth/viability of isolated bovine secondary follicles after 18 days of *in vitro* culture, 6) as well as quantification of levels of messenger RNAs for *GDF-9, Cyclin B1, BMPR1A, BMPR1B, BMPRII, FSHR* and *SMAD1* before and after the culture of these follicles. For *in situ* culture, fragments of ovarian cortex were cultured *in vitro* for 6 days in α-MEM^{+} added with different concentrations of BMP2 or BMP4. Before and after culturing, the ovarian tissue fragments were fixed, analyzed by classical histology and transmission electron microscopy. The follicles were classified as primordial, primary and secondary, as well as in normal or atresic. In addition, oocyte and follicular diameters were evaluated. For follicular culture, follicles were microdissected and cultured by: a) 12 days in α-MEM^{+} with different concentrations of BMP-2 (10, 50 or 100 ng/mL); b) 18 days in α-MEM^{+} supplemented with BMP2 (10 ng/mL) associated or not to sequential FSH (50, 100 or 200 ng / mL) and c) 18 days in TCM199^{+} supplemented with BMP2 (10 ng/mL), BMP4 (100 ng/mL), or the interaction of both. After culturing, the follicles were evaluated morphologically and their diameters were measured. The RT-PCR technique was used to quantify the messenger RNAs. For *in situ* culture, the results showed that BMP2 and BMP4 do not influence the activation of primordial follicles *in vitro*. However, the addition of 100 ng/mL BMP4 increased the oocyte and follicular diameters of primary and secondary follicles included in ovarian tissue cultured *in vitro* for 6 days. BMP4 did not influence the expression of messenger RNAs to *GDF-9, BMP-15, PCNA, Bax* and *Bcl2* after culturing. Regarding the culture of isolated secondary follicles, it was observed that BMP2 at 10 ng/mL, alone or associated with FSH, maintained the follicular ultrastructure after 12 days of culture. In addition, BMP2 (10 ng/mL) promoted follicle growth, antrum formation, maintained viability, and modulated *GDF-9, NLRP-5* and *NMP-2* expression of

follicles after 18 days of *in vitro* culture. BMP4 (100 ng/mL) promoted follicular growth, antrum formation and maintained follicular viability after *in vitro* culture. However, BMPs 2 and 4 together did not influence messenger RNA expression levels for *GDF-9, Cyclin B1, BMPR1A, BMPR1B, BMPRII, FSHR* and *SMAD1*. In conclusion, the addition of BMP2 (10 ng/ml) and BMP-4 (100 ng/ml) to the culture medium promoted growth and contributed to the maintenance of the ultrastructure of bovine preantral follicles after *in vitro* culture.

Keywords: Cow. Culture. Follicular activation. Growth.

LISTA DE ABREVIATURAS E SIGLAS

	Português	**Inglês**
%	Percentagem	Percentage
~	Aproximadamente	Approximately
<	Menor que	Less than
>	Maior que	Higher than
°C	Graus Celsius	Celsius degrees
µg	Micrograma	Microgram
µM	Micromolar	Micromolar
pM	Picomolar	Picomolar
ActR	Receptor para ativina	Activin receptor
ActR-IA	Receptor para ativina tipo IA	Activin receptor type IA
ActR-II	Receptor para ativina tipo II	Activin receptor type II
ActR-IIB	Receptor para ativina tipo IIB	Activin receptor type IIB
ALK	Receptor de ativina semelhante à quinase	Activin receptor-like kinase
ALK-2	Receptor de ativina semelhante à quinase tipo 2	Activin receptor-like kinase type 2
ALK-3	Receptor de ativina semelhante à quinase tipo 3	Activin receptor-like kinase type 3
ALK-5	Receptor de ativina semelhante à quinase tipo 5	Activin receptor-like kinase type 5
ALK-6	Receptor de ativina semelhante à quinase tipo 6	Activin receptor-like kinase type 6
AMH	Hormônio anti-Mulleriano	Anti-Mullerian hormone
ANOVA	Análise de variância	Analysis of variance
as	Anti-senso	Antisense
Bak	Proteína-killer antagonista homóloga à Bcl-2	BCL-2 antagonist killer 1
bax	Proteína X associada às células B de linfoma 2	B-cell lymphoma-2-associated X
bcl-x	Forma longa da proteína X associada	B-cell lymphoma-extra large

	às células B de linfoma 2	
Bcl-2	Linfoma de células B2	B-cell lymphoma 2
Bid	Proteína BH3 semelhante à Bax	Bax like BH3 protein
Bik	Proteína Killer que interage com Bcl-2	BCL2 interacting killer
bFGF	Fator de crescimento fibroblástico básico	Basic fibroblast growth factor
BMP	Proteína morfogenética óssea	Bone morphogenetic protein
BMP-1	Proteína morfogenética óssea 1	Bone morphogenetic protein 1
BMP-2	Proteína morfogenética óssea 2	Bone morphogenetic protein 2
BMP-3	Proteína morfogenética óssea 3	Bone morphogenetic protein 3
BMP-3B	Proteína morfogenética óssea 3b	Bone morphogenetic protein 3b
BMP-4	Proteína morfogenética óssea 4	Bone morphogenetic protein 4
BMP-5	Proteína morfogenética óssea 5	Bone morphogenetic protein 5
BMP-6	Proteína morfogenética óssea 6	Bone morphogenetic protein 6
BMP-7	Proteína morfogenética óssea 7	Bone morphogenetic protein 7
BMP-8a	Proteína morfogenética óssea 8ª	Bone morphogenetic protein 8ª
BMP-8b	Proteína morfogenética óssea 8b	Bone morphogenetic protein 8b
BMP-11	Proteína morfogenética óssea 11	Bone morphogenetic protein 11
BMP-12	Proteína morfogenética óssea 12	Bone morphogenetic protein 12
BMP-13	Proteína morfogenética óssea 13	Bone morphogenetic protein 13
BMP-14	Proteína morfogenética óssea 14	Bone morphogenetic protein 14
BMP-15	Proteína morfogenética óssea 15	Bone morphogenetic protein 15
BMP-16	Proteína morfogenética óssea 16	Bone morphogenetic protein 16
BMPR	Receptor para BMP	BMP receptor
BMPR-IA	Receptor para BMP tipo IA	BMP receptor type IA
BMPR-IB	Receptor para BMP tipo IB	BMP receptor type IB
BMPR-II	Receptor para BMP tipo II	BMP receptor type II
BSA	Albumina sérica bovina	Bovine serum albumin
cDNA	DNA complementar	Complementary DNA
ConA	Concanavalina A	Concanavalin A
CG	Célula da granulosa	Granulosa cell
CGPs	Células germinativas primordiais	Primordial germ cells
CO_2	Dióxido de carbono	Carbon dioxide
COC	Complexo *cumulus*-oócito	Cumulus-oocyte complex

CT	Limiar do ciclo	Cycle threshold
CYP17A1	Enzima P450 17α hidroxilase	Cytochrome P450 17alpha-hydroxylase
D0	Dia 0 do tratamento	Day 0
D6	Dia 6 do tratamento	Day 6
xD12	Dia 12 do tratamento	Day 12
D18	Dia 18 do tratamento	Day 18
dATP	Deoxiadenosina trifosfato	Deoxyadenosine triphosphate
dCTP	Deoxicitidina trifosfato	Deoxycytidine triphosphate
dGTP	Deoxiguanosina trifosfato	Deoxyguanosine triphosphate
DNA	Ácido desoxirribonucleico	Deoxyribonucleic acid
DNAse	Deoxiribonuclease	Deoxyribonuclease
dNTP	Deoxiribonuclease trifosfato	Deoxyribonucleotide triphosphates
DTT	Ditiotreitol	Dithiothreitol
dTTP	2’ deoxitimidina 5’ trifosfato	2’deoxythymidine 5’triphosphate
E2	Estradiol	Estradiol
EGF	Fator de crescimento epidermal	Epidermal growth factor
FGF-2	Fator de crescimento fibroblástico-2	Fibroblast growth factor-2
FGF-10	Fator de crescimento fibroblástico-10	Fibroblast growth factor-10
Figα	Fator de transcrição em linhagem germinativa alfa	Factor in the germline alpha
FIV	Fecundação *in vitro*	*In vitro* fertilization
FOPA	Folículo ovariano pré-antral	Preantral ovarian follicle
FSH	Hormônio folículo estimulante	Follicle-stimulating hormone
G	Gauge	Gauge
GAPDH	Gliceraldeído-3-fosfato-desidrogenase	Glyceraldehyde 3-phosphate dehydrogenase
GDF-9	Fator de diferenciação de crescimento tipo 9	Growth differentiation factor type 9
GnRH	Hormônio liberador de gonadotrofinas	Gonadotropin-releasing hormone
GREL	Células semelhantes ao epitélio da superfície gonadal	Gonadal ridge epithelial-like cell
GS	Sequência rica em glicina e serina	Glycine- and serine-rich sequence
h	Hora	Hour

IA	Inseminação artificial	Artificial insemination
IL1-β	Interleucina 1 beta	Interleukin 1 beta
IGF-1	Fator de crescimento semelhante à insulina-1	Insulin-like growth factor 1
ITS	Insulina-transferrina-selênio	Insulin-transferrin-selenium
kDa	Quilodalton	Kilodalton
KL	Kit ligand	Kit ligand
LIF	Fator inibidor de leucemia	Leukemia Inhibitory Factor
LH	Hormônio luteinizante	Luteinizing hormone
LHR	Receptor para LH	LH receptor
M	Molar	Molar
MAPK	Proteína kinase ativada por mitógenos	Mitogen-activated protein kinase
MAPK-p38	Proteína kinase ativada por mitógenos p38	p38 mitogen-activated protein kinase
Mater	Antígeno maternal	Maternal antigen
MET	Microscopia eletrônica de transmissão	Transmission electron microscopy
mg	Miligrama	Milligram
MEM	Meio essencial mínimo	Minimum essential medium
min	Minuto	Minute
MIV	Maturação *in vitro*	*In vitro* maturation
mL	Mililitro	Millilitre
mm	Milímetro	Millimeter
mM	Milimolar	Millimolar
MOIFOPA	Manipulação de oócitos inclusos em folículos pré-antrais	manipulation of oocytes included in preantral follicles
mRNA	RNA mensageiro	Messenger RNA
n	Núcleo	Nucleus
ng	Nanograma	Nanogram
nL	Nanolitro	Nanoliter
nm	Nanômetro	Nanometer
Npm-2	Nucleoplasmina 2	Nucleoplasmin 2
Oo	Oócito	Oocyte
NUBIS	Núcleo de Biotecnologia de Sobral	Nucleus of Biotechnology of Sobral
P<0.05	Probabilidade menor do que 5%	Probability less than 5%

P>0.05	Probabilidade maior do que 5%	Probability higher than 5%
P4	Progesterona	Progesterone
PCNA	Antígeno nuclear de proliferação celular	Proliferating cell nuclear antigen
PCR	Reação em cadeia da polimerase	Polymerase Chain Reaction
PDK-1	Piruvato dehidrogenase lipoamida kinase isoenzima 1	Pyruvate dehydrogenase lipoamide kinase isoenzyme 1
PF	Folículo primordial	Primordial follicle
PGC	Célula germinativa primordial	Primordial germ cell
PI3K	Fosfoinositida 3-kinase	Phosphoinositide 3-kinase
PKC	Proteína quinase C	Protein kinase C
PrF	Folículo primário	Primary follicle
PHA	Fitohemaglutinina de *Phaseolus vulgaris*	Phitohemaglutinin of *Phaseolus vulgaris*
R-FSH	Receptor para o hormônio folículo estimulante	Receptor for follicle stimulating hormone
RNA	Ácido ribonucleico	Ribonucleic acid
RNAm	RNA mensageiro	Messenger RNA
RNAse	Ribonuclease	Ribonuclease
s	Senso	Sense
SD	Desvio-padrão	Standard deviation
SF	Folículo secundário	Secondary follicle
SMAD-1	Mensageiro intracelular do tipo 1	Mothers against decapentaplegic homolog 1
SMAD-4	Mensageiro intracelular do tipo 4	Mothers against decapentaplegic homolog 4
SMAD-5	Mensageiro intracelular do tipo 5	Mothers against decapentaplegic homolog 5
SMAD-8	Mensageiro intracelular do tipo 8	Mothers against decapentaplegic homolog 8
SMAD	MAD encontrada em vertebrádos	MAD found in vertebrates
StAR	Proteína reguladora aguda esteroidogênica	Steroidogenic acute regulatory protein

TE	Transferência de embriões	Transfer of embryos
TEM	Microscopia eletrônica de transmissão	Transmission electron microscopy
TGF-β	Superfamília de fatores de crescimento transformante beta	Transforming growth factor beta
TNF	Fator de necrose tumoral	Tumor necrosis fator
TNF-α	Fator de necrose tumoral alpha	Tumor necrosis fator alpha
UI	Unidade internacional	International unit
V	Vacúolo	Vacuole
VEGF	Fator de crescimento endotelial vascular	Vascular endothelial growth fator
VIP	Peptídeo intestinal vasoativo	Vasoactive intestinal peptide
ZAR1	Fixador de zigoto 1	Zygote arrest 1
Zp	Zona pelúcida	Pellucid zona
α-MEM	Meio essencial mínimo modificado de Eagle alfa	Minimum Essential Medium Eagle Alpha Modification
α-MEM^{+}	α-MEM suplementado	α-MEM supplemented
Ml	Microlitro	Microliter
Mm	Micrômetro	Micrometer

SUMÁRIO

1 INTRODUÇÃO

É conhecido que os ovários bovinos contêm milhares de oócitos imaturos inclusos predominantemente nos folículos pré-antrais. Estes folículos representam uma fonte potencial de gametas fertilizáveis, sendo de grande interesse assegurar o crescimento *in vitro* e permitir a aquisição da competência dos oócitos provenientes destes folículos (McLAUGHLIN *et al*., 2010). Entretanto, de toda a população folicular presente no ovário, apenas cerca de 0,1% atingirá a ovulação (NUTTINCK; MERMILLOD; DESSY, 1993), enquanto que os demais folículos sofrerão atresia (CARROLL *et al.*, 1990; OTALA *et al.*, 2002).

Nesse contexto, inúmeras pesquisas vêm sendo desenvolvidas a fim de aprimorar as técnicas de cultivo *in vitro* de folículos pré-antrais em bovinos, além de melhor compreender a foliculogênese ovariana nesta espécie, ainda pouco elucidada. O cultivo *in vitro* de córtex ovariano mimetiza as condições *in vivo* para manter a integridade tridimensional dos folículos e das suas interações com as células do estroma. Além disso, os folículos pré-antrais podem ser isolados do tecido ovariano e cultivados *in vitro*, gerando uma grande fonte potencial de oócitos que podem alcançar a competência meiótica. Esses tipos de cultivo permitem avaliar os efeitos de várias substâncias e fatores de crescimento na ativação e posterior desenvolvimento folicular. Isso contribui para uma melhor compreensão dos mecanismos envolvidos no desenvolvimento do oócito, regulação e controle da foliculogênese. Nesse sentido, o modelo de cultivo *in vitro* bovino pode ser aprimorado e ainda posteriormente usado em seres humanos, devido à semelhança da dinâmica do ovário de mulheres e vacas.

Diversas substâncias e fatores de crescimento já foram testados em meios de cultivo de folículos ovarianos. Entretanto, em folículos pré-antrais bovinos, a expressão e as funções biológicas de membros da subfamília das proteínas morfogenéticas ósseas (BMPs), como as BMP-2 e BMP-4, ainda são pouco estudadas. A expressão de BMP-2, BMP-4 e seus receptores (BMPRIA, BMPRIB e BMPRII) foram observados em folículos de vaca, cabra e ovelha, sugerindo o papel dessas BMPs no desenvolvimento folicular (FATEHI *et al*., 2005; COSTA *et al*., 2012; BERTOLDO *et al*., 2014). Além disso, é sabido que a BMP-4, em bovinos, controla um série de respostas biológicas, incluindo proliferação celular, esteroidogênese e supressão da apoptose em células da granulosa cultivadas *in vitro* (GLISTER e KNIGHT, 2004; SHIMIZU *et al*., 2012).

Para uma melhor compreensão deste trabalho, a revisão de literatura a seguir aborda aspectos relacionados à formação, ativação e crescimento dos folículos ovarianos, atresia folicular, sistemas de cultivo *in vitro* de folículos ovarianos e BMPs.

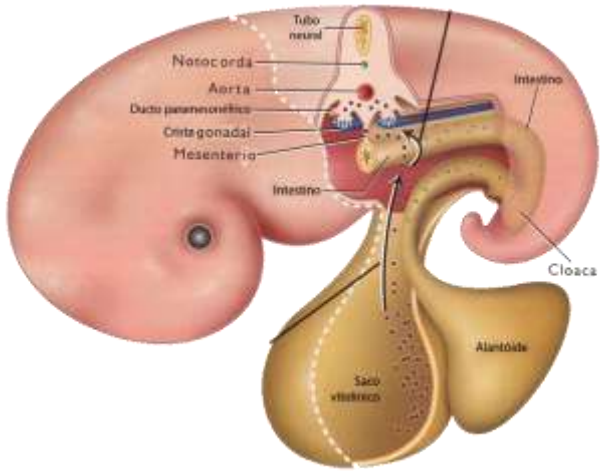
2 REVISÃO DE LITERATURA

2.1 Foliculogênese ovariana e sua regulação

2.1.1 Oogênese e formação do folículo primordial

O processo de formação, desenvolvimento e maturação folicular é denominado de foliculogênese (VAN DEN HURK e ZHAO, 2005). A foliculogênese inicia com a formação do oócito (oogênese) ainda na vida fetal a partir das células germinativas primordiais (CGPs), derivadas do endoderma do saco vitelínico do embrião, seguido da proliferação e migração por movimentos amebóides até a crista genital e colonização da gônada primitiva (Figura 1) (ADAMS *et al.*, 2008). No embrião bovino, as gônadas primitivas se originam por volta do 30° dia de gestação. Por volta de 35 dias de gestação, ocorre a migração de CGPs do saco vitelínico para a região das gônadas primitivas (SHIM e ANDERSON,1998).

Figura 1 – Migração das células germinativas primordiais a partir do saco vitelínico para as cristas gonadais.



Fonte: Adaptado de Senger (2003).

As CGPs são diferenciadas a partir do epiblasto proximal adjacente à ectoderme extraembrionária, sob a influência dos sinais de proteínas morfogenéticas ósseas derivadas do ectoderma (BMP-4 e 8b) e endoderme extraembrionária (BMP-2) (YING e ZHAO, 2001; YING *et al*., 2001). O destino de células germinativas no epiblasto é uma consequência direta da sinalização de BMP-4 da ectoderme extraembrionária, que é antagonizado pelo endoderma

visceral anterior. Em resposta à BMP-4, o epiblasto ativa reguladores de transcrição e adquire características de células germinativas. A BMP-8b do ectoderma extraembrinário restringe o desenvolvimento da endoderme visceral anterior, contribuindo assim para a sinalização da BMP-4 (OHINATA et al., 2009). A ausência das BMP-4 (LAWSON *et al*., 1999) e BMP-8 (YING *et al.,* 2000), em embriões de camundongas, por exemplo, promoveu falha no desenvolvimento das CGPs. Além das BMPs, as moléculas sinalizadoras dos ligantes das BMPs (SMADs 1 e 5) e do fator de crescimento transformante-β (TGF-β), (SMADs 2 e 3), o fator inibidor de leucemia (LIF) e outros fatores repressores e reguladores transcricionais estão envolvidos na formação e proliferação das CGPs (OKTEN e URMAN, 2010).

Acredita-se que a migração das CGPs é orientada pelo contato entre o receptor c-kit, expresso na superfície destas células, e o seu ligante, o Kit ligante (KL) expresso nas células somáticas que formam o substrato para a migração das CGPs para a crista gonadal (FLEISCHMAN, 1993). Alguns fatores, como o LIF, tem papel importante na proliferação das CGPs (CHENG *et al.,* 1994). Além disso, a ausência de Oct4 em CGPs provoca apoptose prematura dessas células antes da colonização da gônada (KEHLER *et al.,* 2004).

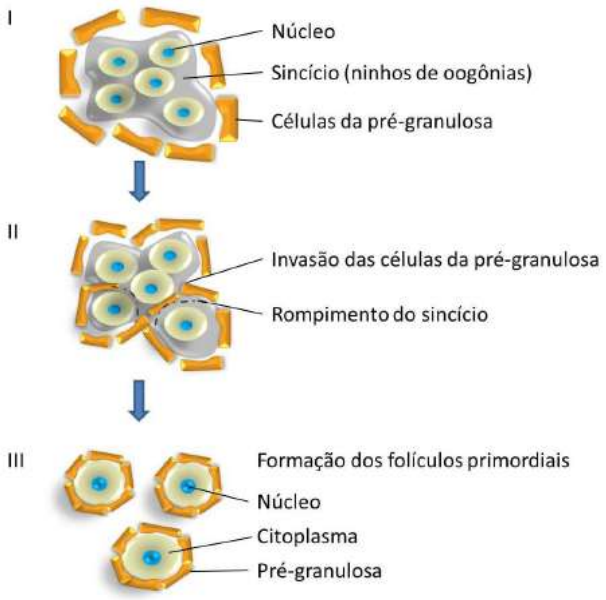
Com a chegada das CGPs na gônada primitiva, ocorre a formação dos cordões sexuais. A partir de então, as CGP perdem a sua motilidade e passam a se multiplicar por mitose, morrendo, entretanto, a grande maioria delas por apoptose (KIM e TILLY, 2004). As demais CGPs são então diferenciadas em oogônias (BAKER e FANCHI, 1967; SATHANANTHAN; SELVARAJ; TROUNSON, 2000) e uma vez diferenciadas, estas irão se dividir sucessivamente por mitose, aumentando significativamente em número e irão formar ninhos de oogônias (sincícios) interligados por pontes intercelulares (causadas por citocinese incompleta durante a divisão celular) (PEPLING e SPRADLING, 1998, 2001; PEPLING, 2006). Dentro das oogônias, ligadas umas às outras com as pontes intercelulares, a meiose começa simultaneamente, sugerindo a propagação do sinal para favorecer o início da meiose através destas pontes (BALTUS *et al.,* 2006). O início da meiose marca o fim da fase oogonial e estas células passam a se chamar oócitos primários. Em ovários de fetos bovinos, o início da meiose ocorre por volta do 82° dia de gestação (RÜSSE, 1983).

Em seguida, ocorre o rompimento das pontes intercelulares dos oócitos primários, através de apotose de oócitos individuais, juntamente com a migração das células da pré-granulosa, as quais podem ser derivadas do mesonéfron ou do epitélio da superfície ovariana (MCNATTY *et al.,* 2000). O rompimento inadequado das pontes intercelulares dos sincícios pode levar à formação de folículos multioocitários (PEPLING *et al.,* 2006). O ovário pode utilizar caminhos alternativos para sobrevivência e morte celular além da apoptose. A

autofagia pode ser um importante regulador da sobrevivência das células germinativas antes da formação do "pool" de folículos primordiais em ovários de murino (GAWRILUK *et al.*, 2011).

Uma vez que o oócito é circundando pelas células somáticas, ocorre uma parada da meiose no estágio de diplóteno da prófase I, também conhecido como estádio de vesícula germinativa (BAKER e FRANCHI, 1967; PICTON; BRIGGS; GOSDEN, 1998), no qual as células da pré-granulosa param de se multiplicar, entram num período de quiescência (SAWYER *et al.*, 2002) e, juntamente com o oócito, formam os folículos primordiais (Figura 2). Os folículos primordiais representam o pool de células germinativas disponíveis durante toda a vida reprodutiva da fêmea (EPIFANO e DEAN, 2002) e correspondem a um total de 95% de todos os folículos presentes no ovário (CORTVRINDT e SMITZ, 2001). A manutenção da quiescência é importante para garantir a fertilidade das fêmeas, pois impede a ativação precoce de todos os folículos e/ou a morte folicular (REDDY, 2008).

Figura 2 – Formação de folículos primordiais. (I) Ninhos de células germinativas; (II) Invasão das células somáticas (pré-granulosa) causando o rompimento do sincício (ninho) acompanhado por uma intensa apoptose oocitária; (III) as células germinativas sobreviventes, no estágio de prófase I (oócitos primários), são circundadas por uma camada de células da pré-granulosa e por uma membrana basal, formando os folículos primordiais.

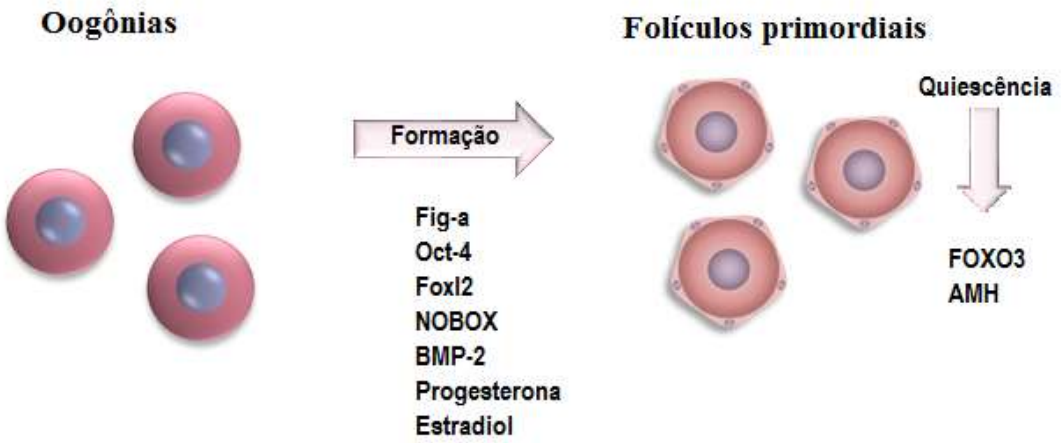


Fonte: Adaptado de Chaves *et al.*, (2011).

Os produtos de vários genes estão envolvidos na montagem e quiescência de folículos primordiais, tais como o Fator de linhagem germinal alpha (Fig-α), Fator de transcrição "Forkhead box" L2 (Foxl2), Fator de transcrição "Forkhead" 3 (FOXO3) e NOBOX (Newborn ovary homebox). Fig-α é um fator transcricional e está envolvido na formação de folículos primários através da ativação de outros genes, como o Oct-4. Em camundongas Knockout para Oct-4, nenhum folículo foi encontrado no ovário, o que foi atribuído a uma apoptose prematura das CGPs antes da colonização da gônada (KEHLER *et al.*, 2004). O Foxl2 é ativo nas células da pré-granulosa e tem papel na diferenciação dessas células durante a formação do folículo primordial. A ausência de atividade do Foxl2 pode afetar a diferenciação de células da granulosa, resultando na perda dos folículos e falha ovariana prematura (FOP) (SHIMIDT *et al.*, 2004). FOXO3 é um potente supressor da ativação de folículos primordiais, contribuindo para a manutenção da reserva folicular. A perda da sua função leva à ativação global dos folículos primordiais que ocorre quase imediatamente depois da formação do folículo, dentro de poucos dias após o nascimento, levando à FOP (GALLARDO *et al.*, 2009). Uma mutação em NOBOX resulta na incapacidade das células da granulosa rodearem os oócitos individualmente, resultando na formação de folículos poliovulares. Adicionalmente ocorre também interações célula-célula que sugerem que as propriedades de reconhecimento e adesão durante a organização folicular estão comprometidas, culminando na sentença de morte dos folículos envolvidos no início da vida pós-natal e comprometendo a reserva folicular (ALBERTINI, 2011).

Além disso, alguns hormônios e fatores de crescimento também influenciam a formação dos folículos primordiais. Já foi demonstrado que a BMP-2 regula a formação de folículos primordiais através da transição de células germinativas para oócitos e da diferenciação de células somáticas em células da pré-granulosa (CHAKRABORTY e ROY, 2015). Estudos têm ainda sugerido que os níveis de progesterona e estradiol fetais e maternos regulam a formação do folículo primordial (KEZELE e SKINNER, 2003; BRITT *et al.*, 2004; NILSSON; STANFIELD; SKINNER, 2006a; CHEN *et al.*, 2007). O Hormônio anti-müleriano (AMH) é um importante regulador da quiescência dos folículos primordiais, sendo utilizado como um marcador hormonal da reserva ovariana em testes clínicos (DURLINGER *et al.*, 2002) (Figura 3).

Figura 3 – Fatores envolvidos na formação e manutenção da quiescência de folículos primordiais.



Fonte: Adaptado de Rossetto *et al.*, (2013).

2.1.2 Transição de folículo primordial para primário (ativação folicular)

Durante a vida reprodutiva das fêmeas, o folículo primordial tem três possíveis destinos: (I) manter-se quiescente, ou em repouso, durante todo o período reprodutivo; (II) ser ativado e fazer parte do *pool* de folículos em crescimento, podendo sofrer atresia ou ovulação em uma fase posterior do desenvolvimento, ou ainda (III) sair da quiescência e sofrer atresia diretamente (McGEE; HSUEH, 2000; BROEKMANS *et al.*, 2007).

A ativação dos folículos primordiais caracteriza-se pela saída destes folículos do estágio de quiescência e entrada destes para o *pool* de folículos em crescimento. A ativação folicular resulta em alterações bioquímicas e funcionais nas células foliculares que levam ao aumento da atividade metabólica e transcricional destas células. As camadas de células achatadas da pré-granulosa tornam-se cubóide e adquirem atividade mitogênica, passando a serem chamadas células da granulosa (SALHA *et al*., 1998). Quando o oócito é circundado por uma camada completa de células da granulosa de morfologia cúbica, os folículos são classificados como primários (BARNETT *et al.*, 2006).

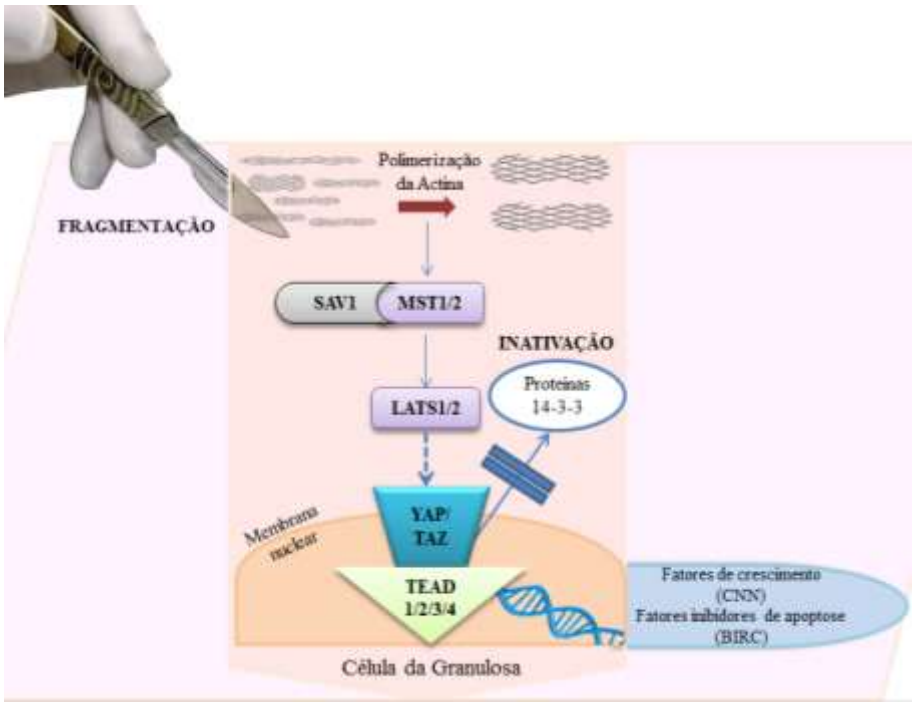
Uma das questões-chave de interesse no processo de ativação é o fato de que um folículo em particular é estimulado a crescer, enquanto outro imediatamente adjacente permanece quiescente. Uma proposta conhecida como hipótese da “linha de produção” sugere que os primeiros oócitos a entrarem na parada meiótica durante o desenvolvimento da gônada embrionária são, de fato, também os primeiros a ativarem na vida adulta (HENDERSON e

EDWARDS, 1968; MCLAUGHLIN e MCIVER, 2009). O crescimento inicial pode depender da proporção de cada tipo de célula que compõe o folículo, em que cada tipo celular pode ter um limiar para determinar o início da ativação de cada folículo (HIRSHIFIELD, 1992). Além disso, em humanos, estudos relacionados ao estoque de gametas sugerem que a proporção de folículos que ativam parece depender do tamanho da reserva folicular ovariana, e existe uma aparente correlação inversa entre a fração de folículos em crescimento e o tamanho do estoque de folículos primordiais (PETERS, 1979; GOUGEON, 1996).

Com ações coordenadas e sinérgicas de sinais resultantes de diferentes compartimentos, tais como oócitos, células somáticas e do estroma, o crescimento é iniciado em folículos primordiais. A diminuição dos níveis de fatores inibitórios ou o aumento dos níveis de fatores estimulatórios, inicia esse processo (WANDJI; PELLETIER; SIRARD, 1992; FORTUNE *et al.*, 2003).

Tem sido sugerido que um inibidor de origem medular regula a ativação folicular *in vivo* e que a separação do córtex da medula provoca ativação espontânea de folículos primordiais *in vitro* (CUSHMAN *et al.*, 2002). No entanto, mais recentemente, Kawamura et al. (2013) demonstraram que a fragmentação do ovário aumentou a polimerização de actina e interrompeu a via Hippo de sinalização, o que leva ao aumento da expressão de fatores de crescimento CCN. O nome CCN denomina os principais membros da família desses fatores, incluindo a proteína angiogênica rica em cisteína (Cyr61 ou CCN1), o fator de crescimento do tecido conjuntivo (CTGF ou CCN2) e NOV (nephroblastoma overexpressed ou CCN3). A secreção de CCN2 e fatores relacionados promoveu o crescimento de folículos primordiais *in vitro* (revisado por HSUEH *et al.,* 2015). A via Hippo de sinalização é essencial para a manutenção ótima do tamanho dos órgãos e é conservada em todos os animais metazoários (PAN, 2007; HALDER e JOHNSON, 2011; HERGOVITCH, 2012). A sinalização Hippo consiste de vários reguladores de crescimento negativos que agem em uma cascata de quinases que, em última análise, fosforila e inativa efetores-chave da via Hippo, proteínas Yes-associated (YAP) /coativador transcricional com domínio de ligação PDZ (TAZ). Quando a sinalização Hippo é interrompida, diminui a fosforilação de YAP e aumenta os níveis de YAP nucleares, bem como aumenta a expressão de fatores de crescimento CNN (HOLBOURN *et al*., 2008) (Figura 4).

Figura 4 – Via de sinalização Hippo no ovário. A fragmentação do córtex ovariano induz a desorganização do citoesqueleto e a polimeração da actina que desencadeia a ativação de quinases, MST1/2. Feito isso, acontece a fosforilação da proteína SAV1 e formação do complexo SAV1_MST1/2, que por sua vez fosforila e ativa as quinases LARS1/2. Em seguida, co-ativadores transcricionais YAP1/TAZ são fosforilados e associados com proteínas 14-3-3, sendo retidos no citoplasma e, portanto inativados. A inativação de YAP1/TAZ é seguido da translocação destes fatores para o núcleo, onde, em conjunto com outros ligantes, como o TEAD1-4, induzem a expressão de fatores de crescimento (Ex: CCN), bem como de fatores que inibem a apoptose, controlando assim a proliferação e/ou sobrevivência.



Fonte: Adaptado de HSUEH *et al.*, (2015).

A via de sinalização PI3K/PTEN/Akt é importante na determinação do curso do desenvolvimento do folículo primordial, incluindo sua ativação, sobrevivência e morte (MARKHOLT *et al*, 2012). A supressão do gene PTEN em ratas causou a ativação prematura de todos os folículos primordiais do ovário, seguido do seu esgotamento no início da idade adulta, resultando em FOP (REDDY *et al.*, 2008). Contudo, quando o gene PTEN foi excluído de folículos primários formados, seu desenvolvimento não foi alterado, atingindo a maturação e gerando crias normais (JAGARLAMUDI *et al.*, 2009). Isso sugere que a PTEN/PI3K regula o crescimento folicular apenas no folículo primordial. Além disso, a proteína alvo da rapamicina em mamíferos - complexo 1 (mTORC1) é uma serina treonina quinase, membro da via PI3K/Akt, é um regulador de funções ovarianas, incluindo

quiescência, ativação e sobrevivência de folículos primordiais (MAKKER *et al.*, 2014). O supressor de tumor (TSC1) está envolvido na manutenção da quiescência do folículo primordial. A proteína TSC1 é parte de um heterodímero (TSC1/TSC2) que inibe a ativação de um complexo de proteínas quinase (mTORC), que por sua vez, atua no crescimento celular (ADHIKARI *et al.*, 2010). A supressão da atividade do complexo mTORC pelo gene *TSC1-TSC2* nos oócitos, é essencial para a manutenção da quiescência, enquanto a elevada atividade do mTORC1 no oócito é necessária para a ativação folicular em camundongos fêmeas (ADHIKARI *et al*., 2009; 2010). Já o NOBOX é expresso em células germinativas e tem papel na indução da ativação folicular. Um estudo utilizando ratas nocaute para o gene *Nobox* indicou a importância desse gene na ativação, uma vez que sua ausência interrompeu a transição dos folículos primordiais para o estágio primário (RAJKOVIC *et al.*, 2004).

Estudos com ovários de ratas demonstraram que o LIF promove transição de folículo primordial para primário e que pode interagir com KL para aumento na ativação folicular (NILSSON *et al*., 2002). Em cultivos *in vitro* de longa duração, que visam promover crescimento oocitário para posterior maturação *in vitro*, foi demonstrado que um meio dinâmico contendo KL e FSH foi capaz de manter a integridade e promover a ativação e crescimento de folículos primários caprinos, após 16 dias de cultivo (LIMA *et al.*, 2012). O papel do fator de crescimento epidermal (EGF) na manutenção da viabilidade (mas não na ativação) de folículos primordiais por estimular fosforilação de Akt foi demonstrado em gatas domésticas pré-púberes (FUJIHARA *et al.*, 2014). Vários membros da superfamília TGF-β, como as proteínas morfogenéticas ósseas -4, -7 e -15 (BMP-4, -7 e -15), são expressos por células do estroma ovariano e/ou células da teca (LEE *et al.*, 2001; NILSSON; SKINNER, 2003) e o fator de crescimento e diferenciação-9 (GDF-9) é expresso em oócitos (VITT *et al.*, 2000) e tem um papel importante na ativação folicular. Em cabras, a adição de BMP15 (100 ng/mL) ao meio de cultivo manteve a integridade e promoveu o crescimento de folículos primordiais cultivados durante sete dias (CELESTINO *et al.*, 2011). Em ovelhas, a imunização contra a proteína BMP-15 resultou em um bloqueio do desenvolvimento folicular inicial (JUENGEL *et al.*, 2002).

Outras substâncias, como o fator de crescimento derivado de plaquetas (PDGF) (NILSSON; DETZEL; SKINNER, 2006), ativina (OKTAY *et al.*, 2000), o fator neurotrópico derivado da glia (GDNF) (DOLE; NILSSON; SKINNER, 2008), as neurotrofinas (NGF), neurotrofina tipo 5 (NTF5) e fator neurotrófico derivado do cérebro (BDFN) (ROMERO et al., 2002; SPEARS et al., 2003; PAREDES et al., 2004; DOLE; NILSSON; SKINNER, 2008), o fator de crescimento epidermal (EGF) (CELESTINO *et al.*, 2009b) e o hormônio

insulina (KEZELE; NILSSON; SKINNER, 2002) podem estimular o início do crescimento folicular.

Em ruminantes, estudos *in vitro* com folículos primordiais caprinos têm demonstrado que o controle do início do crescimento folicular é regulado por diversos fatores, como o KL (CELESTINO *et al.,* 2010), o estradiol (LIMA-VERDE *et al.,* 2010), o peptídeo intestinal vasoativo (VIP) (BRUNO *et al.,* 2010), o fator de crescimento semelhante à insulina (IGF-1) (MARTINS *et al.,* 2010), o VEGF (BRUNO *et al.,* 2009), o fator de crescimento e diferenciação 9 (GDF-9) (MARTINS *et al.,* 2008; K TANG *et al*., 2012), a Ativina-A (SILVA *et al.,* 2006a), o fator de crescimento fibroblástico básico (FGF-2) (MATOS *et al.,* 2007; K TANG *et al*., 2012), o fator de crescimento epidermal (EGF) (SILVA *et al.,* 2004b), que atuam na promoção da ativação de folículos primordiais e no crescimento oocitário. A esfingosina 1-fosfato (S1P), após 7 dias de cultivo na concentração de 1 ng/mL, promoveu a ativação e o desenvolvimento de folículos primordiais caprinos (NÓBREGA Jr. *et al.,* 2014). Estudos *in vitro* demonstraram que o FSH (HULSHOF *et al.,* 1995), a Ativina-A (McLAUGHLIN *et al.,* 2010), e a testosterona (YANG *et al.,* 2006) estimulam o crescimento oocitário e a transição de folículos primários para secundários na espécie bovina. Ademais, o FGF-10 mantêm a viabilidade e a ultraestrutura folicular, promovendo a ativação e o crescimento de folículos pré-antrais caprinos cultivados *in vitro* por 16 dias (ALMEIDA *et al.*, 2015). Em ovinos, o EGF associado ao ácido indolacético (IAA) ou ao FSH foi capaz de promover a ativação de folículos primordiais e manter a viabilidade folicular por até seis dias de cultivo (ANDRADE *et al*., 2005). Em ovinos, o KL promoveu a ativação de folículos primordiais inclusos em tecido ovariano após 7 dias de cultivo *in vitro* (CAVALCANTE *et al*., 2014).

Já se sabe que a ativação é independente de gonadotrofinas. Apesar disso, o Hormônio Folículo Estimulante (FSH) exerce um efeito indireto sobre o desenvolvimento folicular inicial através da liberação de fatores parácrinos produzidos por folículos maiores ou pelas células do estroma ovariano (VAN DEN HURK e ZHAO, 2005). Alguns trabalhos demonstraram que o FSH regula a expressão de vários fatores de crescimento, tais como Kit Ligand (KL), Fator de Crescimento e Diferenciação-9 (GDF-9) e Proteína Morfogenética Óssea-15 (BMP-15) (THOMAS *et al.,* 2005). Entretanto, em bovinos, o FSH não promoveu ativação folicular (TEPEKOY e AKKOYUNLU, 2016).

2.1.3 Transição de folículo primário para secundário

O crescimento do folículo primário é iniciado com um aumento da multiplicação das células da granulosa para formar multicamadas, aumento do oócito e de seu conteúdo protéico, formação da zona pelúcida, formação de uma lâmina basal e formação da camada da teca interna, a partir de células do estroma intersticial (PICTON; BRIGGS; GOSDEN, 1998; KNIGHT; GLISTER, 2006). Durante esta fase, ocorre uma intensa atividade mitótica nas células da granulosa, que é regulada por inúmeros fatores, dentre os quais se destaca o antígeno nuclear de proliferação celular (PCNA). O PCNA é uma proteína nuclear que atua como co-fator para a DNA polimerase e se expressa diferentemente de acordo com o ciclo celular. A sua taxa de síntese é diretamente proporcional à taxa de proliferação celular. Essa proteína constitui-se em um marcador de células em proliferação, sendo expressa durante a replicação do DNA, no início da fase G1, como expressão máxima na fase S e declínio na fase G2 (WANDJI *et al*., 1996). O PCNA atua quando três subunidades dessa proteína se ligam a braçadeira deslizante onde estão unidas as DNAs polimerases durante a replicação do DNA, podendo ser usado como um marcador confiável de crescimento folicular (WANDJI; PELLETIER; SIRARD, 1992).

Quando duas ou mais camadas de células da granulosa se desenvolvem e as células da teca podem ser evidenciadas do estroma circundante, formam-se os folículos secundários. Nesse estágio, os oócitos entram em extensiva fase de crescimento, resultando em uma complexa organização citoplasmática dependente da síntese de novos produtos gênicos e organelas, bem como da modificação e redistribuição das organelas já existentes (PICTON; BRIGGS; GOSDEN, 1998). Além do expressivo aumento no número de ribossomos, mitocôndrias e outras organelas, os oócitos em crescimento acumulam grânulos glicogênicos, proteínas e lipídios, e sofrem ainda um incremento na síntese de RNA e proteínas, considerados importantes para garantir a futura competência meiótica (VAN DEN HURK; ZHAO, 2005).

Com o desenvolvimento folicular, as células da teca se estratificam em duas camadas: uma externa, denominada teca externa, composta por células indiferenciadas; e outra interna, conhecida como teca interna, na qual algumas células diferenciam-se e passam a secretar esteróides (GOUGEON *et al.*, 2010). As células da teca indiferenciadas, não expressam receptores para LH (LHRs) ou enzimas esteroidogênicas e, portanto, não são responsivas ao LH, mostrando que o início da diferenciação das células da teca independe de gonadotrofinas (MAGOFFIN; WEITSMAN, 1994).

Em primatas e ruminantes, o desenvolvimento de um folículo secundário é um processo demorado que leva meses para ser concluído. Este lento processo, parece não ser

mediado pela ação das gonadotrofinas, mas existe uma grande divergência sobre este fato. Embora folículos primários e secundários já expressem os receptores para FSH nas células da granulosa (OKTAY; BRIGGS; GOSDEN, 1997), essa gonadotrofina desempenha apenas um papel responsivo, ao invés de essencial, ao desenvolvimento desses folículos. Entretanto, estudos já mostraram que o FSH promove o crescimento de folículos secundários bovinos cultivados *in vitro* (PASSOS *et al*., 2013). Outras substâncias ainda têm se mostrado importantes para essa fase. Na espécie caprina, estudos em folículos pré-antrais confirmaram a importância da atuação tanto da BMP-15 (CELESTINO *et al.*, 2011b) como do GDF-9 (MARTINS *et al*., 2008) na progressão de folículos primários até o estágio secundário *in vitro*, destacando ainda os efeitos positivos destes dois fatores de crescimento sobre a manutenção da viabilidade folicular durante essa fase. Em contraste, outros estudos apontam o papel negativo para o AMH no desenvolvimento de folículos pré-antrais (KNIGHT; GLISTER, 2006). YANG e FORTUNE (2007) mostraram após o cultivo *in vitro* de folículos pré-antrais bovinos, que a testosterona e o fator de crescimento do endotélio vascular (VEGF) também podem influenciar a transição de folículos primários para secundários.

2.1.4 Transição de folículo secundário para terciário (antral)

Nos folículos secundários, há uma intensa proliferação das células da granulosa, que se organizam em várias camadas. O crescimento dos folículos secundários é primariamente controlado pelos reguladores intraovarianos (fatores de crescimento, citocinas e esteroides gonadais) e não são dependentes de gonadotrofinas (HALPIN *et al.*, 1986). À medida que crescem, secretam água, eletrólitos, proteínas séricas e alta concentração de hormônios esteroides, formando uma cavidade cheia de líquido entre as camadas, chamada cavidade antral ou antro (BARNETT *et al*., 2006). Esse líquido, denomidado fluido folicular antral, é originalmente derivado da vascularização das camadas tecais adjacentes. Já foi evidenciado a presença de grandes moléculas osmoticamente ativas no fluido folicular ovariano, tais como ácido hialurônico, sulfato de condroitina e sulfato de dermatan, que são fortemente hidrofílicas e carregadas negativamente, e assim contribuem diretamente para uma intensa atividade osmótica (CLARKE *et al.*, 2006). Dentre os proteoglicanos que atuam na formação do antro, os mais importantes são a Hialuronidase sintetase 1 e 2 (HAS 1 e 2), Versican e Perlecan. Sabe-se que a HAS 2 pode ser induzida por gonadotrofinas em células da granulosa de bovinos (SCHOENFELDER *et al.*, 2003). Em bovinos, o GDF-9 estimula a

expressão de Versican e Perlecan e interage positivamente com FSH para aumentar a expressão de HAS 2 (VASCONCELOS *et al.*, 2013).

Durante o desenvolvimento folicular, a produção de fluido antral é intensificada pelo aumento no número e na permeabilidade dos vasos sanguíneos foliculares. O folículo desenvolve a sua própria rede vascular dentro da camada tecal circundante (FRASER e DUNCAN, 2009). Existem muitos fatores potenciais envolvidos no controle da angiogênese no folículo em desenvolvimento, mas o fator de crescimento endotelial vascular (VEGF) tem um papel central e tem sido estudado extensivamente. VEGF, um potente mitógeno para as células endoteliais (FERRARA e DAVIS-SMYTH, 1997), estimula a permeabilidade vascular (CONNOLLY 1991, SENGER *et al.*, 1993), e é altamente expresso em células da granulosa e em níveis mais baixos na camada da teca de folículos a partir da fase secundária em ovários de primatas (TAYLOR *et al.*, 2004). Certamente, nos estágios iniciais do desenvolvimento folicular, a inibição do VEGF impede a proliferação de células endoteliais e, portanto, diminui a formação de célula da teca, impedindo o desenvolvimento do folículo (WULFF *et al.*, 2002, FRASER *et al.*, 2005, FRASER e DUNCAN 2009). Além disso, muitos outros fatores também contribuem e modular a angiogênese e vasculogênese em mamíferos, tais como os membros da superfamília do TGF-β e seus antagonistas, angiopoietinas, factor de crescimento de fibroblastos (FGF) e gonadotrofinas (YOUNG e McNEILLY, 2009). Nesta fase, as células da teca sofrem alterações morfológicas e funcionais, e aquelas células localizadas próximas à membrana basal passam a ser denominadas teca interna, enquanto que as localizadas perifericamente são classificadas como teca externa.

Diversos fatores de crescimento influenciam o crescimento de folículos secundários, entre eles o Hormônio do crescimento (GH), o fator de crescimento epidermal (EGF), Kit Ligant (KL), BMP-15 e Ativina A. Estudos *in vitro* usando GH têm demonstrado que esse fator desempenha um importante papel controlando as fases iniciais do desenvolvimento folicular. A adição de GH ao meio de cultivo *in vitro* de folículos pré-antrais estimulou a produção de estradiol e a proliferação das células da granulosa e da teca em murinos (KOBAYASHI *et al.*, 2000), além de promover um aumento no diâmetro de folículos pré-antrais de camundongos fêmeas cultivados *in vitro* (KIKUCHI *et al.*, 2001). Magalhães et al. (2011) demonstraram que o GH estimula o desenvolvimento de folículos secundários caprinos, favorecendo a formação do antro e a posterior maturação oocitária. O EGF promove aumento do crescimento, viabilidade e formação de antro de folículos secundários caprinos (CELESTINO *et al.*, 2011a). Em caprinos e ovinos, foi verificado que o KL estimula a

formação da cavidade antral e o posterior crescimento e retomada da meiose de oócitos oriundos de folículos pré-antrais cultivados *in vitro* (LIMA *et al*., 2011; LUZ *et al.,* 2013). Em bovinos, foi demonstrado que a BMP-15 influencia positivamente a formação de antro, o crescimento e mantém a ultraestrutura de folículos secundários cultivados *in vitro* (PASSOS *et al.,* 2013). A Ativina-A promove proliferação das células da granulosa, formação de antro e manutenção da morfologia do oócito de folículos bovinos (McLAUGHLIN e TELFER, 2010; SILVA *et al.,* 2014).

Com a progressão do desenvolvimento dos folículos antrais, o FSH torna-se criticamente determinante para o crescimento e a sobrevivência dos folículos, os quais passam a ser dependentes desta gonadotrofina. A partir deste ponto, será a concentração de FSH circulante disponível que determinará se o folículo sofrerá atresia ou continuará o seu desenvolvimento até os estágios antrais tardios. O desenvolvimento desses folículos pode ser subdividido nas fases de crescimento, recrutamento, seleção e dominância (VAN DEN HURK e ZHAO, 2005). Em várias espécies domésticas, os folículos antrais são recrutados e crescem simultaneamente em uma onda folicular sob o controle das gonadotrofinas (FORTUNE *et al*., 2001). Em bovinos, já foi demonstrado que a dinâmica folicular acontece observando um padrão de uma, duas, três ou até quatro ondas de desenvolvimento folicular por ciclo estral (BARUSELLI *et al.,* 2007). Uma elevação nas concentrações plasmáticas de FSH estimula o recrutamento folicular e a emergência da onda folicular (FORTUNE, 1994).

Ao iniciar uma nova onda folicular, um determinado grupo de folículos é recrutado, apresentando crescimento simultâneo, sobre estímulo do FSH, mas somente um deles será selecionado (GINTHER *et al*., 2003). Esta etapa estende-se por dois a três dias e os folículos apresentam uma taxa de crescimento constante (MIHM e BLEACH, 2003; PETER *et al*., 2009). A partir de então, este folículo passará a exercer dominância sobre os demais folículos, suprimindo assim o desenvolvimento e levando à inibição do recrutamento de um novo grupo de folículos. Na presença de níveis elevados de progesterona, o folículo dominante tornar-se-á anovulatório, em virtude da frequência na pulsatilidade do hormônio luteinizante (LH) ser baixa (GINTHER; KNOPF e KASTELIC, 1989; GINTHER *et al.,* 1996).

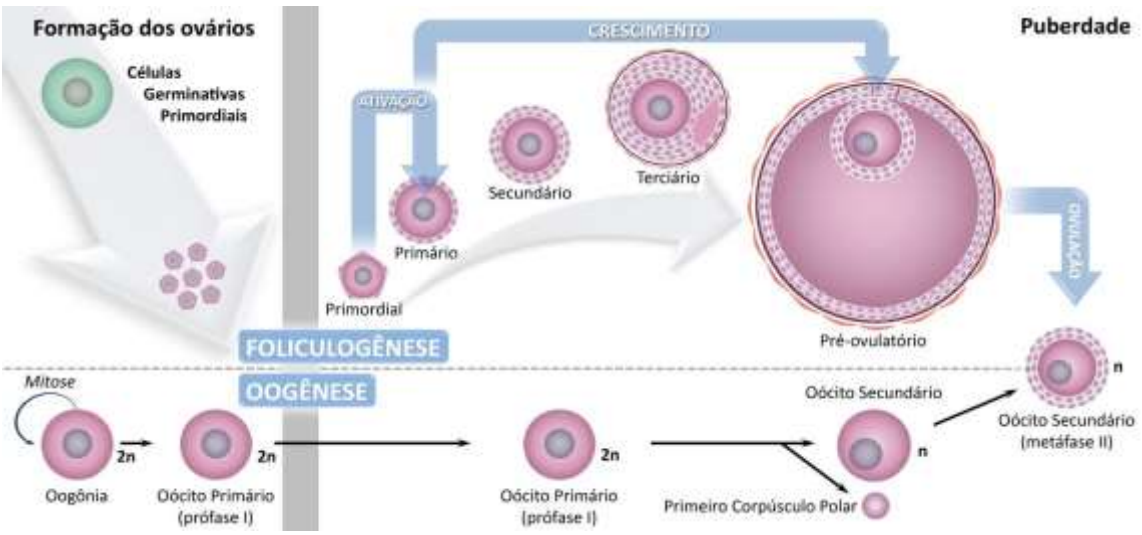
Para a espécie bovina, a formação dos folículos antrais é observada aos 230 dias de gestação (RÜSSE, 1983). A partir deste estágio, o diâmetro do folículo aumentará notavelmente em virtude do crescimento oocitário, proliferação das células da granulosa, das células da teca e pela expansão da cavidade antral (DRIANCOURT, 2001). As células da granulosa que ficam próximas ao oócito sofrem diferenciação para formar as células do cumulus, enquanto que as demais formam as células da granulosa murais. Os folículos antrais

podem ser classificados como pequenos ou grandes folículos antrais. Os pequenos folículos antrais apresentam crescimento rápido em diâmetro e decorrente acúmulo de fluido folicular (BRISTOL-GOULD; WOODRUFF, 2006). A partir deste estágio, os folículos tornam-se dependentes destes hormônios até o estágio de folículos pré-ovulatórios, que tem um diâmetro em torno dede 10 a 12 mm em *Bos indicus* e 12 a 15 mm em *Bos taurus* (GINTHER, 2003).

Quando atingem o estágio de folículos pré-ovulatórios, apresentam o oócito circundando por várias camadas de células do cumulus. Nessa fase final do desenvolvimento folicular, ocorre uma diminuição dos níveis circulantes de FSH em resposta ao alto nível de estradiol e inibina produzidos pelo folículo e observa-se então a formação do folículo pré-ovulatório. Neste estágio, as células da granulosa param de se multiplicar e iniciam o processo final de diferenciação devido à ação do LH (DRIANCOURT, 2001).

Na puberdade, devido à liberação pré-ovulatória do LH, algumas horas antes da ovulação, a meiose é retomada e o núcleo do oócito sai da fase de diplóteno da prófase I e entra em diacinese (ADASHI, 1994). Nesse momento, a meiose progride finalizando a prófase I, metáfase I, anáfase I e telófase I ocorrendo a expulsão do primeiro corpúsculo polar, resultando na formação do oócito secundário, assim denominado por estar na segunda divisão da meiose (SAUMANDE, 1991). Este iniciará a segunda divisão meiótica, em que o núcleo do oócito evolui até o estágio de metáfase II, quando ocorre a segunda interrupção da meiose (GORDON *et al*.,1994). O oócito permanece assim até ser fecundado pelo espermatozóide, quando então completa a meiose e expulsa o segundo corpúsculo polar, formando o oócito haplóide fecundado (Figura 5) (MOORE; PERSAUD, 1994).

Figura 5 – Desenho esquemático dos processos de oogênese e foliculogênese.



Fonte: Rossetto *et al.* (2013).

2.2 Atresia folicular

As fêmeas dos mamíferos, com exceção das murinas, nascem com os ovários contendo sua reserva total de gametas, os quais são mantidos em bloqueio meiótico até a puberdade, quando uma porção deles é estimulada a crescer até culminar na ovulação (BYSKOV, 1982). Entretanto, evidências em murinos sugerem que os oócitos que são constantemente perdidos podem vir a ser substituídos a partir de uma pequena população de células tronco germinativas presente nos ovários adultos, durante a vida reprodutiva do animal (JOHNSON *et al*., 2004). Em humanos, o cultivo do epitélio germinativo ovariano permitiu o desenvolvimento de células da granulosa e oócitos (BUKOVSKY; SVETLIKOVA; CAUDLE, 2005). Em caprinos e em bovinos, o cultivo do epitélio da superfície ovariana gerou células que foram posteriormente diferenciadas em células semelhantes à oócitos (PARTE *et al.*, 2011; SOUZA *et al.*, 2016).

Independente disso, ao longo da vida das fêmeas, há uma significativa redução na população de folículos pré-antrais (SHAW; ORANRATNACHAI; TROUNSON, 2000). Isto se deve a um processo fisiológico degenerativo irreversível conhecido como atresia (morte celular), que acomete cerca de 99,9% dos folículos ovarianos (FIGUEIREDO *et al.*, 2008). Portanto, apesar do expressivo contingente da população folicular no ovário, poucos são os oócitos que serão ovulados e potencialmente serão fecundados, caracterizando a gônada feminina como um órgão de baixo rendimento.

Durante a atresia, a morte celular não se limita a um tipo específico de célula e o folículo todo é degradado durante este processo. Várias formas de morte foram reportadas para as células ovarianas, incluindo apoptose, autofagia, necrose e cornificação (JOLLY et al., 1994, VAN WEZEL *et al.*, 1999, D'HAESELEER *et al.*, 2006).

A atresia está associada a uma série de mudanças morfológicas e bioquímicas, que variam de acordo com o estágio do crescimento folicular e também com a espécie animal (CUNNINGHAM; KLEIN, 2011). Este processo, pelo qual o folículo perde completamente a sua integridade, pode se dar por duas vias distintas: (1) via degenerativa (SAUMANDE *et al.*, 1981), na qual pode ser observada alterações no fornecimento de oxigênio e nutrientes para o ovário, além de ser causada por isquemia, que resulta em algumas alterações na permeabilidade da membrana celular. Essas alterações podem levar ao aumento de água intracelular e do volume das células, vacuolização citoplasmática e, consequentemente, degeneração (BARROS; HERMOSILLA; CASTRO, 2001). Além disso, a atresia folicular pode ocorrer pela (2) via apoptótica (FIGUEIREDO *et al.*, 1995), na qual observa-se um

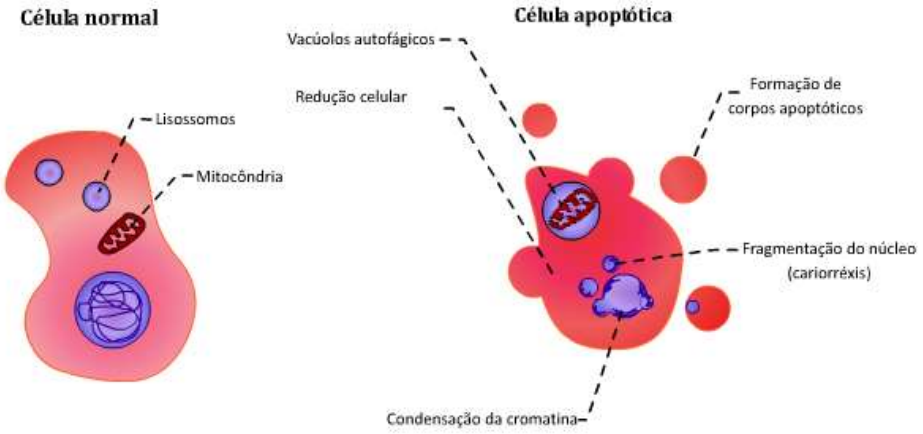
processo de morte celular individual e ativo, caracterizado pela fragmentação nuclear e pela formação de corpos apoptóticos (RACHID; VASCONCELOS; NUNES, 2000), em que o desbalanço entre os genes pró e anti-apoptóticos determinam a morte celular (HURWITZ; ADASHI, 1992). Apesar de causar a perda de vários folículos ovarianos, a atresia é um evento crucial para manutenção da homeostase ovariana em mamíferos, assegurando a ciclicidade dos animais e prevenindo o desenvolvimento de múltiplos embriões durante a gestação (AMSTERDAM *et al.,* 2003; CELESTINO *et al.,* 2009).

A iniciação, execução e regulação da apoptose envolve vários fatores bioquímicos e a família de enzimas caspases desempenham um papel central na rede de sinalização da apoptose. As caspases são membros da família altamente conservada de proteases de cisteína com especificidade a aspartato. As caspases são expressas como pró-enzimas que sofrem processamento proteolítico para gerar a forma ativada após estímulo apoptótico. Existem 14 tipos de caspases identificados como caspase-1, 2, 3 até caspase-14 (TIBBETS *et al.,* 2003). Alguns membros da família funcionam especificamente na morte celular por apoptose e são subdivididos em iniciadoras (caspases-2, -8, -9 e -10) e executoras ou caspases efetoras (caspase-3, -6 e -7; STRASSER *et al.,* 2000). Caspases iniciadoras são clivadas em resposta a estímulos apoptóticos e ativam as caspases efetoras (GREEN, 2003; 2004). Durante a apoptose, as caspases efetoras clivam numerosas proteínas localizadas na membrana da célula, no núcleo e no citoplasma (NAGASE *et al.,* 2003). No sentido de evitar a morte celular por apoptose, existem processos de sobrevivência celular que promovem a transcrição de várias proteínas anti-apoptóticas, tais como alguns membros da família Bcl-2, que bloqueiam a progressão da apoptose em diferentes etapas ao longo deste processo (JOHNSON, 2003). A família Bcl-2 compreende tanto membros anti-apoptóticos, como o Bcl-2 (Linfoma de células B2) e membros pró-apoptóticos, incluindo Bax (Proteína X associada as células B de linfoma 2), Bid (Proteína BH3 semelhante à Bax), Bak (Proteína-killer antagonista homóloga à Bcl-2) e Bik (Proteína Killer que interage com Bcl-2) (SHI et al., 2002).

A apoptose pode ser desencadeada por vários estímulos e condições e é ativada por duas vias principais: a via extrínseca ou via do receptor de morte, e a via intrínseca ou via mitocondrial, que convergem mutuamente através da cascata de reações proteolíticas que envolvem a ativação das caspases (ELMORE, 2007; IGNEY e KRAMMER, 2002). A via extrínseca é mediada pela ligação de ligantes a certos receptores existentes na superfície celular – os receptores de morte (ELMORE, 2007), pertencentes à família dos fatores de necrose tumoral (TNF). Estes receptores de morte incluem uma subfamília que é caracterizada

por terem domínios extracelulares ricos em cisteína e um domínio intracelular – o domínio de morte – que é essencial para a transdução do sinal apoptótico (IGNEY e KRAMMER, 2002). A via intrínseca pode ser desencadeada por diversos fatores, como por exemplo, espécies reativas de oxigênio (EROs), ausência de fatores de crescimento e hormônios. Independente da via, as características morfológicas que indicam apoptose são colapso e enrrugamento do núcleo, segmentando-se em corpúsculos heterocromáticos para o interior do citoplasma (cariorréxis), picnose do núcleo, bem como a condensação da cromatina, edema, irregularidade dos limites celulares e nucleares e fragmentação no citoplasma (ZEISS, 2003). Subsequentemente, as membranas nuclear e citoplasmática se desprendem e formam corpos apoptóticos (cariólise) (Figura 6) (GRIVICICH *et al.*, 2007).

Figura 6 - Alterações morfológicas encontradas em uma célula apoptótica.



Fonte: Adaptado de Grivicich *et al.* (2007).

2.3 Cultivo *in vitro* de folículos pré-antrais inclusos em tecido ovariano (*in situ*)

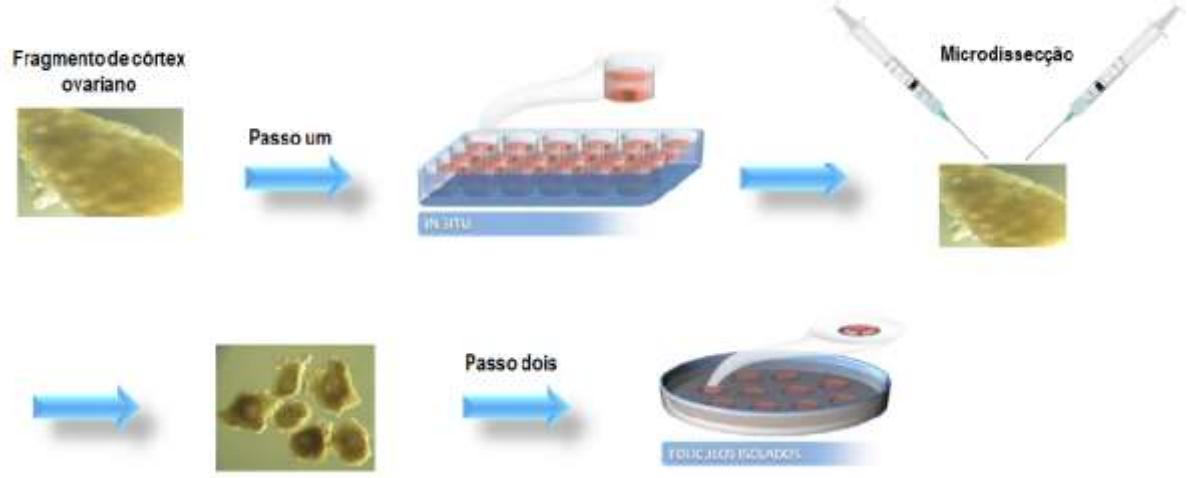
A maioria dos folículos no tecido cortical ovariano estão em repouso, ou seja são folículos primordiais. Por isso, a primeira consideração de um sistema de cultivo *in vitro* deve ser o de otimizar a ativação destes folículos *in vitro* e suportar o desenvolvimento folicular inicial. Os fatores que regulam a ativação folicular e crescimento inicial ainda não estão bem definidos, mas o processo exige uma combinação de inibidores, estimuladores e fatores de manutenção (TELFER e ZELINSKY, 2013; NELSON *et al.*, 2013). O cultivo de folículos inclusos em tecido ovariano (*in situ*) visando a ativação e crescimento de folículos primordiais vem sendo estudado como uma alternativa para otimizar o potencial reprodutivo das fêmeas,

incluindo mulheres. Além disso, o cultivo *in vitro* de folículos pré-antrais é uma ferramenta que permite aperfeiçoar o conhecimento básico sobre os mecanismos envolvidos na foliculogênese ovariana (ARUNAKUMARI; SHANMUGASUNDARAM; RAO, 2010) e ainda constitui um modelo para testes de toxicidade de diversas substâncias.

Em roedores, a pequena dimensão do ovário permite que ele seja cultivado inteiro. Já para animais maiores, o córtex ovariano é fragmentado para o cultivo (TELFER; McLAUGHLIN, 2011). Esse tipo de cultivo é prático, não requer muito tempo para execução, mantém a arquitetura tridimensional dos folículos e preserva as interações entre o folículo e as células do estroma circundante. Entretanto, o tecido cortical pode agir como uma barreira à perfusão do meio de cultivo (FORTUNE, 2003). Outro fator limitante desse tipo de cultivo é que cada peça cortical tem variação em número e fase de distribuição de folículos, dificultando a comparação entre os números de folículos em um fragmento de tecido ovariano do tratamento controle com peças submetidas a diferentes tratamentos *in vitro* (RICE; OJHA; MASON, 2008; TING *et al.*, 2011). Para minimizar essa variação, um processo utilizando o corante vital vermelho neutro foi desenvolvido para a visualização de folículos pré-antrais dentro das peças corticais de ovários ovinos e aplicado com sucesso para a determinação da densidade folicular no tecido cortical humano (CHAMBERS et al., 2010; KRISTENSEN *et al.*, 2011).

Uma vez que o crescimento do folículo é iniciado dentro do tecido cortical, eles podem se desenvolver para os estágios de folículos primários e secundários. No cultivo *in situ*, folículos secundários humanos podem ser detectados após 6 dias de cultivo (TELFER *et al.*, 2008). Entretanto, grandes folículos secundários não sobrevivem bem dentro do ambiente cortical, que parece ter efeitos inibitórios do crescimento a partir desse estágio, resultando em perda da integridade do folículo e sobrevivência (OVATTA *et al.*, 1999; TELFER *et al.*, 2008). Portanto, para apoiar o desenvolvimento, os folículos secundários podem ser isolados do tecido cortical e cultivados individualmente (cultivo de dois passos), minimizando, assim, os efeitos das interações entre os folículos, o estresse oxidativo e a crescente necessidade de nutrientes e fatores de crescimento (Figura 7) (Mc LAUGHLIN e TELFER, 2010).

Figura 7 – Desenho esquemático do cultivo de dois passos.



Fonte: Adaptado de Telfer e Zelinsky (2013).

Diversos suplementos, hormônios e fatores de crescimento já foram testados em sistemas de cultivo *in situ*. A tabela 1 mostra os principais resultados obtidos a partir do cultivo *in situ* desde o ano de 2010.

Tabela 1 – Efeitos de suplementos, hormônios e fatores de crescimento testados no cultivo *in vitro* de folículos pré-antrais inclusos em tecido ovariano de diversas espécies desde o ano de 2010 (em ordem alfabética).

Substância	**Concentração**	**Espécie**	**Período de cultivo**	**Efeitos no folículos**	**Autor/ano**
Ácido Ascórbico	50 ou 100 µg/mL	Equina (e)	6 dias	Promove ativação folicular	GOMES *et al*., 2015
AMH	50 ou 150 ng/mL	Caprina (c)	7 dias	Inibe a ativação	ROCHA *et al*., 2016
Ativina-A	100 ng/mL	Bovina (b)	6 dias	Promove ativação folicular	TEPEKOY E AKKOYUNLU, 2016
bFGF	100 ng/mL	Bovina (b)	14 dias	Promove ativação, crescimento e reduz a apoptose	K TANG *et al*., 2012
BMP-4	50 ng/mL	Ovina (o)	9 dias	Não tem efeito na ativação; reduz a produção de progesterona; aumenta os diâmetros folicular e ocitário de folículos primários	BERTOLDO *et al*., 2014
BMP-6	50 ng/mL	Caprina (c)	7 dias	Não tem efeito na ativação, induz atresia folicular	ARAÚJO *et al*., 2010b
BMP-7	1 ou 10 ng/mL	Caprina (c)	7 dias	Não tem efeito na ativação, reduz a porcentagem de folículos normais, mantém a ultraestrutura folicular	ARAÚJO *et al*., 2010a
BMP-15	50 ou 100 ng/mL	Caprina (c)	7 dias	Não tem efeito na ativação; mantém a viabilidade e a ultraestrutura folicular	CELESTINO *et al*., 2011b

Estradiol	10 pg/mL	Caprina (c)	7 dias	Promove ativação e mantém a ultraestrutura folicular	LIMA-VERDE *et al*., 2010
FGF-10	50 ng/mL	Caprina (c)	7 dias; 16 dias	Promove ativação, mantém a viabilidade e a ultraestrutura folicular	CHAVES *et al*., 2011; ALMEIDA *et al*., 2015
FSH	50 ng/mL (e,b)	Equina (e); Bovina (b)	7 dias (e); 6 dias (b)	Promove ativação e mantém a viabilidade (e, b); Mantém a produção de estradiol e de espécies reativas de oxigênio semelhantes ao controle (e)	AGUIAR *et al*, 2015; TEPEKOY E AKKOYUNLU, 2016;
GDF-9	200 ng/mL (b)	Caprina (c), Bovina (b)	7 dias (c), 14 dias (b)	Não tem efeito na ativação, reduz a percentagem de folículos normais, aumenta os diâmetros folicular e ocitário de folículos primários (c); Promove ativação; aumenta os diâmetros folicular e ocitário de folículos primários (b)	MARTINS *et al*., 2010; K TANG *et al*., 2012
GH	50 ng/mL (b), 10 ng/mL (c)	Bovina (b), Caprina (c)	7 dias	Promove ativação, mantém a viabilidade e promove aumento dos diâmetros folicular e oocitário (b, c);	JIMENEZ *et al*., 2016b; MARTINS *et al*., 2014
IGF-I	50 ng/mL (c), 30 ng/mL (b)	Caprina (c), Bovina (b)	7 dias	Não tem efeito na ativação, mantém a viabilidade, aumenta os diâmetros folicular e ocitário de folículos primários (c); Promove ativação, mantém a viabilidade e a ultraestrutura folicular (b)	MARTINS *et al*., 2010; JIMENEZ *et al*., 2016a
Insulina	10ng/mL	Equina (e)	7 dias	Promove ativação, crescimento e reduz o estresse oxidativo (espécies reativas de oxigênio)	AGUIAR, *et al*, 2016
Interleucina 1-β	10 ou 50 ng/mL	Bovina (b)	6 dias	Promove ativação e mantém a viabilidade	PASSOS *et al*., 2016;
KL	50 ng/mL	Caprina (c)	7 dias	Promove ativação e mantém a ultraestrutura folicular	CELESTINO *et al*., 2010
Melatonina	1000pM	Caprina (c)	7 dias	Não tem efeito na ativação; mantém a ultraestrutura folicular	ROCHA *et al*., 2013
TGF-β	10 ng/mL	Caprina (c)	7 dias	Não tem efeito na ativação; mantém a ultraestrutura folicular	RODRIGUES *et al*., 2014
TNF-α	10 ng/mL	Bovina (b)	6 dias	Reduz a viabilidade; aumenta a apoptose	SILVA *et al*., 2017

Fonte: dados de pesquisa, 2017.

2.4 Cultivo *in vitro* de folículos pré-antrais isolados

Para o cultivo de folículos isolados, métodos mecânicos e/ou enzimáticos são empregados para a dissociação dos folículos (oócito, células da granulosa e da teca) dos demais componentes do estroma ovariano (fibroblastos, fibras colágenas e elásticas, fibronectina, etc.) (VANACKER *et al.*, 2011). A microdissecação, método de isolamento folicular com o auxílio de agulhas, tem se tornado uma das técnicas mais adotadas para o

isolamento de folículos pré-antrais (secundários) em camundongas (CORTVRINDT; SMITZ; VAN STEIRTEGHEM, 1996), ratas (DANIEL; ARMSTRONG; GORE-LANGTON, 1989), gatas (JEWGENOW; STOLTE, 1996), ovelhas (TAMILMANI; RAO; VAGDEVI, 2005), vacas (GUTIERREZ *et al.*, 2000), búfalas (SANTOS *et al.*, 2006), cabras (SARAIVA *et al.*, 2010b) e mulheres (ABIR *et al.*, 1999), por ser uma técnica de baixo custo, comparado ao método ezimático e que permite o adequado isolamento folicular. Esta técnica tem a vantagem de permitir a manutenção da estrutura folicular e da membrana basal, além de preservar os receptores de superfície e a interação entre os compartimentos foliculares (teca-granulosa-oócito) após o isolamento (KURVILA *et al.*, 2010). O método enzimático utiliza enzimas digestivas como a colagenase, tripsina e DNase, que digerem o estroma e permitem o isolamento de grande número de folículos. Apesar disso, a digestão enzimática pode danificar as células da teca e a membrana basal, comprometendo a estrutura e o desenvolvimento folicular (DEMEESTERE *et al.*, 2000).

O cultivo de folículos secundários isolados permite o acompanhamento individual dos folículos ao longo do cultivo, mantém a estrutura folicular e melhora a perfusão do meio para o folículo. Modelos bi e tridimensionais têm sido criados e aperfeiçoados para o cultivo de folículos pré-antrais isolados. Em modelos bidimensionais, o folículo é cultivado diretamente sobre uma placa de cultivo ou sobre uma matriz extracelular, como por exemplo, o colágeno. Esse modelo tem a desvantagem de interferir na integridade folicular, pois o folículo tende a aderir e se achatar no fundo da placa ou matriz (FIGUEIREDO *et al*., 2008). Em modelos de cultivo tridimensionais, onde o folículo geralmente é recoberto por gel de colágeno (BELLI *et al.*, 2012), alginato (PANGAS *et al.*, 2003; XU *et al.*, 2011), fibrina-alginato (SHIKANOV *et al.*, 2009; XU *et al.*, 2013), Polietileno-glicol (AHN *et al.*, 2014), matrigel (XU *et al.*, 2009; HIGUCHI *et al*., 2015), sua estrutura esférica é mantida, o que permite a preservação da integridade estrutural do tecido. As vantagens desse sistema incluem a sua transparência óptica (translúcidos), facilidade de encapsulamento e suas propriedades sincronizáveis, que permitem que ele possa ser modificado para imitar diferentes ambientes in vivo (DESAI *et al.*, 2012). No entanto, os materiais utilizados devem assegurar a manutenção da arquitetura tridimensional do folículo durante seu desenvolvimento *in vitro*, ser permeável ao meio permitindo o acesso dos hormônios e fatores de crescimento às células foliculares e ainda, permitir o crescimento folicular sem prejudicar sua forma (SMITZ *et al.*, 2010). Outras formas de cultivo tridimensional não utilizam gel ou matriz e incluem óleo de imersão (MOUSSET-SIMEON *et al.*, 2005), poços invertidos (WYCHERLEY *et al.*, 2004; NATION

e SELWOOD, 2009) e a técnica de microgotas (BOLAND *et al.*, 1993). Em geral, folículos pré-antrais bovinos isolados são cultivados em sistema 2D usando placas sob óleo mineral.

Em diferentes espécies, avanços têm sido observados no cultivo *in vitro* de folículos pré-antrais. Em humanos, bovinos e caninos, folículos secundários isolados cresceram *in vitro* até o estágio antral (ROY e TREACY, 1993; GUTIERREZ *et al*., 2000; SERAFIM *et al*., 2010; McLAUGHLIN e TELFER 2010; ROSSETTO *et al.*, 2012, 2013). Resultados mais satisfatórios foram obtidos com suínos (WU e TIAN, 2007), bubalinos (GUPTA *et al*., 2008), ovinos (ARUNAKUMARI *et al.*, 2010) e caprinos (SARAIVA *et al*., 2010; MAGALHÃES *et al*., 2011), em que se alcançou a produção de embriões após cultivo *in vitro* de grandes folículos secundários.

Em relações aos hormônios e fatores de crescimentos já testados em cultivo de folículos pré-antrais isolados, destacam-se a Ativina-A (SILVA *et al.*, 2012), EGF (CELESTINO *et al.*, 2012), FGF-2 (SHARMA *et al.*, 2010), GDF-9 (VASCONCELOS *et al.*, 2013), BMP-15 (PASSOS *et al.*, 2013), BMP-4 (ROSSI *et al.*, 2015), Insulina (ROSSETTO *et al.*, 2016), FSH (ROSSETTO *et al.*, 2016), IGF-I (ROSSETTO *et al.*, 2013). Outras substâncias, que não são produzidas pelo ovário, tiveram efeitos positivos no cultivo *in vitro* de folículos secundários isolados, entre elas as lectinas PHA (CUNHA *et al*, 2013), ConA (PORTELA *et al.*, 2014) e Jacalina (RIBEIRO *et al.*, 2014), os antioxidantes Anetol (SÁ *et al.*, 2017), Rutina (LINS *et al.*, 2017) e ácido alpha lipóico (ZOHEIR *et al.*, 2017).

Além disso, para o cultivo de córtex ovariano bovino, o meio de cultivo α-MEM^{+} é o meio mais eficaz para garantir o crescimento, viabilidade e preservação da morfologia e ultraestrutura de folículos pré-antrais (JIMENEZ *et al.*, 2016c). Já para o cultivo de folículos pré-antrais isolados, Rossetto *et al.* (2012) demonstraram que o TCM199 foi o meio mais adequado para preservar viabilidade folicular e ultra-estrutura, resultando em maiores taxas de formação de antro, quando comparado aos meios α-MEM e McCoy.

Apesar dos avanços obtidos no cultivo de folículos pré-antrais bovinos, um meio de cultivo ideal, capaz de promover o crescimento até o estágio pré-ovulatório, ainda não foi estabelecido. Até o momento do início desse trabalho, as BMPs 2 e 4, objetos de estudo dessa tese, ainda não haviam sido testadas em meios de cultivo para folículos bovinos.

2.5 Proteínas morfogenéticas ósseas (BMPs)

As BMPs são fatores de crescimento multifuncionais pertencentes à superfamília do TGF-β e foram inicialmente descobertas em extratos de ossos desmineralizados, que foram

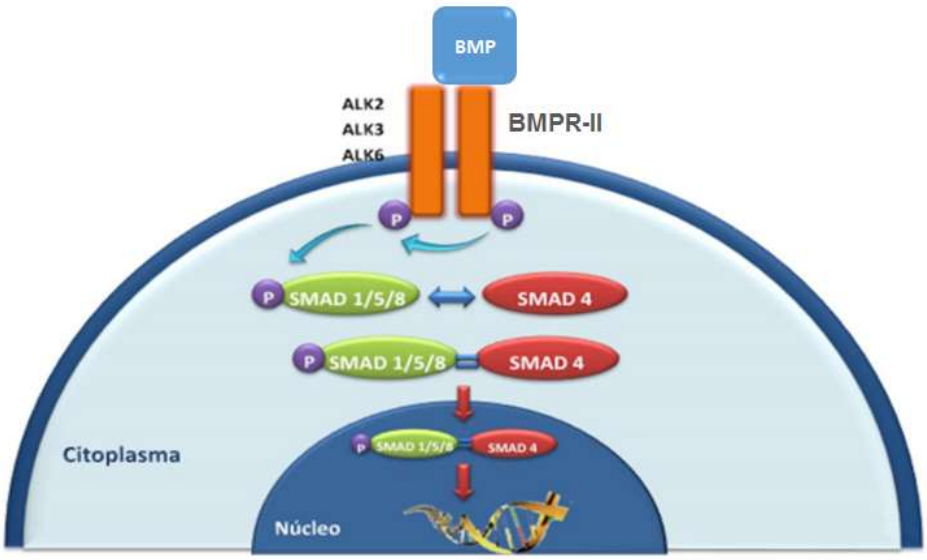
capazes de induzir a formação óssea em locais ectópicos (URIST, 1965). A família BMP é formada pelas seguintes moléculas: BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8A, BMP-8B, Gdf2 (BMP-9), BMP-10, Gdf11 (BMP-11), Gdf7 (BMP-12), Gdf6 (BMP-13), Gdf5 (BMP-14), BMP-15. Apenas a BMP-1 não pertence à superfamília do TGF-β, embora chamada de BMP, não possui a sequência C-terminal que caracteriza a estrutura molecular das BMPs (GRANJEIRO *et al.*, 2005).

As BMPs são glicoproteínas de baixo peso molecular, de estrutura dímera, compostas por duas cadeias polipeptídicas, unidas por pontes dissulfeto, podendo ser homodímeras (duas cadeias idênticas) ou heterodímeras (duas cadeias diferentes) (GRANJEIRO *et al.*, 2005). Ao sofrerem dimerização, suas moléculas são clivadas proteoliticamente na região carboxi-terminal durante sua secreção. Uma vez secretadas, ligantes diméricos maduros com peso de 21-25KDa ligam-se a receptores de membrana plasmática em diferentes tipos celulares, com efeitos autócrinos e parácrinos (KIM e KIM, 2006). A sinalização das BMPs é mediada por receptores transmembrana do tipo treonina/serina quinase, onde três tipos de receptores I podem se ligar às BMPs - receptor activina tipo IA (ActRIA ou Alk2), tipo IA (BMPR1A ou Alk3) e IB (BMPR1B ou Alk6) - e também três tipos de receptores II – receptor tipo II BMP (BMPRII), tipo IIA/ActR-IIA e o tipo IIB/ActR-IIB. Os domínios quinase treonina/serina dos receptores tipo II são constitutivamente ativos e fosforilam os domínios Gly-Ser (GS) nos receptores tipo I após a interação com a BMP ligante, resultando na ativação das quinases dos receptores tipo I (BLANCO CALVO *et al.*, 2009). A especificidade de sinalização da BMP é largamente mediada por receptores tipo I. Estudos *in vitro* têm mostrado que todos os membros das BMPs ligam-se a receptores BMPRII em combinação com BMPR1A, embora exista uma combinação preferencial entre ligantes e receptores (HSU *et al.*, 2005; KIM e KIM, 2006).

A via de atuação das BMPs depende da natureza de receptor ao qual a BMP se liga. Se o complexo de receptores tipo I e II forem heterodímeros, a via da BMP sinaliza a ativação de mensageiros intracelulares conhecidos como Smads (via canônica). Entretanto, se o complexo for homodímero, ocorre a ativação mitógena da via de proteínas quinases (MAPK) (WAN e CAO, 2005). Os receptores BMPR-I/BMPR-II fosforilam as Smads, as quais convertem o sinal para o núcleo modificando a expressão gênica, alterando a atividade celular, incluindo crescimento, diferenciação e síntese de matriz extracelular. As Smads constituem três subfamílias: Smads receptoras (R-Smads: Smad-1, Smad-5 e Smad-8); Smad mediadora comum (Co-Smad: Smad4); e Smads inibidoras (I-Smads: Smad-6 e Smad-7). A via Smad é regulada pelo co-mediador Smad-4 e pelas Smads inibidoras (Smad-6 e Smad-7;

ten DIJKE; HILL, 2004), sendo as BMPs ativadoras as Smad-1, Smad-5 e Smad-8 (MIYAZAWA *et al.*, 2002). As R-Smads são fosforiladas pelo BMPR-I, enquanto que as I-Smads exercem um feedback negativo, uma vez que competem com as R-Smads pelas interações com os receptores levando-os à degradação (MOUSTAKAS *et al.*, 2001). As R-Smads que forem fosforiladas interagem com a Smad-4 e são translocados ao núcleo para ativar a maquinaria transcricional e modular a transcrição dos genes de BMP (WANG *et al.* 2010) (Figura 8).

Figura 8 – Via de sinalização das BMPs/Smads. As BMPs se ligam inicialmente ao receptor tipo II, que sofre fosforilação, e recruta o receptor tipo I, que também é fosforilado. Em seguida, o receptor tipo I promove a fosforilação do complexo SMADs 1, 5 e 8 que se unem à SMAD 4, formando um complexo (co-SMAD 4), que é translocado para o núcleo, ligando–se aos fatores de transcrição (FT) e co-reguladores (CO) inibindo ou ativando a expressão gênica.



Fonte: Adptado de Costa *et al.* (2012).

Outra via de sinalização é a MAPK-p38, que pode ser Smad-independente e é induzida pela oligomerização dos receptores através da BMP-2. A BMP-2 pode inicialmente mediar a homodimerização do receptor I e posteriormente recrutar o receptor II. Cada diferente receptor oligômero pode estar associado a diferentes proteínas citoplasmáticas, que podem regular vias de sinalização específicas (NOHE *et al.*, 2001).

O controle da via de sinalização da BMP ocorre em vários níveis, por fatores intra e extracelulares, incluindo inibição da interação da BMP com seu receptor por proteínas

extracelulares que se ligam à BMP, presença de pseudorreceptores de membrana, bloqueio da sinalização da BMP pela ligação intracelular das Smads e degradação proteossomal de moléculas de sinalização das BMPs (BALEMANS e VAN HUL, 2002; GAZZERRO e CANALIS, 2006). Em nível extracelular, algumas proteínas podem se ligar seletivamente às BMPs, impedindo a interação destas com os seus receptores serina/treonina quinase afins. Esses antagonistas possuem em sua estrutura grupamentos cisteína (assim como os membros do TGF-β) e são classificados em três subfamílias: a família DAN, a Tsg (*Twisted gastrulation*) e a Chordin e Noggin, que ligam-se às BMPs com a mesma afinidade de seus receptores específicos, bloqueando a transdução de sinal e a atividade biológica das BMPs (HARISSON *et al.*, 2004; YANAGITA, 2005). Outro tipo de regulação é feita através do pseudorreceptor BAMBI (*BMP and Activin membrane-bound inhibitor*), que é uma glicoproteína transmembrana com domínio extracelular semelhante aos receptores da BMP. Este pseudorreceptor neutraliza os receptores tipo II, sem contato direto com as BMPs (NOHE *et al.*, 2004).

2.5.1 Ações das Proteínas Morfogenéticas Ósseas 2 e 4 (BMP-2 e BMP-4) no ovário

A expressão de BMP-2 tem sido relatada no ovário em desenvolvimento, bem como seu papel no desenvolvimento de células germinativas primordiais (YING e ZHAO, 2001; SOUZA *et al.*, 2002). Já foi demonstrado que a BMP-2 regula a formação de folículos primordiais através da transição de células germinativas para oócitos e da diferenciação de células somáticas em células da pré-granulosa (CHAKRABORTY e ROY, 2015). Em ratos, a BMP-2 interage com a BMP-4 e tem um efeito aditivo na estimulação da formação de CGPs (YING e ZHAO, 2001). O cultivo *in vitro* de células da granulosa a partir de folículos antrais ovinos tem mostrado que a BMP-2 aumenta a produção de estrógeno e inibina A após a estimulação com FSH, promovendo, assim, a diferenciação de células da granulosa (SOUZA *et al.*, 2002). Além disso, a BMP-2 é capaz de aumentar a produção de estradiol pelas células da granulosa (VINOD KUMAR *et al.*, 2014). No entanto, em suínos, a BMP-2 suprime a síntese de estradiol e androstenediona (BRANKIN *et al.*, 2005).

A presença de BMP-4 foi detectada nas células da teca de folículos antrais bovinos e a expressão de BMPR-II foi encontrada em folículos em primordial, primário e secundário, tanto nas células da granulosa e oócitos (FATEHI *et al.*, 2005). Além disso, RNAm para BMP-4 e seus receptores (BMPR-IA e BMPR-IB) foram observados em folículos pré-antrais de cabra e ovelhas, sugerindo que a BMP-4 medeia o desenvolvimento

nesta fase da foliculogênese (COSTA *et al,* 2012; BERTOLDO *et al.,* 2014). A BMP-4 controla um série de respostas biológicas, incluindo proliferação celular e esteroidogênese (GLISTER e KNIGHT, 2003) e supressão da apoptose em células da granulosa cultivadas *in vitro* (SHIMIZU *et al.,* 2012). A BMP-4 promove proliferação de CGPs em gônadas de ratos (ROSS et al., 2003) e humanos (CHILDS *et al.,* 2010), e atua como um regulador da gametogênese nas várias fases de desenvolvimento. Em camundongos, a BMP-4 promove ativação de folículos primordiais e previne apoptose no oócito (DING *et al.,* 2013). Foi demonstrado ainda que a BMP-4 potencializa a ação do FSH através do aumento da produção de estradiol e inibição da síntese de progesterona em células da granulosa de bovinos (MULSANT *et al.,* 2001). Entretanto, as ações das BMPs 2 e 4 no cultivo de folículos pré-antrais bovinos ainda não foram totalmente estudadas.

3 JUSTIFICATIVA

No contexto da reprodução animal, avanços significativos foram obtidos a partir do cultivo de folículos pré-antrais em várias espécies (camundongo: O'BRIEN *et al.*, 2003; ovino: ARUNAKUMARI *et al.,* 2010; caprino: SARAIVA *et al.,* 2010). No entanto, na espécie bovina, estudos que envolvem a utilização de folículos pré-antrais apresentam resultados limitados à formação da cavidade antral (ROSSETTO *et al.,* 2016, JIMENEZ *et al.*, 2016c; ROSSI *et al.,* 2015). Nesse sentido, se faz necessária a realização de estudos envolvendo fatores de crescimento que modulam o desenvolvimento folicular em bovinos, tais como a BMP-2 e BMP-4 a fim de se compreender os mecanismos moleculares que controlam as etapas específicas de desenvolvimento folicular, deste a ativação até a fase de folículos em desenvolvimento.

As BMPs 2 e 4 são fatores que atuam na regulação da formação de folículos primordiais através da diferenciação de células germinativas para oócitos e de células somáticas em células da pré-granulosa (CHAKRABORTY e ROY, 2015). Resultados de ativação folicular em tecido ovariano tratados com BMP-2 ou BMP-4 foram obtidos em ratos (NILSSON e SKINNER, 2003), camundongos (DING *et al.,* 2013), e ovelhas (BERTOLDO *et al.,* 2015). Além disso, é sabido que a BMP-4, em bovinos, controla um série de respostas biológicas, incluindo proliferação celular, esteroidogênese e supressão da apoptose de células da granulosa (KNIGHT e GLISTER, 2003; SHIMIZU *et al.*, 2012). Entretanto, estudos que envolvem os efeitos desses fatores no cultivo *in vitro* de folículos pré-antrais bovinos ainda não foram descritos. Desta forma, este trabalho justifica-se pela necessidade de compreender como estes fatores interagem durante o desenvolvimento folicular *in vitro*, sobretudo sua relação com a expressão de genes envolvidos nos processos de crescimento e ativação folicular, como o FSHR, bem como fatores envolvidos nas vias de ativação das BMPs (BMPRIA, BMPRIB, BMPRII e SMAD1) que promovem um estímulo ao crescimento desses folículos *in vivo*. Além disso, a comparação da expressão destes genes antes e após o cultivo *in vitro*, fornecerá informações importantes para a compreensão dos mecanismos moleculares que controlam este processo. Portanto, o desenvolvimento de um meio de cultivo eficiente que utilize as BMPs 2 e 4 poderá ser importante tanto para o desenvolviemnto de programas de reprodução assistida quanto para uma melhor compreensão da foliculogênese.

4 HIPÓTESES

I. O efeito das BMP-2 e BMP-4 na ativação, crescimento e sobrevivência de folículos pré-antrais bovinos cultivados *in vitro* inclusos em tecido ovariano é concentração dependente.

II. A BMP-4 modula a expressão de RNAm para *GDF-9, BMP-15, PCNA, Bax* e *Bcl2* em folículos pré-antrais bovinos inclusos em tecido ovariano após 6 dias de cultivo *in vitro*.

III. O efeito da BMP-2 no desenvolvimento e viabilidade de folículos secundários bovinos cultivados *in vitro* por 12 dias é concentração dependente.

IV. A BMP-2 modula a expressão de RNAm para *GDF-9, NPM-2* e *NLRP-5* em folículos secundários bovinos cultivados *in vitro* por 18 dias.

V. A interação entre as BMPs 2 e 4 melhora as taxas de crescimento e sobrevivência e modula a expressão de RNAm para *GDF-9, Ciclina B1, BMPRIA, BMPRIB, BMPRII, FSHR* e *SMAD1* de folículos secundários bovinos cultivados *in vitro* durante 18 dias.

5 OBJETIVOS

5.1 Objetivo geral

- Avaliar os efeitos das BMPs 2 e 4 durante o desenvolvimento *in vitro* de folículos pré-antrais bovinos.

5.2 Objetivos específicos

- Avaliar o efeito de diferentes concentrações das BMPs 2 e 4, utilizando como parâmetros a sobrevivência, a ativação e o crescimento de folículos pré-antrais bovinos inclusos em tecido ovariano cultivados *in vitro* por 6 dias;

- Avaliar a expressão de RNAs mensageiros para *GDF-9, BMP-15, PCNA, Bax* e *Bcl2* em tecido ovariano bovino após 6 dias de cultivo *in vitro* na presença de BMP-4;

- Avaliar o efeito de diferentes concentrações de BMP-2 sobre o desenvolvimento *in vitro* de folículos secundários bovinos durante 12 dias de cultivo;

- Avaliar a expressão de RNAs mensageiros para *GDF-9, NPM-2* e *NLRP-5* de folículos secundários bovinos após 18 dias de cultivo *in vitro* na presença de BMP-2 sozinha ou em associação com o FSH;

- Avaliar o efeito da associação de ambas BMP-2 e BMP-4 sobre o desenvolvimento *in vitro* e expressão de RNAs mensageiros para *GDF-9, Ciclina B1, BMPRIA, BMPRIB, BMPRII, FSHR* e *SMAD1* em folículos secundários bovinos durante 18 dias de cultivo;

- Avaliar o efeito do cultivo *in vitro* sobre a expressão de RNAm para *GDF-9, Ciclina B1, BMPRIA, BMPRIB, BMPRII, FSHR* e *SMAD1* de folículos

secundários bovinos frescos (~200 µm), folículos bovinos crescidos *in vivo* (~350 µm) e folículos bovinos crescidos *in vitro* (~350 µm).

6 INFLUÊNCIA DA BMP-2 NO DESENVOLVIMENTO FOLICULAR INICIAL E A EXPRESSÃO DE RNAM DE GENES ESPECÍFICOS DO OÓCITO EM FOLÍCULOS PRÉ-ANTRAIS BOVINOS CULTIVADOS *IN VITRO*

Influência da BMP-2 no desenvolvimento folicular inicial e a expressão de RNAm de genes específicos do oócito em folículos pré-antrais bovinos cultivados *in vitro*

Influence of BMP-2 on early follicular development and mRNA expression of oocyte specific genes in bovine preantral follicles cultured *in vitro*

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Influência da BMP-2 no desenvolvimento folicular inicial e a expressão de RNAm de genes específicos do oócito em folículos pré-antrais bovinos cultivados *in vitro*

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Resumo

Este estudo avaliou o efeito de diferentes concentrações (0, 10, 50 e 100 ng/mL) da proteína morfogenética óssea-2 (BMP-2) no desenvolvimento de folículo primordial e secundário. Ele também investiga os efeitos do FSH e BMP-2 no crescimento, morfologia, ultraestrutura e expressão de RNAm para os genes GDF9, NLRP5 e NPM2 em folículos secundários cultivados por 18 dias. A presença de BMP-2 em todas as concentrações testadas aumentou o desenvolvimento de folículos primordiais *in vitro*, mas a maior concentração de BMP-2 (100 ng/mL) reduziu a porcentagem de folículos normais quando comparados com os tecidos cultivados com 10 ng/mL de BMP-2. Durante o cultivo de folículos secundários, em contraste com as maiores concentrações (50 ou 100 ng/mL), a concentração de BMP-2 (10 ng/mL) manteve a morfologia dos folículos durante os estágios iniciais do cultivo *in vitro*. Esta concentração de BMP-2 também beneficia a manutenção da ultraestrutura de folículos

cultivados de 18 dias. A presença de BMP-2 e FSH no meio de cultura resultou em um aumento significativo (P <0,05) no diâmetro folicular após 18 dias de cultura. No entanto, tanto FSH quanto BMP-2 reduziram a expressão de mRNA folicular de GDF9 e NLRP5 quando comparados aos folículos cultivados em meio contendo apenas FSH. Em combinação com FSH, BMP-2 reduziu os níveis de mRNA de NPM2, quando comparados aos folículos cultivados em meio de controle. Conclui-se, a partir destes dados, que 10 ng/mL de BMP-2 promove o crescimento de folículos primordiais *in vitro* e ajuda a manter a ultra-estrutura de folículos secundários, enquanto que o FSH é mais importante para uma melhor expressão de marcadores foliculares como GDF9 e NLRP5.

Palavras-chave: Folículos ovarianos, BMP-2, FSH, vaca, cultivo.

Influence of BMP-2 on early follicular development and mRNA expression of oocyte specific genes in bovine preantral follicles cultured *in vitro*

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Abstract

This study evaluates the effect of different concentrations (0, 10, 50 and 100ng/mL) of bone morphogenetic protein-2 (BMP-2) on primordial and secondary follicle development. It also investigates the effects of FSH and BMP-2 on the growth, morphology, ultrastructure and expression of mRNA for *GDF9*, *NLRP5* and *NPM2* genes in secondary follicles cultured for 18 days. The presence of BMP-2 at all tested concentrations increased the development of primordial follicles *in vitro*, but the highest concentration of BMP-2 (100 ng/mL) reduced the percentage of normal follicles when compared with tissues cultured with 10 ng/mL BMP-2. During culture of secondary follicles, in contrast to higher concentrations (50 or 100 ng/mL), 10 ng/mL BMP-2 kept the morphology of follicles during initial stages of *in vitro* culture.

This concentration of BMP-2 also benefits maintenance of the ultrastructure of 18-day cultured follicles. The presence of both BMP-2 and FSH in culture medium resulted in a significant ($P<0.05$) increase in follicular diameter after 18 days of culture. However, both FSH and BMP-2 reduced follicular mRNA expression of *GDF9* and *NLRP5* when compared to follicles cultured in media containing only FSH. In combination with FSH, BMP-2 reduced the mRNA levels of *NPM2*, when compared to follicles cultured in control medium. It is concluded from these data that 10 ng/mL BMP-2 promotes the growth of primordial *in vitro* and it helps to maintain the ultrastructure of secondary follicles, while FSH is more important for better expression of follicular markers like *GDF9* and *NLRP5*.

Key-words: ovarian follicles, BMP-2, FSH, cow, culture

1. Introduction

Knowledge about antral follicular development is very extensive (Richards, 2001; Webb et al., 2003), but information on the role of growth factors in the earliest stages of bovine folliculogenesis remains limited. The elucidation of the main factors controlling different stages of follicular growth is important to optimize female gamete utilization and to enhance reproductive efficiency of highly valuable animals and endangered species. Additionally, this knowledge could provide a potential solution for some fertility problems in humans (Fortune, 2003).

Culturing preantral follicle is an important tool to evaluate the role of hormones and growth factors, like FSH and bone morphogenetic proteins (BMPs), during initial folliculogenesis, which can contribute to understand the mechanisms involved in oocyte development and granulosa cell proliferation and differentiation (Thomas et al., 2003). Studies on expression and biological activities of BMPs showed that BMP-2 plays an important role in the formation of primordial germ cells (Ying and Zhao, 2001) and in ovarian follicular development. Expression of mRNA for BMP-2 was demonstrated in granulosa cells

from primary, secondary and antral follicles (Erickson and Shimasaki, 2003), as well as in theca cells and oocytes from antral follicles (Fatehi et al., 2005). BMP-2 act through a family of transmembrane serine/threonine kinase receptors and based on their structural and functional properties, the receptors are divided into two subfamilies: receptor type I and type II (Wrana et al., 1994). Previous studies have reported the expression of BMP receptors in different follicular compartments of preantral and antral follicles of ruminants (Fatehi et al., 2005).

The presence of BMP-2 during *in vitro* culture of cells from antral follicles has been associated with an increase in the production of estrogen and inhibin-A in sheep granulosa cells after stimulation with FSH (Souza et al., 2002), but inhibited the production of FSH-induced progesterone in rat granulosa cell-oocyte cocultures (Inagaki et al., 2009). Besides, BMP-2 appears to promote down-regulation of luteinizing hormone receptor (LHR) and steroidogenic acute regulatory (StAR) mRNA in human granulosa cells (Shi et al., 2011), and to inhibit granulosa cell apoptosis in caprine granulosa cells (Zhu et al., 2013). Despite the importance of BMP-2 for primordial germ cell formation and for the regulation of steroidogenesis, studies related to its influence on bovine early follicular development have not yet been performed.

It is well-known that GDF9 (Carabatsos et al., 1998) has a key role during follicle development and maturation. During follicular growth *in vitro*, the levels of mRNA for this growth factor are an important marker of oocyte quality (Li et al., 2014). Furthermore, oocyte specific genes, such as *NLRP5* (Tong et al., 2000) and *NPM2* (Burns et al., 2003) are involved in the early embryonic development. Previous studies have demonstrated that mRNA for these factors are expressed in oocytes enclosed in mice preantral follicles (Sánchez et al., 2009). Although these factors are considered important markers for oocyte competence, it is unknown if their expression is influenced by BMP-2, FSH or both in cultured bovine oocytes.

The aim of this study was to evaluate the *in vitro* effect of different concentrations of BMP-2 on primordial and secondary follicle development and to investigate the interaction between BMP-2 and FSH on secondary follicles, i.e. on their morphology, ultrastructure, growth and mRNA expression of *GDF9* and the oocyte specific genes *NLRP5* and *NPM2*.

2. Material and methods

2.1. Experiment 1: Effect of different concentrations of BMP-2 on primordial follicle development

Ovaries (n=10) from adult cycling cows (n=5) were collected at a local abattoir. After collection, the ovaries were washed once in 70% ethanol for about 10 sec, and then twice in 0.9% saline solution supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL). In the laboratory, ovarian cortical tissue from the same ovarian pair was cut in slices (3 mm x 3 mm x 1 mm) using scissors and scalpel under sterile conditions. After fragmentation, some pieces of ovarian cortex were directly fixed for histology and the remaining fragments were cultured *in vitro* for 6 days in 24-well culture dishes 1 mL of culture media. Culture was performed at 38.5°C in 5% CO_2 in a humidified incubator. The basic culture medium consisted of α-MEM (pH 7.2-7.4) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxantine, antibiotics 100 IU/mL penicillin and 100 mg/mL streptomycin, 50 µg/mL ascorbic acid, 3.0 mg/mL of bovine serum albumin (α-MEM^+). The ovarian cortical fragments were cultured in control medium (α-MEM^+) alone or supplemented with 10, 50 or 100 ng/mL of recombinant human bone morphogenetic protein 2 (Sigma-Aldrich, USA). Every 2 days, the culture medium was replaced with fresh medium. After six days of culture, the pieces of ovarian

tissue were fixed overnight at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for histological studies.

After fixation, the ovarian fragments were processed for classical histology and 7 µm sections were mounted on slides and stained with eosin and hematoxylin. Coded anonymized slides were examined under a microscope (Nikon, Tokyo, Japan) at x100 and x400 magnification. The developmental stages of follicles were classified as primordial or growing follicles, as well as histologically normal or degenerated as described previously (Ribeiro et al., 2014). The percentages of morphologically normal follicles relative to the total number of follicles counted in all categories, and those of primordial and developing follicles after 6 days of culture were subjected to Fisher´s exact test ($P<0.05$). The differences were considered significant when $P<0.05$.

2.2. Experiment 2: Effect of different concentrations of BMP-2 on secondary preantral follicle development

Ovaries (20 pairs) from adult cows were collected at a local abattoir as described in experiment 1. In the laboratory, the ovaries from each animal were stripped of surrounding fat tissue and ligaments, whereupon fine slices of the ovarian cortex (1 - 2 mm) were cut from the ovarian surface using a sterile scalpel blade. The slices were subsequently placed into fragmentation medium, consisting of Minimum Essential Medium Eagle Alpha Modification (α-MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. Secondary follicles of approximately 200 µm of diameter were identified under a stereo-microscope (SMZ 645 Nikon, Tokyo, Japan), magnified 100x, and manually microdissected from strips of ovarian cortex using 26 gauge (26 G) needles. Only follicles exhibiting a visible oocyte, surrounded by two or more layers of granulosa cells and an intact basal membrane, and without an antral cavity within the granulosa, were selected for *in vitro* culture.

After selection, follicles were individually cultured in 100 μL of culture medium under mineral oil in petri dishes (60 x 15 mm, Corning, USA). The control culture medium was α-MEM$^+$, as described in experiment 1. The preantral follicles (n = 234) were randomly distributed over the following treatments: α-MEM$^+$ alone (control medium) or supplemented with 10, 50 or 100 ng/mL of recombinant BMP-2 (Sigma-Aldrich, USA). The follicles were incubated at 38.5^{o}C, with 5% CO_2 in air, for 12 days. On days 2, 4, 8 and 10 of culture, 60 μL of medium was replaced with fresh medium, whereas on day 6, the culture medium (100 μL) was totally replaced. Morphological normal follicles had a spherical oocyte, surrounded by granulosa cells that limited by an intact basement membrane and an outer thecal-stromal layer, respectively. Follicles with an opaque and/or extruded oocyte and opaque granulosa cells were considered degenerative (Figure 1). The percentage of morphologically normal follicles was evaluated on days 0, 6 and 12. Follicles were measured using a software Motic Images Plus 2.0 ML software (Motic, Causeway Bay, Hong Kong). Two perpendicular measurements were performed in the normal follicles using an inverted microscope with 200 x magnification.

Data from follicular diameters were submitted to Kolmogorov-Smirnov test to confirm normal distribution. The data did not show homogeneity of variance and were analyzed by Kruskal-Wallis non-parametric test. The percentages of normal follicles were analyzed by frequency dispersion by Fisher´s exact test. The differences were considered significant when $P<0.05$.

2.3. Experiment 3: Effect of BMP-2 and FSH on secondary follicle growth and expression of mRNA for GDF9, NLRP5, NPM2

To investigate a possible interaction between BMP-2 and FSH, the concentration of 10 ng/mL BMP-2 was used to culture preantral follicles. Ovaries (20 pairs) from adult cows

were collected at a local abattoir. Protocols and the control culture medium were the same as used in experiment 1. The preantral follicles (n=286) were randomly distributed and cultured for 18 days in the following treatments: α-MEM^{+} alone (culture control) or supplemented with 10 ng/mL of BMP-2 (Sigma-Aldrich, USA), sequential FSH (FSH from sheep pituitary, Sigma, St. Louis, USA) or both BMP-2 and FSH. The sequential FSH consisted of 50 ng/mL FSH from day 0 to day 6, 100 ng/mL FSH from day 7 to day 12, and 200 ng/mL FSH from day 13 to day 18, according to a previous study of Silva et al. (2014).

To evaluate the effects of BMP-2, FSH and their combination on mRNA expression of *GDF9*, *NLRP5* and *NPM2*, morphologically normal follicles that had been cultured in each treatment had their oocytes denudated and three groups of 6 to 8 oocytes were collected and then stored at -80°C until extraction of total RNA. Total RNA extraction was performed using Trizol purification kit (Invitrogen, Sao Paulo, Brazil) as described previously (Ribeiro et al., 2014). Reverse transcription was performed in a total volume of 20 µL, which was comprised of 10 µL of sample RNA, 4 µL 5X reverse transcriptase buffer (Invitrogen, Sao Paulo, Brazil), 8 units RNAse out, 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 1h at 42^{o}C, for 5 min at 80^{o}C, and then stored at -20^{o}C. Negative controls were prepared under the same conditions, but without the inclusion of the reverse transcriptase.

Quantification of RNA was performed by reverse transcription followed by quantitative real-time PCR 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 by using the PrimerQuestSM program (http://www.idtdna.com) to perform amplification of mRNA for *GDF9*, *NLRP5*, *NPM2* and housekeeping gene GAPDH (Table 1). This

housekeeping gene has shown highest stability in bovine preantral follicles (Rebouças et al., 2014) and, thus, was used to normalize expression of target genes. The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 10 min at 95ºC, followed by 50 cycles of 15 sec at 95ºC, 30 sec at 58ºC, and 30 sec at 72ºC. Primer efficiency was determined by using serial dilutions of the target cDNA, and their efficiency varied from 0.96 to 1.02. The final extension was for 10 min at 72ºC. All reactions were performed in a real time PCR Realplex (Eppendorf, Germany). The delta-delta-CT method was used to transform CT values into normalized relative expression levels (Livak and Schmittgen, 2001).

In order to better examine follicular morphology, transmission electron microscopy (TEM) was performed to analyze the ultrastructure of bovine preantral follicles from day 18 of *in vitro* culture. Isolated follicles (n=6 to 10 per treatment) were fixed in Karnovsky solution (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2) for at least 4h at room temperature (approximately 25°C). After fixation, cultured follicles were embedded in drops of 4% low melting agarose, and kept in sodium cacodylate buffer. Specimens were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1h at room temperature, washed in sodium cacodylate buffer and counterstained with 5% uranyl acetate. The samples were then dehydrated through a gradient of acetone solutions and thereafter embedded in epoxy resin (Epoxy-Embedding Kit, Fluka Chemika-BioChemika). After wards, semi-thin sections (2 µm) were cut, stained with toluidine blue and analyzed by light microscopy at a 400x magnification. Ultra-thin sections (70 nm) were obtained from bovine preantral follicles identified in semi-thin sections. Subsequently, ultra-thin sections were counterstained with

uranyl acetate and lead citrate, and examined under a Morgani-FEI transmission electron microscope.

Data from follicular diameters were submitted to Kolmogorov-Smirnov test to confirm normal distribution. The data did not show homogeneity of variance and were analyzed by Kruskal-Wallis non-parametric test. ANOVA and Dunn's multiple comparisons (GraphPad Instat) were used to compare the levels of messenger RNA for *GDF9*, *NLRP5* and *NPM2* after *in vitro* culture. The percentages of normal follicles were analyzed by frequency dispersion by Fisher´s exact test. The differences were considered significant when $P<0.05$.

3. Results

3.1. Experiment 1: Effect of different concentrations of BMP-2 on primordial follicle development

When compared to fresh non-cultured control tissues, a significant ($P<0.05$) reduction in the percentage of primordial follicles (Figure 2A) and increase of developing follicles (Figure 2B) was observed in tissues that were cultured in medium supplemented with different concentrations of BMP-2 for 6 days. In addition, the presence 10, 50 or 100 ng/mL BMP-2 significantly reduced the percentage of primordial and increased those of developing follicles when compared with tissues cultured in control medium. After culture, all treatments reduced the percentage of normal follicles, when compared to those in uncultured control tissues ($P<0.05$). In addition, the presence of 100 ng/mL BMP-2 significantly decreased the percentage of normal follicles when compared to those cultured in presence of 10 ng/mL BMP-2 (Figure 3).

3.2. Experiment 2: Effect of different concentrations of BMP-2 on secondary follicle development

The initial diameter (day 0) of selected preantral follicles was approximately 200μm (Table 2). Compared to this diameter, a significant diameter increase ($P<0.05$) was observed at culture day 6 of follicles from all experimental groups. Compared to culture day 6, a progressive and significant increase in follicular diameters ($P<0.05$) was observed at culture day 12 in all treatments. However, no significant differences were observed among treatments.

As shown in Table 3, the percentages of normal follicles after 6 days of culture in medium supplemented with 10 ng/mL of BMP-2 was similar to day 0. However, those follicles cultured in medium containing 50 or 100 ng/mL of BMP-2 had significantly reduced percentages of follicular survival ($P<0.05$) after this period of culture. A reduction in the percentages of morphologically normal follicles was found when increasing the culture period from 6 to 12 days in all tested media ($P<0.05$). However, no significant differences were observed among treatments.

3.3. Experiment 3: Effect of BMP-2 and FSH on secondary follicle growth and expression of mRNA for GDF9, NLRP5, NPM2

Compared to day 0, follicles cultured in medium with FSH for 6 days and in medium supplemented with BMP-2, FSH or both for 12 days had significantly increased follicular diameters (Table 4). At 12 and 18 days of culture, the follicles cultured with BMP-2 had smaller diameters compared to those cultured in α-MEM^{+} alone or in presence of FSH or the mixture of BMP-2 and FSH. The only exception was for follicles cultured for 18 days in presence of either FSH or BMP-2, which had similar diameters. The supplementation of culture medium with BMP-2 alone or in a mixture with FSH presented higher percentages of morphologically normal follicles after 6 and 12 days of culture, when compared with those of

follicles from control medium or medium with FSH (Table 5). After 18 days of culture, follicular survival rates were similar in all experimental groups ($P>0.05$).

Culturing of follicles for 18 days in medium containing FSH increased ($P<0.05$) the relative expression of *GDF9* mRNA compared to follicles cultured in presence of both BMP-2 and FSH (Figure 4A). In addition, FSH increased ($P<0.05$) the level of mRNA for *NLRP5* (Figure 4B) when compared to follicles cultured in medium supplemented with BMP-2 or both BMP-2 and FSH. Furthermore, the presence of both FSH and BMP-2 in culture medium reduced ($P<0.05$) the relative expression of *NPM2* mRNA when compared with control medium (Figure 4C).

Ultrastructural analysis showed that follicles cultured for 18 days in control medium (α-MEM^{+}) had oocytes with vacuolated ooplasm, having a great area in which the organelles were no longer recognizable. Despite the presence of a regular zona pellucida around the oocyte, the enveloping granulosa cells had condensed chromatin and badly preserved organelles (Figure 5A). Follicles cultured in medium in which BMP-2 (Figure 5B) or both FSH and BMP-2 (Figure 5D) was added, presented a well-preserved oocyte, with visible organelles, such as a Golgi complex, endoplasmic reticulum and mitochondria. Follicles, cultured in medium supplemented with only FSH (Figure 5C) had ultrastructurally normal granulosa cells, but the oocyte was irregularly shaped and its ooplasm vacuolated.

4. Discussion

The present study shows that 10 ng/mL BMP-2 promotes primordial follicle development *in vitro* and contributes to keep the morphology of secondary follicles, having a positive effect in the maintenance of ultrastructural integrity of follicular components. So far, it was not known the effect of BMP-2 on *in vitro* development of bovine early follicles, which reinforces the importance of this work. Despite BMP-2 having a positive effect on activation

of primordial follicles, most of the follicles started growth spontaneously during culture in the control medium. In bovine species, similar results were described in cortical pieces cultured in serum-free medium (Wandji et al., 1996, Braw-Tal and Yossef, 1997). It was suggested that an inhibitor of medullary origin regulates activation in vivo and that separation of the cortex from medulla causes primordial follicles to activate *in vitro* (Cushman et al., 2002). More recently, Kawamura et al. (2013) demonstrated that ovarian fragmentation increased actin polymerization and disrupted the Hippo signaling pathway, leading to increased expression of CCN growth factors. The name CCN is derived from major family members including cysteine-rich angiogenic protein (CYR61 or CCN1), connective tissue growth factor (CTGF or CCN2), and NOV (nephroblastoma overexpressed or CCN3). Secreted CCN2 and related factors promoted primordial follicle growth *in vitro* (reviewed by Hsueh et al. 2015). In other species different from bovine, BMP-2 was shown to be involved in primordial germ cell formation (murine: Ying and Zhao, 2001), as well as in promoting follicular differentiation (ovine: Souza et al., 2002) and to inhibit luteinization (swine: Brankin et al., 2005; murine: Nakamura et al., 2010) but did not stimulate granulosa cell proliferation (Brankin et al., 2005; Campbell et al., 2006).

On average, bovine follicles cultured in medium with both FSH and BMP-2 had larger diameters than those cultured in control medium or media containing BMP-2. It is known that FSH (Spicer and Alpizar, 1994) and BMP-2 (Selvaraju et al., 2013) stimulate granulosa cell estradiol production *in vitro* and that bovine oocytes express estrogen receptor (Burkhart et al., 2010). Thus, the simplest explanation to understand how these substances stimulate follicle growth is that they induce estradiol production, which, in turn, acts directly on oocytes and granulosa cells to stimulate their growth. Hulshof et al. (1995) reported that FSH and/or estradiol stimulate the growth of bovine preantral follicles cultured *in vitro*. These authors demonstrated that, with FSH, the growth was due to an increase in cell proliferation,

while with estradiol this was caused by an increase in granulosa cell size. In species different from bovine, BMP-2 regulates mRNA expression of FSH receptors and steroidogenic enzimes in preantral follicles, as was previously demonstrated for human granulosa cells (Shi et al., 2011). Previous studies showed that a mixture of BMP-2 and FSH induced differentiation of ovine granulosa cells (Souza et al., 2002), and increased the expression of P450 aromatase and phosphorylation of p38 mitogen-activated protein kinase (MAPK) pathway in rat granulosa cells, but did not stimulate their proliferation after *in vitro* culture (Inagaki et al., 2009).

The current study showed that in absence of BMP-2, FSH had no effect on the growth of bovine follicles and in the maintenance of ultraestructural parameters. Like follicles cultured in control medium, those cultured in presence of only FSH showed extreme ooplasmic vacuolization, which is a characteristic sign of degeneration and may represent endoplasmic reticulum swelling (Tassel and Kennedy, 1980; Silva et al., 2000), altered mitochondrial structure (Fuku et al., 1995), or cellular necrosis (de Bruin et al., 2002). Conflicting effects of this gonadotropin were described in the literature on growth and differentiation of follicles from this species (no effects: Nuttinck et al., 1996; positive effects: Saha et al., 2000; Itoh et al., 2002). However, FSH appeared non-essential for human *in vivo* follicular growth until antrum formation (Wright et al., 1999). In regard to non-bovine follicles, various studies described a stimulating role of FSH in their *in vitro* growth (e.g. sheep: Cecconi et al., 1999; pig: Mao et al., 2002; human: Yuan et al., 1999), as well as on the maintenance of morphologically normal granulosa cells (Matos et al., 2007). Other *in vitro* studies with non-bovine mammalian species demonstrated that the early follicle development occurs in the presence or absence of FSH (e.g. mice: Cortvrindt et al., 1998; pig: Mao et al., 2004; goat: Saraiva et al., 2010a).

Real-time PCR showed that FSH increased the expression of GDF9 mRNA in cultured bovine preantral follicles. This finding may indicate that FSH can act via GDF9. Alone or in combination with FSH, GDF9 was recently found to promote follicular growth in cultured bovine (Vasconcelos et al., 2013) and caprine (Alves et al., 2013) preantral follicles. Previously, results from GDF9 gene ablation studies in mice showed that GDF9 plays an autocrine role in the regulation of oocyte development and maturation, as well as a paracrine role in the regulation of granulosa cell differentiation and proliferation (Nath et al., 2013). However, Thomas et al. (2005) reported that FSH did not influence the expression of GDF9 mRNA in oocyte–granulosa cell complexes from mouse preantral follicles. These findings show that the effects of FSH on the expression of GDF9 mRNA are species dependent.

The present findings further showed that FSH stimulated mRNA expression of the oocyte specific gene *NLRP5*, and both FSH and BMP-2 reduced mRNA expression of another oocyte specific gene *NPM2*. *NLRP5* transcript was previously detected in bovine oocytes from secondary and antral follicles (Pennetier et al., 2004), while a reduction of mRNA expression for *NLRP5* and *NPM2* was demonstrated during the final stages of mouse oocyte growth (Sánchez et al., 2009). Recently, Nath et al. (2013) showed that the expression levels of *NLRP5* during *in vitro* maturation of buffalo oocyte were dependent on gonadotropins. Indeed, mice early secondary follicles cultured for 9 days had lower levels of *NLRP5* than those follicles that were exposed to FSH and cultured for only 3 or 6 days, and that these follicles had similar levels of transcript compared to *in vivo* grown follicles (Sánchez et al., 2012).

In conclusion, addition of 10 ng/mL of BMP-2 to culture medium promotes the growth of primordial follicles and contributes to keep the ultrastructure of secondary follicles cultured *in vitro*. These follicles grow even better when confronted with a mixture of FSH and

BMP-2. BMP-2 and FSH alone or in combination modulate the expression of *GDF9*, *NLRP5* and *NPM2*.

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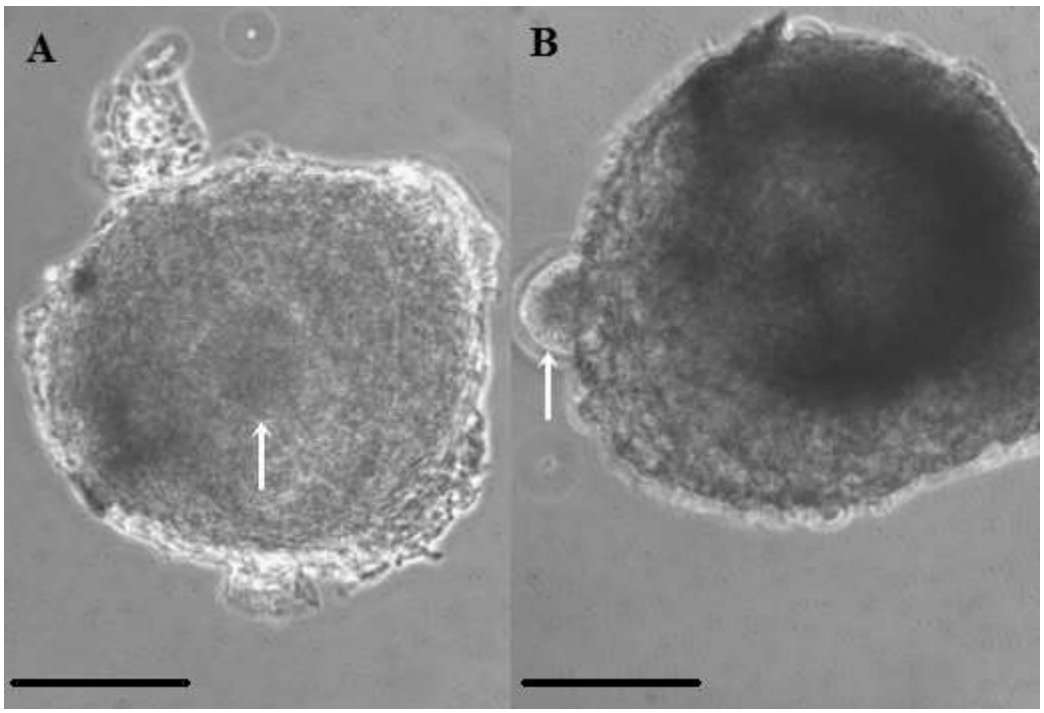

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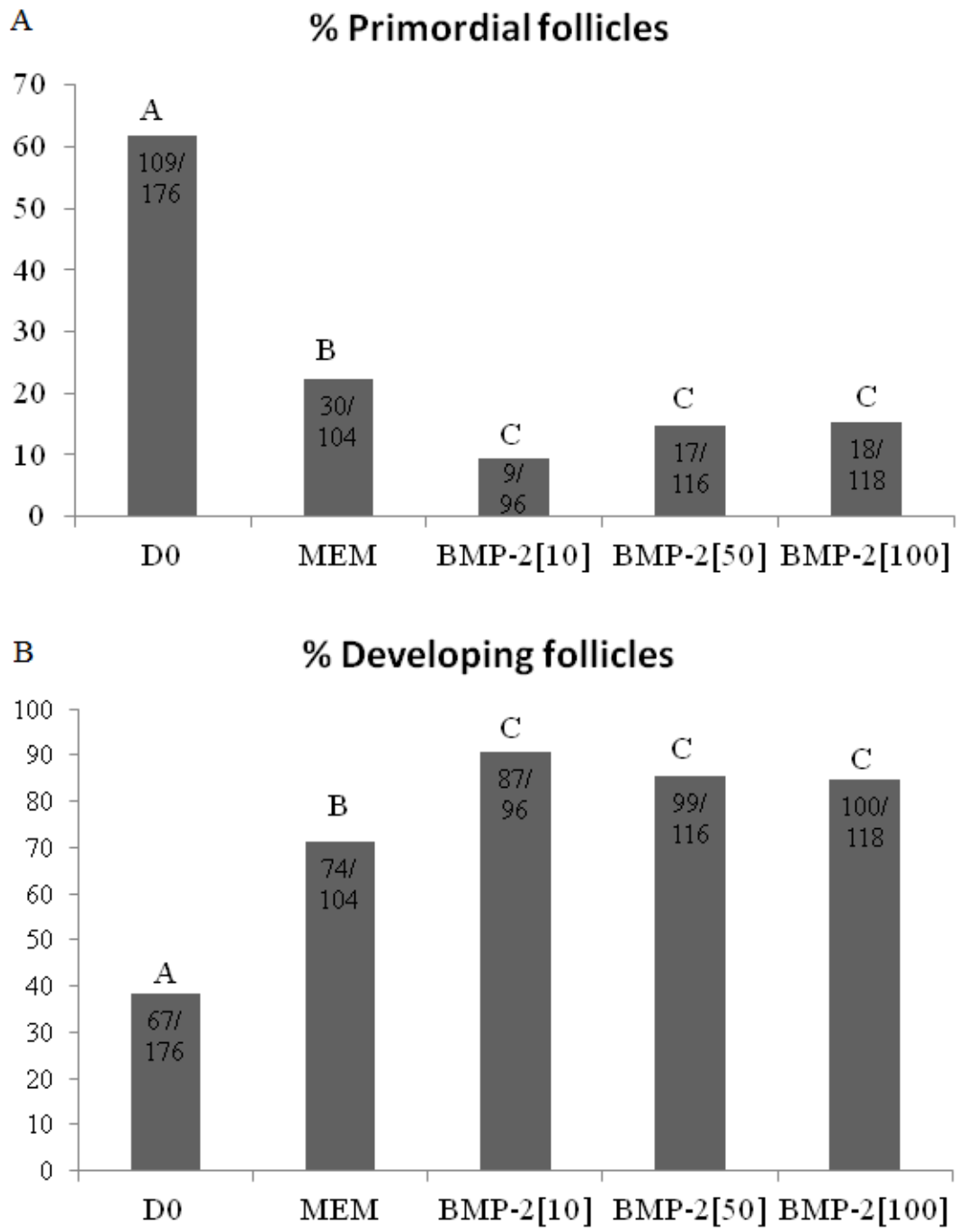
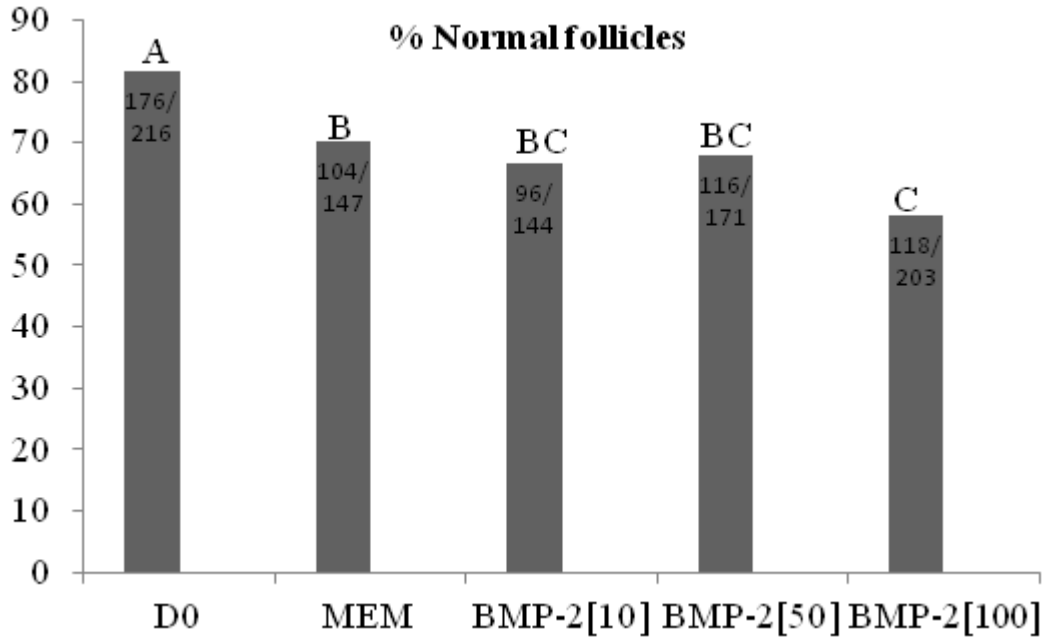


Figure 3. Percentage of normal follicles in non-cultured tissues (control) and in tissues cultured for 6 days in α-MEM^{+} alone or with different concentrations of BMP-2. A,B,C - significant difference between treatments ($P<0.05$).



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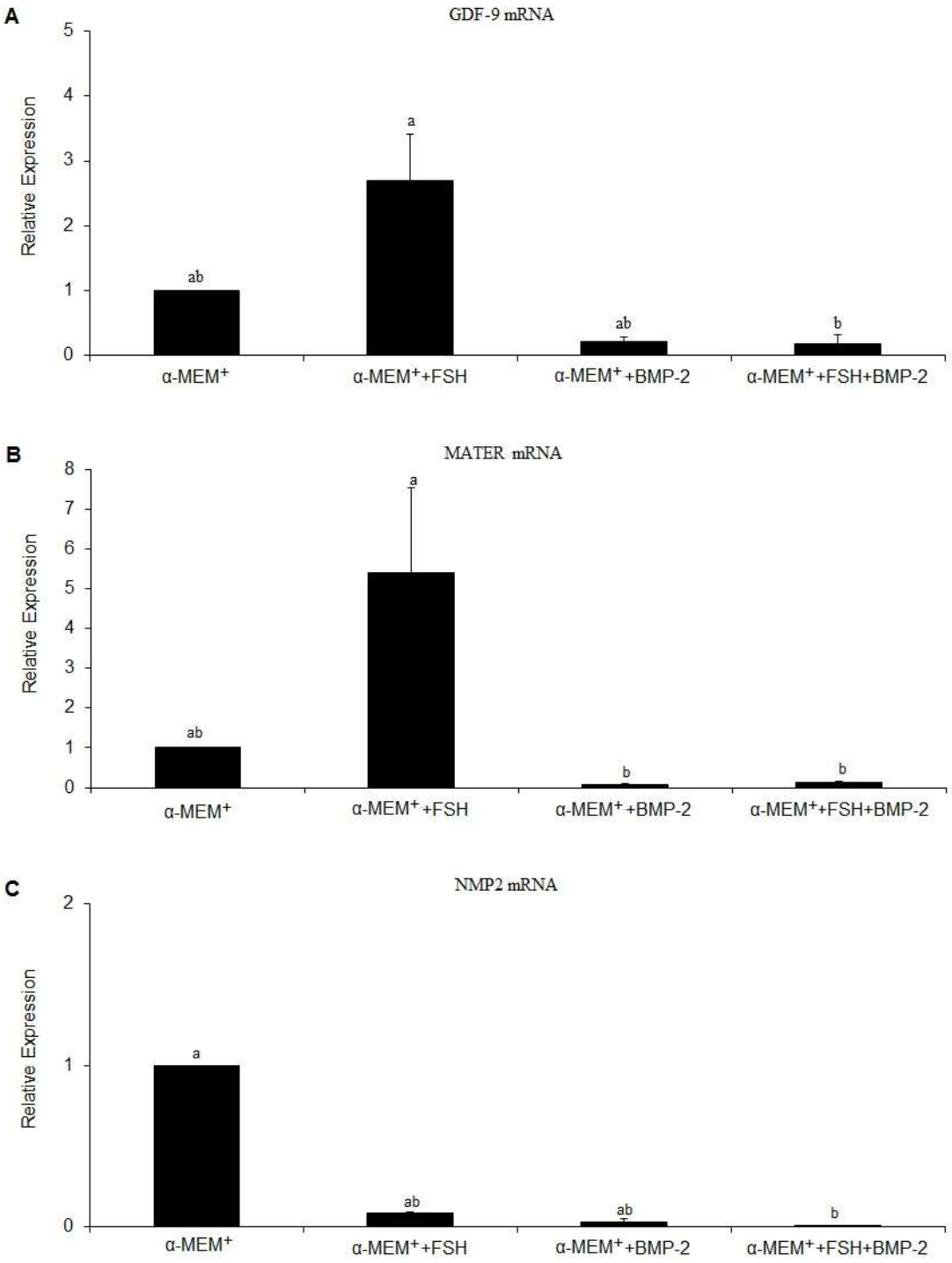

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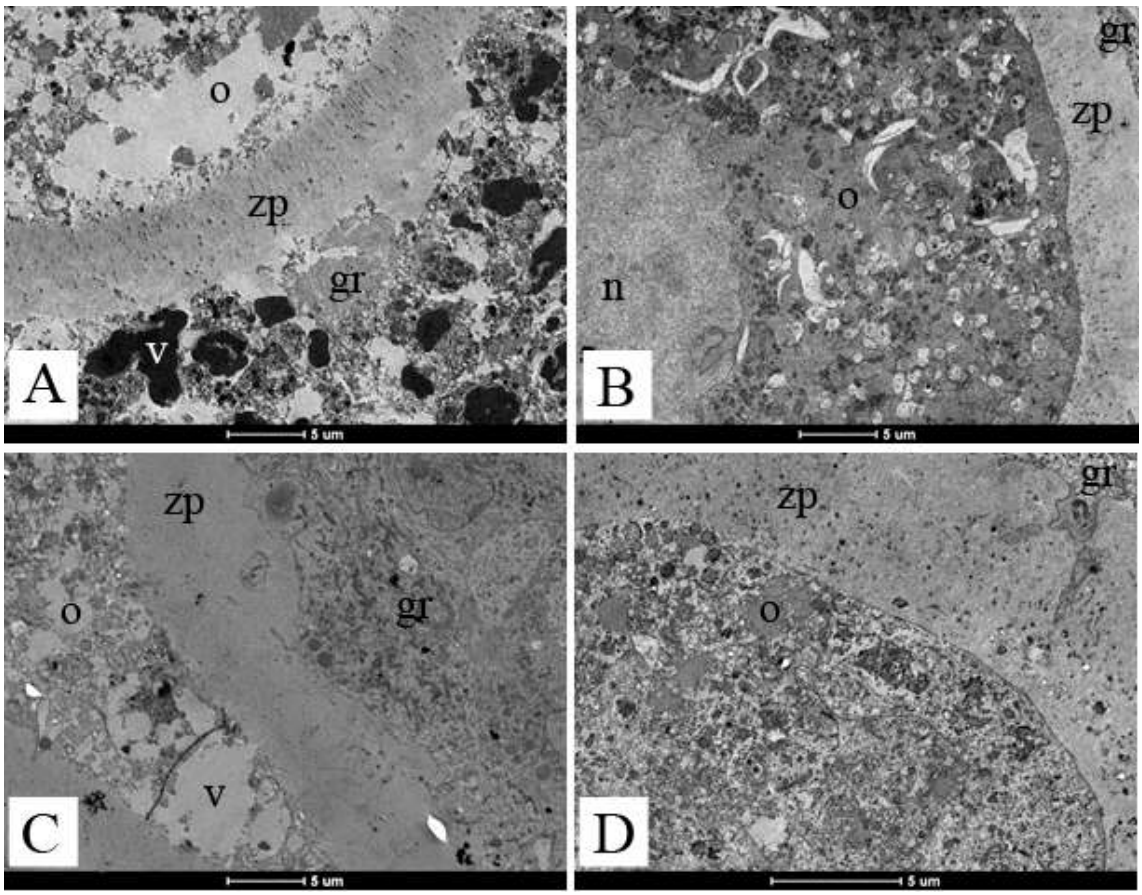

Table 1. Primer pairs used to real-time PCR

Target gene	Primer sequence (5´→ 3´)	Sense (s), ınti-sense (as)	Position	GenBank accession no.
GAPDH	TGTTTGTGATGGGCGTGAACCA	s	288- 309	Gi:27525390
	ATGGCGTGGACAGTGGTCATAA	as	419–440	
GDF9	ACAACACTGTTCGGCTCTTCACCC	s	332 – 356	Gi:51702523
	CCACAACAGTAACACGATCCAGGTT	as	426-451	
NPM2	TCTGGACCTGTGTTCCTCTGT	s	374 - 395	Gi:280967451
	ATCGTCGTCGTCATCATCTTC	as	461 - 481	
NLRP5	AATGACGACGCTGTGTTCTG	s	3107-3127	NM_001007814
	GCGGTTCTCAGGTTCTTCAG	as	3294-3313	

Table 2. Follicular diameters of bovine preantral follicles cultured for 0, 6 or 12 days in α-MEM$^+$ alone or in α-MEM$^+$ supplemented with 10, 50 or 100 ng/mL of BMP-2 (means ± SD).

Day of culture	α-MEM$^+$ (n=60)	α-MEM$^+$ + 10 ng/mL BMP-2 (n=59)	α-MEM$^+$ + 50 ng/mL BMP-2 (n=57)	α-MEM$^+$ + 100 ng/mL BMP-2 (n=58)
D0	203.5 ± 3.9 [a]	204.2 ± 3.2 [a]	206.0 ± 3.7 [a]	199.5 ± 4.8 [a]
D6	225.3 ± 6.2 [b]	230.9 ± 5.9 [b]	234.7 ± 7.3 [b]	220.7 ± 6.2 [b]
D12	254.9 ± 10.7 [c]	255.2 ± 8.5 [c]	266.4 ± 10.7 [c]	264.1 ± 9.5 [c]

a, b, c Values within a column without a common superscript significantly differ (P<0.05).

n = number of follicles at the beginning of culture period.

Table 3. Percentages of follicular survival after culture of bovine preantral follicles in α-MEM$^+$ alone or in MEM$^+$, supplemented with 0, 10, 50 or 100 ng/mL BMP-2.

Day of culture	α-MEM$^+$	α-MEM$^+$ + 10 ng/mL BMP-2	α-MEM$^+$ + 50 ng/mL BMP-2	α-MEM$^+$ + 100 ng/mL BMP-2
D0	100% (60/60) [a]	100% (59/59) [a]	100% (57/57) [a]	100% (58/58) [a]
D6	93.3% (56/60) [a]	94.9% (56/59) [a]	87.7% (50/57) [b]	89.6% (52/58) [b]
D12	53.6% (30/56) [b]	60.7% (34/56) [b]	54.0% (27/50) [c]	48.0% (25/52) [c]

a, b, c Values within a column without a common superscript significantly differ (P<0.05).

Table 4. Follicular diameters after 18-days culture of bovine preantral follicles in α- α-MEM$^+$ alone or in MEM$^+$ supplemented with 10 ng/mL of BMP-2, FSH or a mixture of FSH and 10 ng/mL of BMP-2 (means ± SD).

Days of culture	α-MEM$^+$ (n=75)	α-MEM$^+$ + FSH (n=72)	α-MEM$^+$ + BMP-2 (n=69)	α-MEM$^+$ + BMP-2 + FSH (n=70)
D0	199.1 ± 23.2^{Aa}	201.2 ± 13.6^{Aa}	198.0 ± 23.7^{Aa}	199.7 ± 19.7^{Aa}
D6	210.4 ± 23.7^{ABab}	222.3 ± 19.6^{Bb}	202.5 ± 25.5^{Aa}	212.9 ± 23.0^{Bab}
D12	231.5 ± 59.8^{Bab}	227.0 ± 61.4^{Bb}	208.4 ± 27.7^{Aa}	225.0 ± 90.6^{Bb}
D18	236.0 ± 61.7^{Bab}	227.3 ± 61.5^{ABab}	212.2 ± 28.3^{Aa}	233.8 ± 94.6^{Bb}

n = number of follicles at the beginning of culture period.

a, b, c Values within a column; values without a common superscript significantly differ (P<0.05). A, B, C Values within a row; values without a common superscript significantly differ (P<0.05).

Table 5. Percentages of follicular survival after culture of bovine preantral follicles in α-MEM^{+} or in α-MEM^{+} supplemented with 10 ng/mL of BMP-2, FSH or a mixture of FSH and 10 ng/mL of BMP-2.

Day of culture	α-MEM^{+}	FSH	BMP-2	BMP-2 + FSH
D0	100%^{Aa} (75/75)	100%^{Aa} (72/72)	100%^{Aa} (69/69)	100%^{Aa} (70/70)
D6	60.0%^{Ab} (45/75)	59.7%^{Ab} (43/72)	85.5%^{Bb} (59/69)	88.6%^{Bb} (62/70)
D12	46.7%^{Ab} (35/75)	58.3%^{ABb}(42/72)	73.9%^{Bb} (51/69)	65.7%^{Bb} (46/70)
D18	40.0%^{Ab} (30/75)	40.0%^{Ab} (40/72)	44.9%^{Ab} (31/69)	50.0%^{Ab} (35/70)

a, b, c Values within a column; values without a common superscript significantly differ ($P<0.05$).

A, B, C Values within a row; values without a common superscript significantly differ ($P<0.05$).

7 EFEITOS DA PROTEÍNA MORFOGENÉTICA ÓSSEA 4 (BMP-4) NO DESENVOLVIMENTO E SOBREVIVÊNCIA *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS BOVINOS INCLUSOS EM FRAGMENTOS DE TECIDO OVARIANO

Efeitos da proteína morfogenética óssea 4 (BMP-4) no desenvolvimento e sobrevivência *in vitro* de folículos pré-antrais bovinos inclusos em fragmentos de tecido ovariano

Effects of bone morphogenetic protein 4 (BMP4) on *in vitro* development and survival of bovine preantral follicles enclosed in fragments of ovarian tissue

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(Qualis B2 em Biotecnologia)

Efeitos da proteína morfogenética óssea 4 (BMP-4) no desenvolvimento e sobrevivência *in vitro* de folículos pré-antrais bovinos inclusos em fragmentos de tecido ovariano

Ellen de Vasconcelos da Cunha[1], Glaucinete Borges de Souza[1], José Renato de Sousa Passos[1], Anderson Weiny Barbalho Silva[1], Andressa Minussi Dau[2], Márcia Viviane Alves Saraiva[1], Raimundo Nonato Braga Lobo[3], José Roberto Viana Silva[1,4]

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Resumo

O objetivo deste estudo foi avaliar os efeitos de diferentes concentrações de BMP-4 sobre a ativação, desenvolvimento e expressão de mRNA de GDF-9, BMP15, PCNA, Bax e Bcl2 em folículos bovinos cultivados em tecidos ovarianos. Os fragmentos de tecido ovariano foram cultivados durante 6 dias em α-MEM$^+$ sozinho ou suplementado com diferentes concentrações de BMP-4 (10, 50 e 100 ng/mL). A histologia clássica foi realizada para analisar o crescimento e a morfologia foliculares, enquanto PCR em tempo real foi utilizada para analisar os níveis de mRNA em tecidos frescos e cultivados. Após 6 dias, o cultivo de tecido ovariano em α-MEM$^+$ sozinho ou suplementado com BMP-4 nas concentrações de 10, 50 e 100 ng/mL promoveu a ativação folicular. As diferentes concentrações de BMP-4 mantiveram a percentagem de folículos normais semelhante ao controle. A presença de 100 ng/mL de BMP-4 no meio de cultivo aumentou os diâmetros foliculares e do oócito de folículos primários e secundários quando comparados aos folículos do controle não cultivado

ou cultivados em α-MEM^{+} sozinho (P <0,05). Apesar dos tecidos cultivados na presença de concentrações crescentes de BMP-4 terem um aumento na expressão de mRNA dos genes testados, as diferenças não foram estatisticamente significativas. Em conclusão, 100 ng/mL de BMP-4 promove um aumento nos diâmetros foliculares e de oócitos de folículos primários e secundários após 6 dias de cultivo *in vitro*.

Palavras-chave: Vaca. Ativação. Folículo primordial. Ovário. Cultivo.

Effects of bone morphogenetic protein 4 (BMP4) on *in vitro* development and survival of bovine preantral follicles enclosed in fragments ovarian tissue

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Summary

The aim of this study was to evaluate the effects of different concentrations of BMP4 on activation, development and mRNA expression of *GDF9, BMP15, PCNA*, *Bax* and *Bcl2* in cultured bovine follicles enclosed in ovarian tissues. Ovarian tissue fragments were cultured for 6 days in α-MEM$^+$ alone or supplemented with different concentrations of BMP4 (10, 50 and 100ng/mL). Classical histology was performed to analyze follicle growth and morphology, while real time PCR was used to analyze mRNA levels in fresh and cultured tissues. After 6 days, culture of ovarian tissue in α-MEM$^+$ alone or supplemented with 10, 50 and 100ng/mL BMP4 promoted follicular activation. The different concentrations of BMP4 maintained the percentage of normal follicles similar to control. The presence of 100 ng/mL

BMP-4 in culture medium increased oocyte and follicular diameters of primary and secondary follicles when compared to those follicles from uncultured control or cultured in α-MEM$^+$ alone ($P < 0.05$). Despite tissues cultured in presence of increasing concentrations of BMP4 have an increase in mRNA expression of the tested genes, the differences were not statistically significant. In conclusion, 100ng/mLBMP4 promotes an increase in diameters of follicles and oocytes of primary and secondary follicles after 6 days of *in vitro* culture.

Key words: Cow. Activation. Primordial follicle. Ovary. Culture.

1. Introduction

The control of primordial follicle activation involves two-way communication between the oocyte and its surrounding somatic cells (Cortvrindt and Smitz, 2001), but the factors and mechanisms responsible for the activation and growth of these follicles have not been fully elucidated (Kerr et al., 2013). The development of an *in vitro* culture system able to promote the growth of primordial follicles is extremely important to optimize female reproductive potential, as well as to a better understanding of early folliculogenesis.

Several substances and growth factors have been tested in *in vitro* studies, including bone morphogenetic protein 4 (BMP4). This protein binds to heterogeneous complexes of transmembrane serine/threonine (Ser/Thr) kinase receptors, known as the BMP type IA and IB receptors (BMPR-IA and BMPR-IB) (Chen et al., 2004). BMP4 is derived from the thecal tissue and has been observed to increase the proliferation of ruminant granulosa cells *in vitro* (Glister et al., 2004; JuengeL et al., 2006) and to regulate the action of FSH on progesterone and oestradiol production (Shimasaki et al., 1999; Pierre et al., 2004). In bovine species, the presence of BMP4 was demonstrated in theca cells of antral follicles and the expression of BMPRII was found in in primordial, primary and secondary follicles both in granulosa cells

and oocytes (Fatehi et al., 2005). In addition, BMP4 mRNA and its receptors (BMPR-IA and BMPR-IB) have been observed in goat and sheep preantral follicles, suggesting that BMP4 mediates development during this stage of folliculogenesis (Costa et al., 2012; Bertoldo et al., 2014). In mono-ovulatory species, *in vitro* culture of granulosa cells demonstrated that BMP-4 and other members of BMP family have a major role in modulating proliferative and differentiative responses (Campbell et al., 2006). Rossi et al. (2015) reported that BMP-4 contributes to preserve the ultrastructure of bovine secondary follicles cultured *in vitro* and that, in combination with FSH, BMP4 increases the expression of mRNA for BMP15. Despite the role BMP4 on primordial germ cell formation (humans: Park et al., 2013; buffalo: Shah el at., 2015; goat: Singhal et al., 2015) and secondary follicles (Rossi et al., 2015), has already been reported, it is still not known if BMP4 regulates primordial follicle activation and development in bovine species.

During follicle development, expression of oocyte-secreted factors, like growth and differentiation factor 9 (GDF9, Carabatsos *et al.*, 1998) and BMP-15 (Otsuka *et al.*, 2000), is an important event that contributes to the slow maturation process observed in domestic species (Van den Hurk and Zhao, 2005). GDF9 play a role during early follicle development and maturation (Carabastos et al., 1998) and treatment with GDF9 enhances primary and preantral follicular growth *in vitro* and *in vivo* (Hayashi et al., 1999; Vitt et al., 2000). Furthermore, BMP15 contributes to the growth during the different phases of folliculogenesis, including the process of follicular activation (Juengel et al., 2004). In sheep, immunization against BMP-15 resulted in a blockage of follicular growth (Juengel et al., 2002). Cuboidal granulosa cells from growing follicles express proliferating nuclear antigen (PCNA), which is a nuclear protein essential for follicular growth and thus is considered marker of proliferating granulosa cells (Wandji et al., 1996). *Bax* and *Bcl2* are pro-apoptotic and anti-apoptotic genes, respectively, which are involved in growth regulation and follicular apoptosis (Choi et al.,

2004). However, it is still not known if BMP4 influences the expression of all these factors in ovarian cortical tissue cultured *in vitro*.

The aim of this study was to evaluate the effects of different concentrations of BMP4 on activation and survival of bovine follicles after culture of ovarian cortical tissue. Moreover, the influence of different concentrations of BMP4 on mRNA expression of *GDF9, BMP15, PCNA, Bax* and *Bcl2* in follicles cultured *in vitro* was evaluated.

2. Materials and methods

2.1. Chemicals

Unless mentioned otherwise, the culture media, BMP4 and other chemicals used in the present study were purchase from Sigma Chemical (St Louis, MO, USA).

2.2. Source of ovaries

Bovine ovaries (n =20) were collected from females obtained from a local slaughterhouse. Immediately postmortem, the surrounding fat tissue and ligaments were removed and the ovaries were washed in 70% alcohol followed by two washes in sterile saline solution. The ovaries were placed into tubes containing 20 mL alpha minimum essential medium (α-MEM), supplemented with 100 IU/mL penicillin and 150 mg/mL streptomycin and then transported to the laboratory at 4°C within 1 h.

2.3. Experimental protocol

Briefly, ovarian tissue samples from each ovarian pair were cut into slices (3×3×1 mm) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultrastructural analysis (fresh control) or placed in culture for 6 days. Bovine tissues were transferred to 24-well culture dishes containing 1 mL culture

medium. Culture was performed at 39◦C in 5% CO^2 in a humidified incubator and all media were incubated for 1 h before use. The basic culture medium (cultured control) was called α-MEM^+ and consisted of α-MEM (pH 7.2–7.4) supplemented with ITS (insulin 6.25 ng/mL, transferrin 6.25 ng/mL and selenium 6.25 ng/mL), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumin (BSA), 100 IU/mL penicillin and 150 mg/mL streptomycin. Different concentrations of BMP4 (0, 10, 50 or 100 ng/ml) were added to the MEM^+ to test the effects of this growth factor. Each treatment was repeated four times and the culture medium was replenished every other day.

2.4. Morphological analysis and assessment of in vitro *follicular growth*

Before culture (fresh control) and after 6 days of culture, the pieces of ovarian tissue were fixed overnight at room temperature in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) for histological studies. After fixation, the ovarian fragments were dehydrated in a graded series of ethanol, clarified with xylene, and embedded in paraffin wax. For each piece of ovarian cortex, 7µm sections were mounted on slides and stained with hematoxylin and eosin. Coded anonymized slides were examined under a microscope (Nikon, Tokyo, Japan) at ×100 and ×400 magnification. The developmental stages of follicles were classified as primordial (one layer of flattened or flattened and cuboidal granulosa cells around the oocyte) or growing follicles (primary: one layer of cuboidal granulosa cells, and secondary: two or more layers of cuboidal granulosa cells around the oocyte). These follicles were classified further individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells that are well organized in one or more layers, and have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, that has a pyknotic nucleus and/or is surrounded by disorganized granulosa cells, which are detached from the basement membrane. Overall, from 140–212 follicles were evaluated for

each treatment. The percentages of healthy primordial and developing follicles were calculated before (fresh control) and after culture in a particular medium. Follicle and oocyte diameters were only measured in healthy follicles. Follicle diameter was recorded from one edge of the granulosa cell membrane to the other edge, or from the outside edge of the theca cell layer when present. Oocyte diameter was recorded from edge to edge of the oocyte membrane. Two perpendicular diameters were recorded for each and the average was reported as the follicle and oocyte diameters, respectively.

2.5. Expression of mRNA for GDF9, BMP15, PCNA, Bax and Bcl2 in bovine ovarian cortical tissue

For mRNA isolation, bovine ovarian cortex from fresh control, as well as after culture in the different treatments were collected and stored at -80°C until RNA extraction. Total RNA extraction was performed using a Trizol ® purification kit (Invitrogen, São Paulo, Brazil).In accordance with the manufacturer's instructions,800 µL 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 min 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 min at room temperature. After washing the column three times, the RNA was eluted with 30 µL RNase-free water. The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (AmershamBiosciences, Cambridge, UK) and 2 µL of total RNA was used for reverse transcription. Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70°C and then cooled in ice. Reverse transcription was performed in a total volume of 20 µL, which was comprised of 10 µL of sample RNA,4 µL 5× reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8

units RNase out, 150 units SuperscriptIII reverse transcriptase, 0.036 U random primers (Invitrogen, São Paulo, Brazil), 10 mM DTT, and0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C, for 5 min at 80°C, and then stored at -20°C. Negative controls were prepared under the same conditions, but without the inclusion of the reverse transcriptase. Quantification of mRNA 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,USA), 5.5 µL of ultra-pure water, and 0.5 µM of each primer. The primers were designed by using the PrimerQuestSM program (http://www.idtdna.com) to perform amplification of *GDF9, BMP15, Bcl2, Bax* and the housekeeping gene *Ubiquitin (UBQ)* (Table 1).This housekeeping gene has shown highest stability in bovine preantral follicles (Rebouças et al., 2012) and, thus, was used to normalize expression of target genes. The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The efficiency amplification for all genes was verified according to Pfaffl et al. (2001). The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 10min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real-time PCR Realplex (Eppendorf, Germany). The ΔCt method was used to transform the Ct values into normalized relative expression levels (Livak and Schmittgen, 2001).

2.6. Statistical analysis

Data of follicular development were subjected to potential transformation ($x^{3,9}$), while data of degeneration received logarithmic transformation (log10 (x)) and then, evaluated by ANOVA. The mean values of degeneration were contrasted with fresh control means, before culture, by Dunnett's test ($P < 0.05$). The mean values of follicular activation were compared by Student-Newman-Keuls (SNK, $P < 0.05$).The diameters of oocytes and follicles under the

various treatments were subjected to ANOVA followed by SNK. Levels of mRNA for *GDF9, BMP15, Bcl2* and *Bax* in cultured fragments were analyzed by using the non-parametric Kruskal-Wallis test ($P < 0.05$). Data were expressed as mean ± standard error of the mean (S.E.M.). Differences were considered to be significant when $P<0.05$ and data were expressed as mean ± standard error of means (S.E.M.).

3. Results

3.1. Effect of BMP4 concentration on follicular survival

Histological analysis showed that degenerated and normal follicles were found in non-cultured and cultured ovarian cortical pieces. Degenerated follicles show a pyknotic nucleus, shrunken oocyte or unorganized granulosa cells. A total of 1513 follicles were counted to evaluate follicular morphology, activation and growth. After 6 days of culture, there is an increase of degenerated follicles cultured in all treatments compared with fresh control. However, the percentage of viable follicles was not influenced by treatments (Fig. 1).

3.2. Effect of BMP4 concentration on follicular activation and development

After 6 days of culture, a decrease in primordial follicles and increase in primary and secondary follicles was observed in cultured tissues when compared to fresh control (Table 2, $P<0.05$). However, no significant differences were found among tissues cultured in the different treatments. Follicle and oocyte diameters at different follicular categories before and after *in vitro* culture are shows in Table 3. At day 6 of culture, follicular and oocyte diameters of primordial follicles cultured in α-MEM^{+} alone or supplemented with BMP4 (10, 50 or 100ng/mL) had no differences in size when compared to fresh control. In addition, no differences among treatments were seen ($P <0.05$). On the other hand, primary follicles showed a significant increase in their diameter after culture in presence of 100ng/mL BMP4,

when compared to follicles from uncultured control, or cultured in α-MEM$^+$ alone or supplemented with 10ng/mL BMP4. In addition, an increase in the diameters of secondary follicles was observed in ovarian tissues cultured with 100ng/mL when compared to follicles from uncultured control or cultured in α-MEM$^+$ ($P<0.05$). Furthermore, oocytes from these follicles increased their diameters after culture in the presence of 100ng/mLBMP4, when compared to follicles cultured in α-MEM$^+$ alone or added with 10ng/mLBMP4.

3.3. Expression of mRNA for PCNA, GDF9, BMP15, Bax and Bcl2 in bovine preantral follicles

The levels of mRNA for *PCNA*, *GDF9*, *BMP15*, *Bax* and *Bcl2* in tissues cultured for 6 days in α-MEM$^+$ alone or supplemented with different concentrations of BMP4 are showed in Fig. 1 (A-F). Tissues cultured in the presence of BMP-4 at concentration of 100ng/mL had an increase in the levels of *PCNA, GDF9, BMP15*, *Bax* and *Bcl2* mRNA, compared to those cultured in medium supplemented with α-MEM$^+$ alone. However, the differences were not statistically significant ($P>0.05$).

4. Discussion

The present study demonstrates that BMP4 does not influence the transition from primordial to developing follicles in bovine species, but it stimulates the growth of primary and secondary follicles *in vitro*. Various studies have shown that the transition from resting primordial follicles to growth stages occurs spontaneous when cortical ovarian tissue is cultured *in vitro* (Cushman et al., 2002). It has been suggested that ovarian fragmentation increases actin polymerization and stops Hippo signaling pathway, which leads to increased expression of growth factors, including connective tissue growth factor (CTGF or CCN2) and nephroblastoma overexpressed (NOV or CCN3) (Kawamura et al., 2013). Hsueh et al, (2015) reported that secretion of CCN2 and related factors promotes the growth of primordial

follicles *in vitro*. In ovine species, BMP4 also does not influence activation of primordial follicles *in vitro* (Bertoldo et al., 2014). On the other hand, BMP4 promoted an increase on primordial to primary follicle transition in mouse (Ding et al., 2013) and rat cultured ovaries (Nilsson and Skinner, 2003). In mouse ovary, it was detected BMP4 protein in all stages of follicular development including primordial follicles, suggesting that BMP4 might act in paracrine/autocrine manner to affect the transition of primordial to primary follicles (Tanwar et al., 2008, Tanwar and McFarlane, 2011**)**.

Regarding to follicular diameter, after *in vitro* culture, BMP4 increased the diameters of primary and secondary follicles after culture of ovarian tissue, suggest the existence of functional BMP4 signaling in the preantral follicle. A study in sheep demonstrated that BMP4 increases the diameter of follicles cultured in ovarian tissue fragments (Bertoldo et al., 2014). Nilsson and Skinner (2003) reported an increase in the number of developing follicles compared to controls when rat ovaries were treated with BMP4. Park et al. (2013) reported a direct effect of BMP4 on mice oogonial stem cell differentiation into oocyte, sustaining hypothesis of functional BMP signaling in early steps ofoogenesis/folliculogenesis. *In vivo*, BMP4 is synthesized by the theca cells and acts on nearby granulosa cells and oocytes in a paracrine manner (Young and McNeilly, 2010). Possibly BMP4 acts by increasing the capacity of the granulosa cells to secrete factors of which the oocyte is a target, promoting the increase in diameter of primary and secondary follicles.

This study shows that follicles enclosed in ovarian tissues cultured in presence of BMP4 had their morphology preserved after six days of culture. A previous study suggested that BMP4 may be associated with the survival of oocytes and the development of primordial follicles in neonatal pig ovaries (Shimizu et al., 2004). It was also demonstrated that treatment of neonatal rat ovaries with anti-BMP4 antibody resulted in apoptosis of ovarian stromal-interstitial cells, as well as apoptosis of follicular granulosa and oocytes, which indicate the

role of BMP4 as a cell survival factor (Nilsson and Skinner, 2003). Ding et al. (2013) demonstrated that BMP4 enhanced the phosphorylation of SMAD1/5/8 and prevented oocyte apoptosis via up-regulation of Sohlh2 and c-kit in primordial follicles. Childs et al. (2010) reported that BMP4 reduces apoptosis levels in human granulosa cells cultured *in vitro*. BMP4 is also associated with the inhibition of apoptosis in bovine granulosa cells through the PI3K/ PDK-1/ PKC pathway (Shimizu et al., 2012). Spicer et al. (2006) reported that BMP4 has no effect on granulosa cell proliferation, but prevented premature differentiation of the granulosa cells during growth of follicles. In addition, Fabre et al. (2003) reported that BMP4 have no effect on ovine granulosa cell proliferation while significantly affecting steroidogenesis *in vitro*.

In this study, exogenous addition of BMP4 did not affect mRNA expression for *GDF9, BMP15, PCNA, Bax* and *Bcl2*, perhaps a longer *in vitro* culture period is necessary to have a more pronounced increase in gene expression. Sadeu and Smitz (2008) observed follicular activation and increased expression of *GDF9* after 28 days of culture of ovarian cortex in humans. It has been proposed that the addition of BMP4 to culture medium promotes a balance between various factors involved in the mechanisms of folliculogenesis (Pierre et al., 2004). *GDF9* expression has been found in oocytes from bovine follicles at early stages of follicular development (Bodensteiner et al., 1999). It is known that GDF9 promotes follicular activation after seven days of *in vitro* culture. GDF9 also stimulates the transition from primary to secondary follicles while maintaining their ultrastructural integrity (Martins et al., 2008). In goats, high levels of mRNA for BMP15 were found during the transition from primary to secondary follicle stages (Celestino, et al., 2011). PCNA performs the essential function of providing replicative polymerases with the high processivity required to duplicate the entire genome and has been used as a marker of granulosa cell proliferation (Maga and Hubscher, 2003; Muskhelishvili et al., 2005). Bax is a pro-apoptotic protein

involved in granulosa cell apoptosis and is an important regulator of follicle growth (Tilly et al., 1995). The Bcl2 expression is found in the granulosa cells of both fetal and adult ovaries (Hussein, 2005; Hussein et al., 2006).

In conclusion, 100ng/mL BMP4 promotes increase in follicular and oocyte diameters of primary and secondary follicles after 6 days of *in vitro* culture. Furthermore, BMP4 is able to promote the maintenance of follicular viability.

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Statement of Interest

None. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Tables

Table 1. Primer pairs used to real-time PCR.

Target gene	Primer sequence (5´→ 3´)	Sense (s), anti-sense (As)	Position	GenBank accession no.
UBQ	GAAGATGGCCGCACTCTTCTGAT	S	607-631	GI: 57163956
	ATCCTGGATCTTGGCCTTCACGTT	As	756-780	
GDF9	ACAACACTGTTCGGCTCTTCACCC	S	332-356	GI:51702523
	CCACAACAGTAACACGATCCAGGTT	As	426-451	
BMP15	AAGTGGACACCCTAGGGAAA	S	237-257	GI: 8925958
	TTGGTATGCTACCCGGTTTGGT	As	362-384	
PCNA	TGCCGAGATCTCAGTCACAT	S	566-586	GI:77735938
	TATGGCAACAGCTTCCTCCT	As	695-715	
BCL-2	GGTAGGTGCTCGTCTGGATG	S	2317-2336	GI: 22652876
	GGCCACACACGTGGTTTTAC	As	2440-2421	
Bax	GCCCTTTTCTACTTTGCCAGC	S	334-354	GI: 41386763
	GGCCGTCCCAACCACCC	As	481-465	

Table 2. Percentages (mean±S.E.M.) of primordial and growing follicles (primary and secondary) in uncultured tissues and tissues cultured for 6 days in α-MEM^{+}(control medium) and α-MEM^{+} supplemented with various concentrations of BMP4.

Treatments	**Primordial follicles**	**Growing follicles**
Uncultured tissue (Fresh control)	75.66±7.50*	24.34±7.50*
Cultured tissue	Day 6	Day 6
α-MEM^{+}	16.73±6.98	83.27±6.98
BMP4 [10]	15.88±7.52	84.12±7.52
BMP4 [50]	15.48±5.63	84.52±5.63
BMP4 [100]	9.90±5.85	90.10±5.85

*P<0.05, significantly different from uncultured ovarian cortex tissue (fresh control).

Table 3. Follicle and oocyte diameter (µm, mean±SD) in uncultured (fresh control) tissues and tissues cultured for 6 days in α-MEM$^+$(control medium) and α-MEM$^+$ supplemented with various concentrations of BMP4.

Treatments	**Primordial follicles**		**Primary follicles**		**Secondary follicles**	
Uncultured (Fresh control)	**Oocyte diameter**	**Follicle diameter**	**Oocyte diameter**	**Follicle diameter**	**Oocyte diameter**	**Follicle diameter**
	23.98±5.60	35.05±4.56	30.34±7.76	43.98±8.50	36.59±8.57	82.11±9.42
Cultured	**Oocyte diameter**	**Follicle diameter**	**Oocyte diameter**	**Follicle diameter**	**Oocyte diameter**	**Follicle diameter**
α-MEM$^+$	23.30±7.50^a	33.27±5.64^a	29.27±8.10^a	43.27±6.05^a	37.10±9.66^a	83.27±9.80^a
BMP4[10]	24.65±4.45^a	34.12±6.44^a	30.12±7.22^a	44.12±7.26^a	37.83±7.24^a	84.18±10.55^{ab}
BMP4[50]	26.73±6.50^a	32.52±7.03^a	31.52±9.63^a	49.52±8.34^{ab}	38.35±8.33^{ab}	87.82±10.87^{ab}
BMP4[100]	28.10±5.81^a	36.86±5.85^a	33.10±8.77^a	54.26±8.55^{b}*	47.20±6.25^{b}*	94.10±11.36^{b}*

*P<0.05, significantly different from uncultured ovarian cortex tissue (fresh control).

a,b - Values within columns with different letters among treatments are significantly different (P<0.05).

Figure 1. Percentages of normal and degenerated follicles in uncultured tissue (fresh control) and in tissues cultured for 6 days in α-MEM^{+} alone or supplemented with various concentrations of BMP4.* P<0.05, significantly different from cultured ovarian cortex tissue (fresh control).

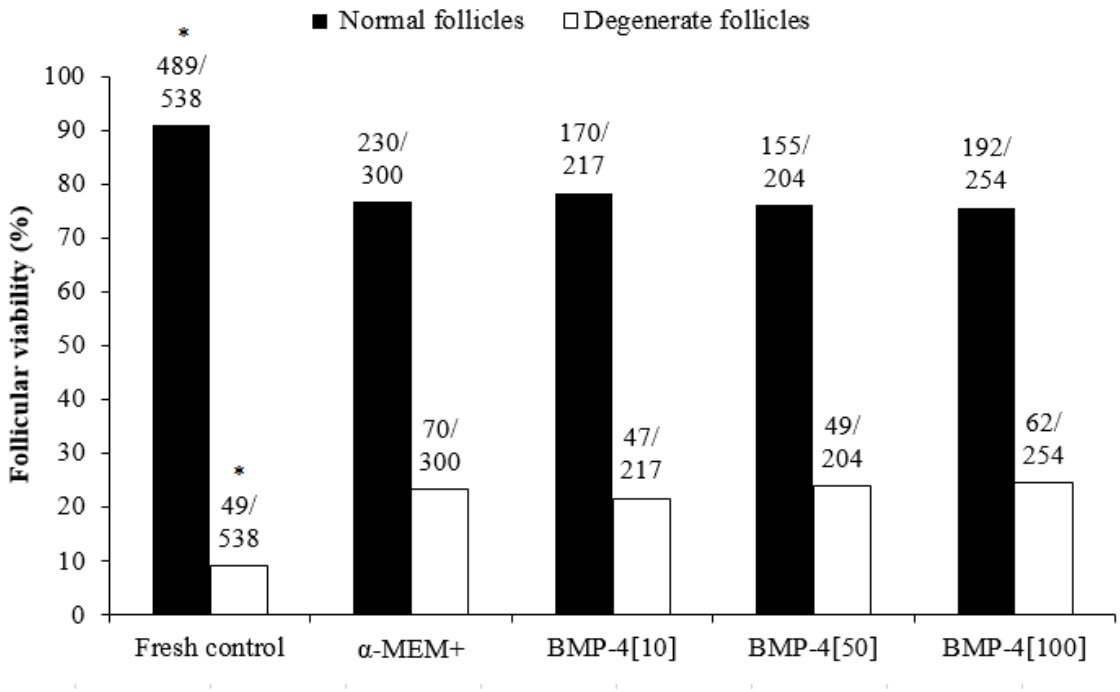
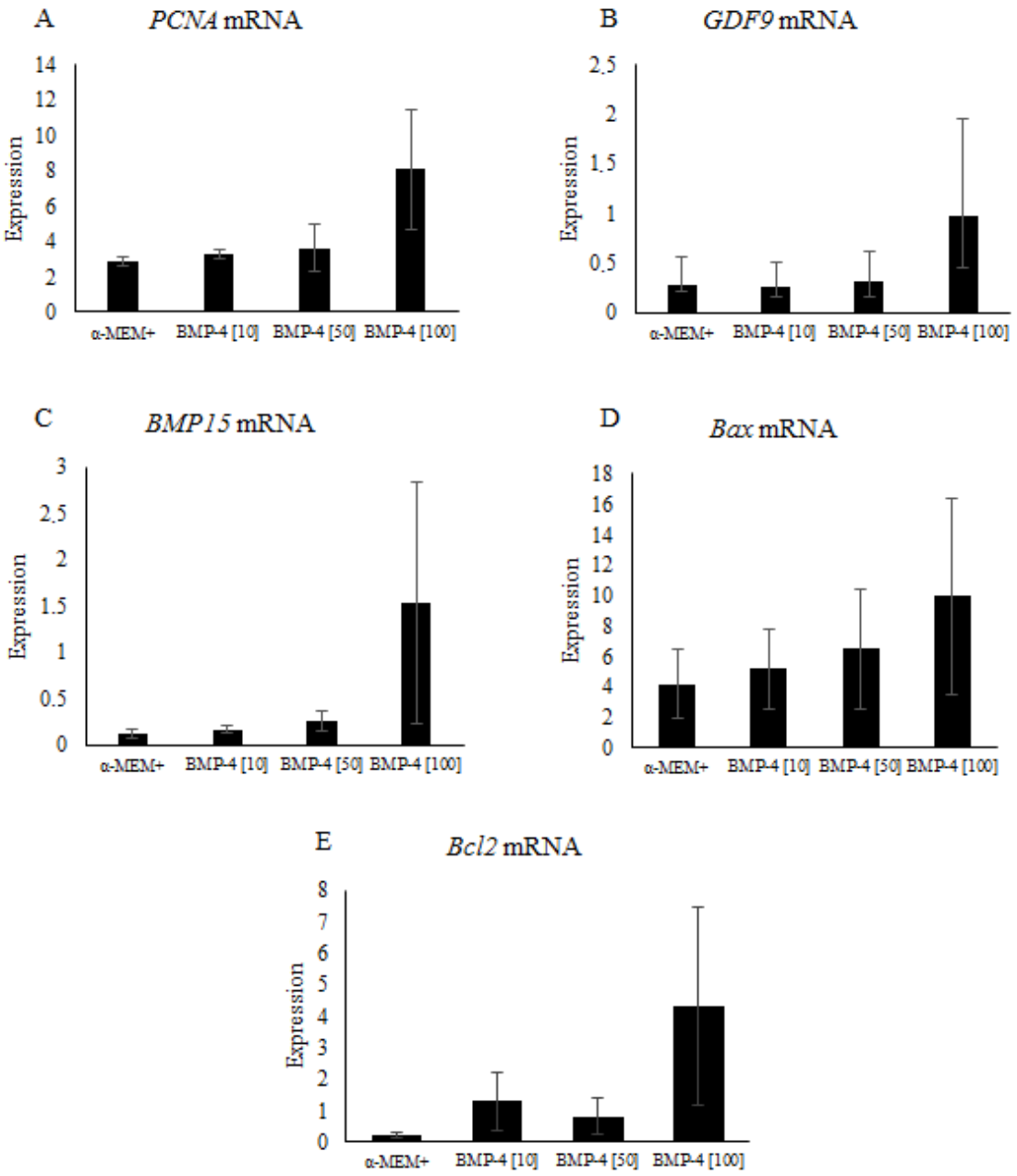


Figure 2. Levels of mRNA for *PCNA* (A), *GDF9* (B), *BMP15* (C), *Bax* (D) and *Bcl2* (E) in tissues cultured for 6 days in α-MEM^{+} alone or supplemented with various concentrations of BMP4.



8 EFEITO DAS PROTEÍNAS MORFOGENÉTICAS ÓSSEAS 2 E 4 NA SOBREVIVÊNCIA E DESENVOLVIMENTO DE FOLÍCULOS SECUNDÁRIOS BOVINOS CULTIVADOS *IN VITRO*

Efeito das Proteínas Morfogenéticas Ósseas 2 e 4 na sobrevivência e desenvolvimento de folículos secundários bovinos cultivados *in vitro*

Effect of bone morphogenetic proteins 2 and 4 on survival and development of bovine secondary follicles cultured *in vitro*

Submetido ao periódico: *Theriogenology*
(Qualis B1 em Biotecnologia)

Efeito das Proteínas Morfogenéticas Ósseas 2 e 4 na sobrevivência e desenvolvimento de folículos secundários bovinos cultivados *in vitro*

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Resumo

Este estudo avaliou o efeito das proteínas morfogenéticas ósseas 2 (BMP2) e 4 (BMP2) no desenvolvimento e na expressão do RNAm para GDF9, Ciclina B1, BMPR1A, BMPR1B, BMPRII, FSHR e SMAD1 em folículos secundários bovinos cultivados *in vitro*. Os folículos secundários isolados foram cultivados durante 18 dias em meio TCM199^{+} sozinho ou suplementado com BMP2 (10 ng/mL), BMP4 (100 ng/mL) ou a combinação de ambas BMP2 e 4. A PCR em tempo real foi utilizada para analisar os níveis de RNAm em folículos frescos e cultivados. Após 18 dias de cultivo, os folículos cultivados com BMP2 sozinha ou com BMP4 sozinha tiveram diâmetros maiores quando comparados ao controle ($P < 0,05$). Além

disso, todos os tratamentos promoveram a formação de antro e mantiveram uma alta taxa de viabilidade após o período de cultivo. A presença de BMP2, BMP4 ou ambas BMP2 e 4 não influenciou a expressão de RNAm para os genes testados. No entanto, o cultivo *in vitro* induz a uma baixa regulação para a expressão de RNAm para BMPR1A. Em conclusão, a adição de BMP2 ou BMP4 no meio promove o crescimento e a formação de antro em folículos bovinos após 18 dias de cultivo *in vitro*.

Palavras-chave: Ovário, vaca, pré-antral, expressão, antro, viabilidade.

Effect of bone morphogenetic proteins 2 and 4 on survival and development of bovine secondary follicles cultured *in vitro*

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Abstract

This study evaluated the effect of bone morphogenetic proteins 2 (BMP2) and 4 (BMP2) on follicle development and mRNA expression for *GDF9*, *Cyclin B1*, *BMPR1A*, *BMPR1B*, *BMPRII*, *FSHR* and *SMAD1* in bovine secondary follicles cultured *in vitro*. Isolated secondary follicles were cultured for 18 days in TCM199^{+} medium alone or supplemented with BMP2 (10 ng/mL), BMP4 (100 ng/mL) or combination of both BMP2 and 4. Real-time PCR was used to analyze mRNA levels in fresh and cultured follicles. After 18 days of culture, follicles cultured with BMP2 alone or with BMP4 alone had larger diameters when compared to control ($P < 0.05$). In addition, all treatments promoted antrum formation and maintained a high viability rate through the growing period. The presence of BMP2, BMP4 or both together did not influence mRNA expression for the tested genes. However, the *in vitro* culture induces down-regulation for mRNA expression of *BMPR1A*. In conclusion, the

addition of BMP2 or BMP4 alone in cultured medium promotes follicular growth and antrum formation in bovine follicles after 18 days of *in vitro* culture.

Keywords: Ovary, cattle, preantral, expression, antrum, viability.

1. Introduction

Preantral follicles are a potential source of oocytes that can reach meiotic competence and that can be used in assisted reproduction programs, including *in vitro* maturation, embryo production and transgenesis. These follicles can be isolated from ovarian tissue and cultured *in vitro*, allowing the evaluation of the effects of various substances and growth factors on follicular development. This contributes to a better understanding of the mechanisms involved in oocyte development, regulation and control of folliculogenesis. In this context, the development of an *in vitro* culture system that is capable of promoting follicle growth from early stages to maturation is important for assisted reproduction programs in women, for the dissemination of animals of high genetic value or those in danger of extinction, as well a model for basic research. Various hormones and growth factors have been tested in *in vitro* culture, including BMP2 and BMP 4. BMPs are members of the transforming growth factorβ (TGFβ) superfamily and play an important role in regulating the development of ovarian follicles [1]. BMPs interact with two classes of serine-threonine kinase transmembrane receptors, type I and type II BMP receptors. In mammals, three type I receptors (BMPRIA/Alk3, BMPRIB/Alk6 and ActRI/Alk2) and three type II receptors (BMPRII, ActRIIA, and ActRIIB) were identified [2]. The BMP receptors phosphorylate intracellular effectors, called Smads, which are classified into three subfamilies: receptor-activated Smads (R-Smads: Smad1, Smad5, and Smad8), phosphorylated through BMPRI, the common mediator Smad (Co-Smad: Smad4), and inhibitory Smads (I-Smads: Smad6 and Smad7).

Inhibitory Smads exert a negative feedback effect through competition with R-Smads for receptor interactions, which may leading the receptors for degradation [3].

Regarding to BMPs 2 and 4, these are detected in the bovine inner theca of antral follicles and their receptor, BMPRII, was demonstrated in granulosa cells and oocytes of primordial, primary and secondary follicles [4, 5]. BMP2 expression has been reported in the developing ovary, as well as its role in the development of primordial germ cells [6, 7]. BMP2 has been shown to regulate the formation of primordial follicles through the transition of germ cells to oocytes and differentiation of somatic cells into pre-granulosa cells [8]. Recently, we have demonstrated that BMP2 promoted growth of primordial follicles and maintained the ultrastructure of secondary follicles after 18 days of *in vitro* culture in bovine species [9]. BMP4, in cattle, controls a number of biological responses, including cell proliferation, steroidogenesis and suppression of apoptosis in granulosa cells grown *in vitro* [10, 11]. BMP4 potentiates the action of follicle-stimulating hormone (FSH) by increasing estradiol production and inhibiting progesterone synthesis in bovine granulosa cells [12]. In addition, in bovine preantral follicles cultured in the presence of BMP4 and FSH, there was an increase in mRNA expression for BMP15 and maintenance of the ultrastructure for 18 days of culture [13]. However, the effects of the interaction between BMPs 2 and 4 on the development, viability and gene expression of isolated bovine preantral follicles has not yet been tested.

During folliculogenesis, expression of oocyte secreted factors, like growth and differentiation factor 9 (GDF9) play a role during early follicle development [14]. In the bovine species, GDF9 has been shown to increase the percentage and diameter of primary and secondary follicles cultured *in vitro*. In addition, GDF9 maintains the viability of these follicles over 14 days of culture [15]. Ovarian follicles undergo exponential growth in response to FSH, largely as a result of the proliferation of granulosa cells [16]. In the presence of FSH and activin, granulosa cells exhibit enhanced expression of cyclin, indicating an

increase in cell proliferation [16]. It has already been shown that BMP2, BMP4, BMP7 and BMP6 all induce FSHR expression in mammals [17, 18, 19, 20, 21]. BMP/Smad signaling regulate many biological processes, including cell proliferation, differentiation and apoptosis during embryonic development and follicle development [22, 23]. It is known that BMP2 and BMP4 preferentially bind to two kinds of Type I receptors (BMPRIA/Alk3 and BMPRIB/Alk6) and one kind of Type II receptor (BMPRII) [24]. Although BMPs have been implicated in the paracrine regulation of ovarian follicular development, the effects of both BMPs 2 and 4 in the development and mRNA expression of their receptors and intracellular effectors (Smads) in bovine secondary follicles is not clearly understood.

Thus, the objective of this study was to evaluate the effect of the interaction of both BMP2 and 4 on the development and maintenance of viability of isolated bovine secondary follicles cultured in vitro. It also evaluates the effects of both BMP2 and 4 on the expression of mRNA for *GDF9, Cyclin B1*, *BMPRIA*, *BMPRIB*, *BMPRII*, *FSHR* and *SMAD1* in bovine secondary follicles cultured *in vitro*.

2. Materials and Methods

All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise indicated in the text.

2.1 Collection of ovaries

Ovaries (n = 40) were collected at a local slaughterhouse, washed twice with 70% alcohol and twice in sterile 0.9% saline solution. The ovaries were transported to the laboratory in tubes containing 20 mL TCM199 medium supplemented with 100 IU/mL penicillin and 10 μg/mL streptomycin at 4°C within 1 hour [25].

2.2 Isolation and *in vitro* culture of secondary follicles

In the laboratory, the ovarian cortex was fragmented (1-2mm) and the fragments were washed in TCM199 medium supplemented with HEPES (0.05 mM/mL), 100 IU/mL penicillin and 10 μg/mL streptomycin. Preantral follicles (~0.2 mm) were visualized under a stereomicroscope (SMZ 645, Nikon, Tokyo, Japan, 40x magnification) and manually dissected with needles (26G). Follicles with visible oocytes, surrounded by two or more layers of granulosa cells, without antral cavity and intact basement membrane were selected for culture. After selection, the follicles were individually cultured in 100μL drops of medium under mineral oil in petri dishes (60x15mm, Corning, USA) for 18 days at 38.5°C in an atmosphere of 5% CO_2. The base medium consisted of TCM199 medium supplemented with 0.05 mM/mL HEPES, 3.0 mg/mL bovine serum albumin, 1% ITS (10 μg/mL insulin, 5.5 μg/mL transferrin, and 5 ng/mL of selenium), 2 mM glutamine, 2 mM hypoxanthine, 50 μg/mL ascorbic acid, 100 IU/mL penicillin, 10 μg/mL streptomycin and 100 ng/mL FSH (Sigma, St. Louis, MO , USA), then called $TCM199^{+}$ (adapted from Rossetto *et al*. [26]). The treatments evaluated were $TCM199^{+}$ (control), BMP2 at 10 ng/mL, BMP4 at 100 ng/mL and the interaction of both BMP2 and BMP4. BMP concentrations were chosen based on previous studies in which these concentrations improved the *in vitro* development of secondary follicles [9, 27]. A partial exchange (60 μL) of medium was performed every two days. The osmolarity of the medium was measured at each exchange and varied between 260 and 300 mOsm.

2.3 Morphological evaluation of follicular development after *in vitro* culture

After the culture period of 18 days, the follicles were considered (1) morphologically normal when they had intact oocytes, with no damage to the basement membrane, (2) degenerated when they had dark or retracted oocytes and (3) extrused follicles when they had

rupture of the basement membrane. The antrum formation was determined by the visualization of a translucent cavity between the granulosa cell mass. The follicular diameter (μm) was calculated only from morphologically normal follicles using two perpendicular measurements using NIS elements 2.4 software (Nikon, Nikon Instruments Inc., Americas). To confirm the morphological analysis, the follicles were incubated with 4 μM of calcein-AM (Molecular Probes, Invitrogen, Germany) and 2 μM of ethidium homodimer-1 (Molecular Probes, Invitrogen, Germany) in a darkroom for 10 minutes. After exposure to fluorescent markers, the follicles were observed under fluorescence inverted microscopy (NIKON, Eclipse, TS 100, 40x magnification), with emission / excitation waves of 488 and 598 nm for calcein-AM and ethidium homodimer-1, respectively. Oocytes and granulosa cells were considered alive when the cytoplasm positively marked for calcein-AM (green) and dead when marked in red by ethidium homodimer-1. Images were obtained and analyzed using NIS-Elements 2.4 software (Nikon, Nikon Instruments Inc., Americas).

2.4 Expression of mRNA for *GDF9, Ciclin B1, BMPRIA, BMPRIB, BMPRII, FSHR* and *SMAD1* in bovine secondary follicles after *in vitro* culture

Morphologically normal follicles that have been cultured in each treatment, uncultured normal follicles with 0.2 mm or 0.35 mm in size were collected and then stored at -80°C until extraction of total RNA. Evaluation of gene expression in in vivo grown follicles was performed to investigate the effect of the culture system itself on gene expression. Total RNA extraction was performed using a Trizol® purification kit (Invitrogen, São Paulo, Brazil). In accordance with the manufacturer's instructions, 800 μL of Trizol solution was added to each frozen sample and the lysate was aspirated through a 20 needle before centrifugation at 10.000g for 3 min 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 min at room temperature. After washing the column three times, the RNA was eluted with 30 µL RNase-free water. 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, UK) and 2 µL of total RNA was used for reverse transcription. Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70°C and then cooled in ice. Reverse transcription was performed in a total volume of 20 µL, which was comprised of 10 µL of sample RNA, 4 µL reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNase out, 150 units SuperscriptIII 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 h at 42°C, for 5 min at 80°C, and then stored at -20°C. Negative controls were prepared under the same conditions, but without the inclusion of the reverse transcriptase. Quantification of mRNA 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, USA), 5.5 µL of ultra-pure water, and 0.5 µM of each primer. The primers were designed by using the PrimerQuestSM program (http://www.idtdna.com) to perform amplification of *GDF9, Cyclin B1, BMPRIA, BMPRIB, BMPRII, FSHR, SMAD1,* and housekeeping gene *GAPDH* (Table 1). This housekeeping gene have shown highest stability in bovine preantral follicles [28] and, thus, it was used to normalize expression of target genes. The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The efficiency amplification for all genes was verified according to Pfaffl *et al*. [29]. The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The

ΔΔCt method was used to transform the Ct values into normalized relative expression levels [30].

2.5 Statistical analysis

The data for follicular survival and antrum formation after *in vitro* culture in each treatment were compared using the Chi-square test, with the results expressed as percentages. The data for the follicular diameters were subjected to analysis using the Kolmogorov-Smirnov and Bartlett tests to confirm normal distribution and homoscedasticity, respectively. ANOVA was subsequently performed, and the treatments were compared using the Student-Newman-Keuls (SNK) test. Analysis was carried out with GraphPad Prism software (GraphPad version 5.0). For the data of mRNA expression, values of Delta-CT were analyzed using the Tukey test. Differences were considered to be significant when $P<0.05$ and data were expressed as mean ± standard error of means (S.E.M.)

3. Results

3.1 Morphological evaluation of follicular development after in vitro culture

After 6, 12 and 18 days of culture in TCM 199^{+} alone or supplemented with BMP2 or BMP4 alone or both BMP2 and BMP4, a significant increase in follicular diameter was observed when compared to day 0. Only at day 18 of culture, follicles cultured in the presence of BMP2 or BMP4 had significantly higher follicular diameter than those grown in the TCM 199^{+} control medium alone or supplemented with both BMP2 and BMP4 (Table 2) ($P<0.05$).

The mean rates of daily follicle growth between days 0, 6, 12 and 18 are shown in Table 3. It was possible to observe that between days 0 and 12 of culture, there was no significant difference between treatments. However, from day 12 to day 18, the mean daily growth of treatments with BMP2 or BMP4 was significantly higher than seen in follicles

cultured in control medium (TCM199^{+}) alone or supplemented with both BMP2 and 4 ($P<0.05$). Similarly, it was observed at total growth rate (D18-D0/18), in which the treatments with BMP2 or BMP4 were significantly higher than TCM199^{+} alone or supplemented with both BMP2 and 4.

Concerning follicular viability, after 18 days of culture, all treatments were able to maintain high follicular survival rates (> 80%) and there was no significant difference between them (Table 4). Fluorescence microscopy evaluation using calcein and ethidium homodimer confirmed the results of the morphological evaluation. All follicles considered morphologically normal were positively stained by calcein (green) after 18 days of culture (Figure 1).

Regarding to antrum formation rate, no significant differences were observed after the culture period (Table 5). At the end of the growing period, the follicular extrusion rate was considered low and there was no significant difference between the treatments (Table 6).

3.2 Levels of mRNA for *GDF9, Cyclin B1, BMPRIA, BMPRIB, BMPRII, FSHR* and *SMAD1* in bovine secondary follicles

The levels of mRNA expression for *GDF9, Cyclin B1, BMPRIA, BMPRIB, BMPRII, FSHR* and *SMAD1* in bovine secondary follicles after 18 days of culture in TCM 199^{+} alone or supplemented with BMP2, BMP4 and both BMP2 and BMP4 were evaluated (Figure 2). No significant differences were observed between the treatments for the expression of the studied genes ($P>0.05$).

In order to evaluate whether the culture conditions altered the expression of these genes, the mRNA levels were evaluated in uncultured follicles of two sizes, 0.2 mm (diameter of follicles at the beginning of culture) and 0.35 mm (in vivo grown follicles). The mRNA expression of these follicles was compared to those follicles that had grown in vitro after

culture in control medium (TCM 199^{+}) (Figure 3). It was observed that, for all genes, *in vivo* grown follicles tended to have increased gene expression those follicles of 0.2mm (fresh control) or those follicles culture in vitro (0.35mm). However, this increase was not statistically significant except for the *BMPRIA* gene. The mRNA expression for *BMPRIA* was higher ($P<0.05$) in *in vivo* grown follicles when compared to those of 0.2mm (fresh control) or those follicles that had been grown *in vitro*.

4. Discussion

The present study shows that BMP2 or BMP4 promote growth and maintains follicular viability after 18 days of *in vitro* culture, but the interaction between BMP2 and BMP4 did not potentiate these effects. In general, all treatments increased follicular diameter throughout the culture period, but BMP2 or BMP4 alone promoted an increase in follicular diameter and growth rate after 18 days of *in vitro* culture, when compared to the other treatments. Previous studies demonstrated that BMP2 (bovine: [9]) and BMP4 (bovine: [13, 27], ovine: [31]) stimulate preantral follicle growth *in vitro*. The absence of interaction between these two factors may be due to the fact that BMP2 and BMP4 share greater than 80% amino acid homology in the ligand domain [32]. Both BMP2 and BMP4 preferentially bind to the type I BMP receptors, BMPR1A and BMPR1B, but can also signal through ActRI [33]. It has been demonstrated that BMP receptors, to which BMPs 2 and 4 bind, was found in oocyte, granulosa and theca cells of bovine secondary follicles [5]. Competition of these two BMPs by the same receptors can explain why these BMPs do not interact to potentiate follicular growth. It was previously described that BMP2 and BMP4 elicit the same biological responses in various in vitro and in vivo assays [34, 35]. Bandyopadhyay et al. [36] also reported redundant roles of BMP2 and BMP4 in osteogenesis. During the culture of bovine ovarian tissue, BMP4 increased the diameters of primary and secondary follicles, emphasizing

the existence of functional BMP4 signaling in preantral follicles [27]. In addition, the presence of BMP2 significantly reduced the percentage of primordial and increased those of developing follicles when compared with tissues cultured in control medium [9]. To emphasize the similar action of BMPs 2 and 4 in ovarian follicles, BMP4 maintained the survival of primordial follicles cultured *in vitro* (rats: [37], sheep: [31], pig: [38] and bovine: [13]) and similar results were obtained for BMP2 in bovine secondary follicles [9]. *In vivo* studies have demonstrated that the expression of BMP4 mRNA in theca cells of atretic follicle is very low or undetectable, suggesting the role of this BMP in maintaining follicular viability [39]. Previous studies have reported that BMP4 is able to reduce the levels of apoptosis in granulosa cells cultured *in vitro* [40]. Furthermore, Kayamori *et al*. [41] demonstrated that BMP-4 increases the expression of survivin mRNA in cultured granulosa cells and results in the inhibition of cell apoptosis. In cultured developing hamster ovaries, BMP2 also reduces the overall levels of apoptosis in the ovary [8]. However, in bovine secondary follicles, neither BMP-2 nor BMP-4 influenced follicle survival *in vitro*.

In this study, a small rate of antrum formation at the end of the growing period was observed. Similarly, Rossi et al. [13] observed that few bovine secondary follicles formed antral cavity after 18 days of culture *in vitro*. Possibly the presence of hormones and other growth factors in culture medium, like GDF-9 [42], VEGF, IGF, GH, FSH [44] is required to potentiate antrum formation in cultured bovine secondary follicles. Moreover, for the some species, such as primates, humans and bovine, the development of preantral follicles until ovulation requires a prolonged culture period, which may extend for months [43], as demonstrated by Araújo et al. [44, 45], which cultured bovine secondary follicles for 32 days.

Concern to PCR analysis, the addition of exogenous BMP2, BMP4 or both did not regulate the expression of the mRNA for *GDF9, Cyclin B1, BMPRIA, BMPRIB, BMPRII, FSHR* and *SMAD1*. Perhaps a longer *in vitro* culture period is necessary to have a more

pronounced increase in gene expression. Oocyte-secreted paracrine factors promote the proliferation, differentiation, and function of granulosa cells [46]. Particularly, oocyte-specific growth factors, like BMP15 and GDF9, play crucial roles in development of oocyte and granulosa cells [47]. Previous studies have shown that BMP2 or BMP4 does not influence GDF9 expression in bovine follicles after *in vitro* culture [13, 9]. FSH-stimulated granulosa cells proliferation in intact animals requires increased expression of cyclin. It is already known that follicles of cyclin-null mice do not proceed beyond the secondary stage of development [48]. Smad complexes regulate many biological processes, including cell proliferation, differentiation and apoptosis during embryonic development, as well as adult tissue homeostasis [22, 51]. BMP receptors phosphorylate receptor SMADs (SMAD1/5), which associate with the co-SMAD4 and translocate to the nucleus. Typically, BMP2 and 4 binds its type-I and -II receptors in a cooperative manner. In bovine specie, BMPRII was detected in oocytes and granulosa cells from primary and secondary non-cultured follicles [5]. In sheep and caprine, *BMPRII* mRNA was demonstrated both in oocytes and granulosa cells of uncultured antral follicles [49, 51], which demonstrate demonstrates its role in the BMP signaling pathway. In sheep, Bertoldo et al. [31] demonstrated presence of all three receptors in the oocytes and granulosa cells of all follicle sizes from the primordial to the small antral stage. In our experiment, *in vivo* grown follicles of 0.35 mm in size had higher mRNA expression for *BMPRIA* than those follicles at the beginning of culture (0.2mm) or that had grown *in vitro* (0.35mm). Previous experiments have also demonstrated a down-regulation of *BMPRIA* [50], *BMPRII* [50, 51], and *SMAD1* expression [51] after the *in vitro* culture of caprine secondary follicles.

In conclusion, BMP2 or BMP4 promotes bovine secondary follicles growth after 18 days of *in vitro*, but these BMPs did not interact to potentiate their action. Moreover, the *in vitro* grown follicles have lower levels of mRNA of *BMPRIA* than those grown *in vivo*.

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Figures and tables:

Figure 1. Viable follicles after *in vitro* culture for 18 days in TCM 199^{+} alone (A,B) or supplemented with BMP2 (C,D), BMP4 (E,F), or with both BMP2 and 4 (G.H). (A,C,E,G – Follicles observed in inverted microscope (10x) and B, D, F, H - follicles stained with calcein-AM and ethidium homodimer-1 under a fluorescence microscope (10x)).

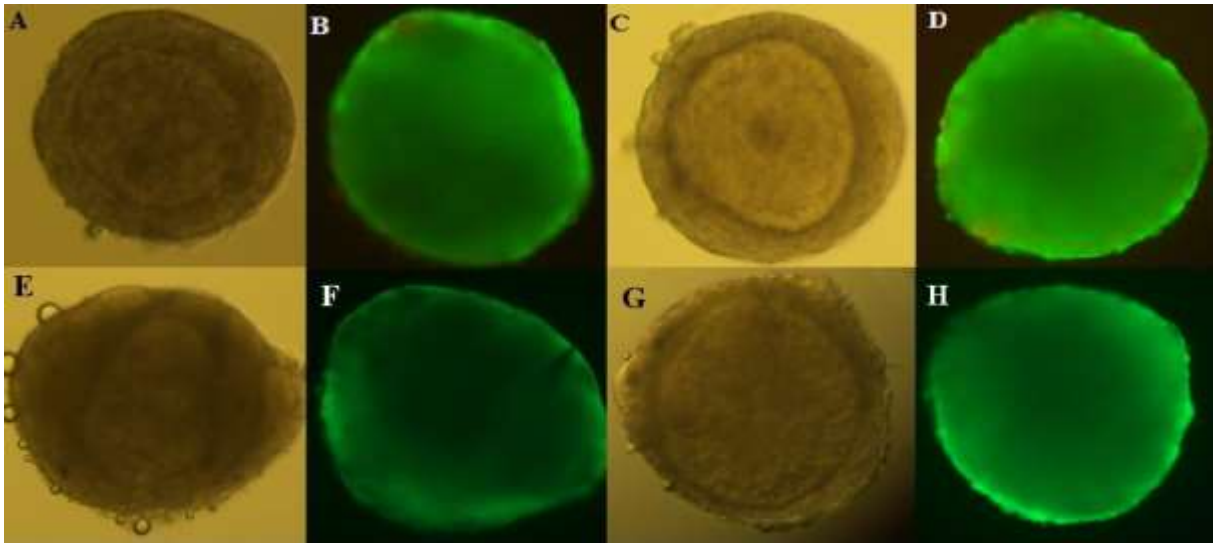

Figure 2. Levels of mRNA (means±SD) for (A) *GDF9*, (B) *Ciclin B1*, (C) *BMPRIA*, (D) *BMPRIB*, (E) *BMPRII*, (F) *FSHR* and (G) *SMAD1* in bovine follicles after *in vitro* culture for 18 days in TCM 199^{+} alone or supplemented with BMP2, BMP4 or both BMP2 and 4.

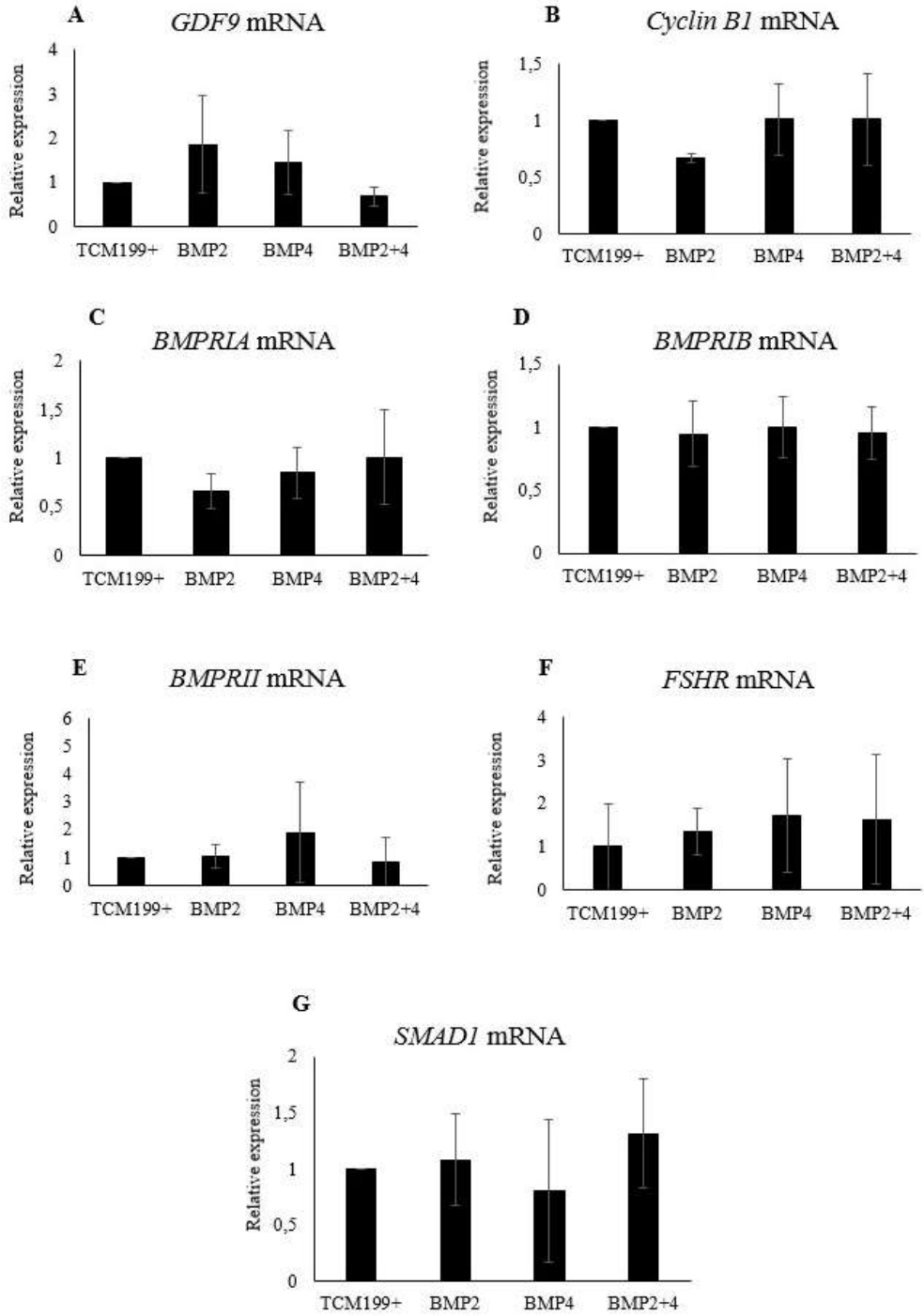

Figure 3. Levels of mRNA (means±SD) for (A) *GDF9*, (B) *Ciclin B1*, (C) *BMPRIA*, (D) *BMPRIB*, (E) *BMPRII*, (F) *FSHR* and (G) *SMAD1* in uncultured bovine follicles (fresh control 0.2), in in vivo grown follicles (0.35mm) and in in vitro grown follicles (0.35mm).

* Represent statistically significant differences between treatments (P<0.05).

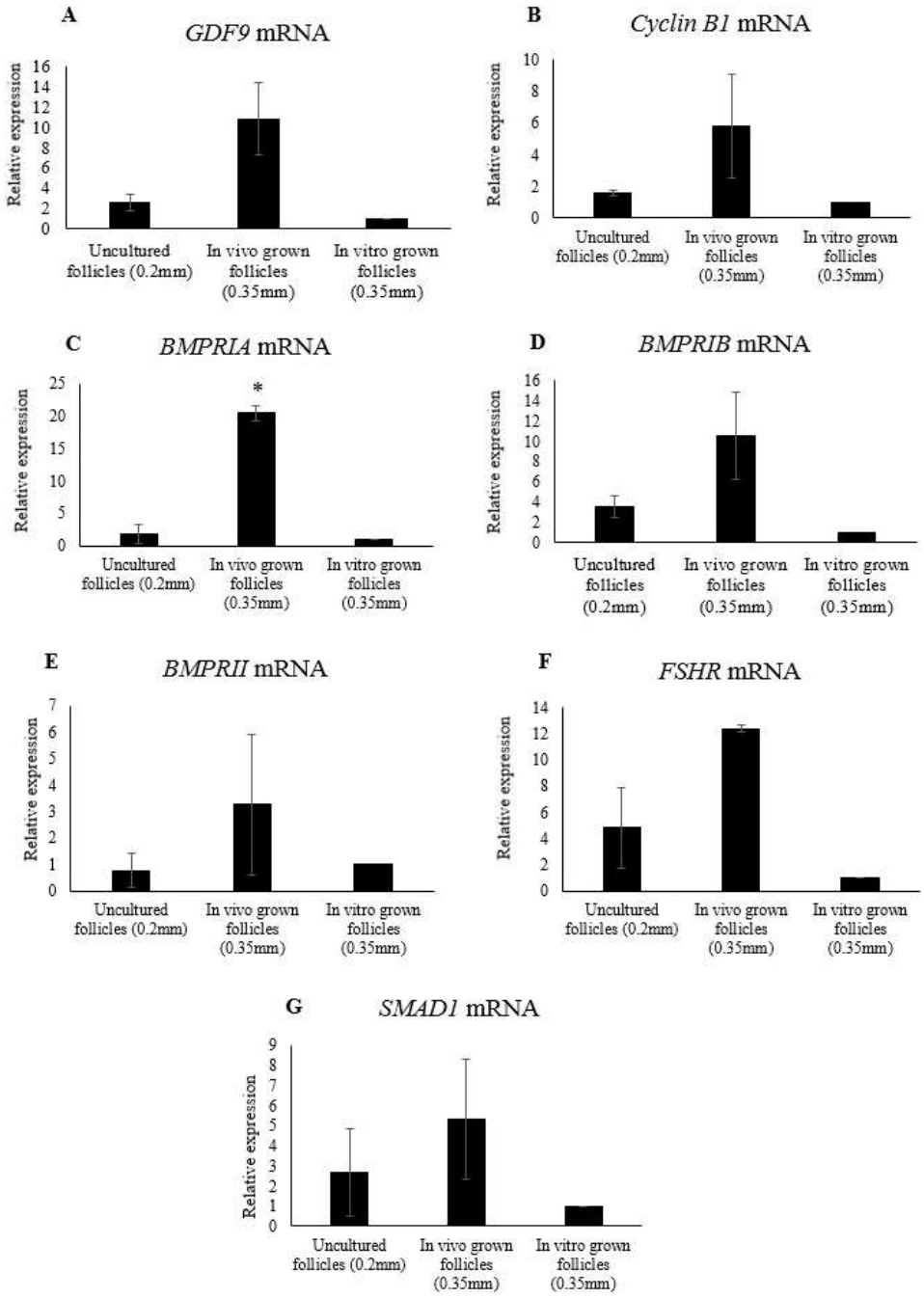

Table 1. Primer pairs used to real-time PCR.

Target gene	Primer sequence (5´→ 3´)	Sense (s), anti-sense (As)	Position	GenBank accession no.
GDF9	ACAACACTGTTCGGCTCTTCACCC	S	332-356	GI:51702523
	CCACAACAGTAACACGATCCAGGTT	As	426-451	
Ciclina B1	CTCCAGTGCTCTCCTCCTCACT	S	601-661	NM_001045872.1
	CTAATCTTCGTGTTCCTGGTGATCC	As	721-781	
BMPR1A	ACGTTTGCGGCCAATTGTGT	S	1664-1685	GI:116003816
	TTGTGAGCCCAGCATTCTGACA	As	1753-1774	
BMPR1B	TTTGGATGGGAAAGTGGCGT	S	653-672	GI:297471905
	TGCAGCAATGAAGCCCAAGA	As	792-811	
BMPRII	TGTGCCAAAGATTGGCCCTT	S	1602-1621	GI:297471905
	TGCTTGCTGCCGTTCATAGT	As	1776-1795	
FSHR	AGGCAAATGTGTTCTCCAACCTGC	S	250-274	GI:95768228
	TGGAAGGCATCAGGGTCGATGTAT	As	316-340	
SMAD1	TTGGAATGCTGCGAGTTTCCCTTC	S	132–155	GI:14486390
	AACTGAGCTAAGAGGCTGTGCTGA	As	283–306	
GAPDH	TGTTTGTGATGGGCGTGAACCA	S	288–309	GI: 402744670
	ATGGCGTGGACAGTGGTCATAA	As	419–440	

Table 2. Follicular diameter of bovine follicles after 0, 6, 12 and 18 days of culture in TCM 199^{+} alone or supplemented with BMP2, BMP4 or both BMP2 and 4. Data are reported as means (µm) ± S.E.M. Distinct lowercase letters (a /b/c) indicate statistically significant differences between days of *in vitro* culture and distinct capital represent statistically significant differences between treatments ($P<0.05$).

Period (days)	TCM 199^{+}	BMP2	BMP4	BMP2+4
0	$202.20\pm4,42^{a}$	193.12 ± 3.56^{a}	193.84 ± 2.35^{a}	196.70 ± 3.56^{a}
6	254.19 ± 7.93^{b}	249.45 ± 6.99^{b}	253.28 ± 3.18^{b}	259.23 ± 6.35^{b}
12	296.28 ± 10.25^{c}	307.48 ± 8.72^{c}	300.46 ± 3.75^{c}	292.96 ± 8.85^{c}
18	321.61 ± 11.78^{dA}	354.38 ± 9.63^{dB}	344.15 ± 9.18^{dB}	321.18 ± 8.57^{dA}

Table 3. Growth rate (μm/day, media ± S.E.M) of bovine follicles after 0, 6, 12 and 18 days of culture in TCM 199^{+} alone or supplemented with BMP2, BMP4 or both BMP2 and 4. Data are reported as means (μm) ± S.E.M. Distinct capital represent statistically significant differences between treatments (P<0.05).

Growth rate	**TCM 199^{+}**	**BMP2**	**BMP4**	**BMP2+4**
0 to 6 days	8.66±0.8^{A}	9.38±0.6^{A}	9.90±0.5^{A}	10.11±0.6^{A}
6 to 12 days	7.01±0.7^{A}	9.6±0.9^{A}	7.86±0.8^{A}	5.83±0.5^{A}
12 to 18 days	4.22±0.5^{A}	7.81±0.7^{B}	7.28±0.7^{B}	4.64±0.6^{A}
Growth rate (D18-D0/18)	6.63±0.6^{A}	8.95±0.5^{B}	8.35±0.4^{B}	6.86±0.4^{A}

Table 4. Percentage of viable follicles after *in vitro* culture for 18 days in TCM 199^{+} alone or supplemented with BMP2, BMP4 or both BMP2 and 4.

Morphologically normal follicles (%)				
Period (days)	**TCM 199^{+} (N=60)**	**BMP2 (N=64)**	**BMP4 (N=66)**	**BMP2+4 (N=64)**
Day 6	100.00 (60/60)	100.00 (64/64)	100.00 (66/66)	95.31(61/64)
Day 12	93.33 (56/60)	95.31 (61/64)	95.45 (63/66)	92.18(59/64)
Day 18	85.00 (51/60)	81.25 (52/64)	89.40 (59/66)	87.50 (56/64)

Table 5. Percentage of antrum formation in bovine follicles after *in vitro* culture for 18 days in TCM 199^{+} alone or supplemented with BMP2, BMP4 or both BMP2 and 4.

Antrum formation (%)				
Period (days)	**TCM 199^{+} (N=60)**	**BMP2 (N=64)**	**BMP4 (N=66)**	**BMP2+4 (N=64)**
Day 6	3.33 (2/60)	3.12 (2/64)	0.00 (0/66)	0.00 (0/64)
Day 12	6.66 (4/60)	7.81 (5/64)	3.03 (2/66)	0.00 (0/64)
Day 18	10.00 (6/60)	7.81 (5/64)	4.54 (3/66)	3.12 (2/64)

Table 6. Percentage of extrusion rate after *in vitro* culture for 18 days in TCM 199^{+} alone or supplemented with BMP2, BMP4 or both BMP2 and 4.

	Extrusion Rate (%)			
Period (days)	**TCM 199^{+} (N=60)**	**BMP2 (N=64)**	**BMP4 (N=66)**	**BMP2+4 (N=64)**
Day 6	0.00 (0/60)	1.56 (1/64)	0.00 (0/66)	0.00 (0/64)
Day 12	3.33 (2/60)	1.56 (1/64)	1.51 (1/66)	1.56 (1/64)
Day 18	5.00 (3/60)	4.68 (3/64)	3.03 (2/66)	1.56 (1/64)

9 CONCLUSÕES

- A BMP-2 nas concentrações de 10, 50 e 100 ng/mL influencia a ativação de folículos primordiais cultivados por 6 dias inclusos em tecido ovariano;

- A BMP-4 na concentração de 100 ng/mL aumenta os diâmetros folicular e oócitário de folículos primários e secundários, mantém a viabilidade e a ultraestrutura folicular após 6 dias de cultivo de tecido ovariano *in vitro;*

- A BMP-2 na concentração 10 ng/mL melhora a viabilidade, mantém a ultraestrura e modula a expressão de RNAm para *GDF-9*, *NLRP-5* e *NMP-2* de folículos secundários bovinos cultivados *in vitro* por 18 dias;

- A BMP-2, na concentração de 10 ng/mL, e a BMP-4, na concentração de 100 ng/mL, aumentam o diâmetro de folículos secundários bovinos após 18 dias de cultivo *in vitro*;

- A associação de ambas BMPs 2 e 4 não influencia no crescimento de folículos secundários bovinos após 18 dias de cultivo *in vitro*;

- A BMP-2, BMP-4 e sua interação não influenciam a expressão de RNAm para *GDF-9, Ciclina B1, BMPIA, BMPRIB, BMPRII, FSHR* e *SMAD1* em folículos secundários bovinos após o cultivo *in vitro*;

- O cultivo *in vitro* de folículos secundários bovinos induz uma regulação negativa da expressão de RNAm para *BMPR1A.*

10 PERSPECTIVAS

A compreensão dos fenômenos fisiológicos associados ao crescimento folicular e maturação oocitária é fundamental para aperfeiçoar os sistemas de cultivo de folículos pré-antrais *in vitro* e, consequentemente, ter impactos positivos para as biotécnicas aplicadas a reprodução animal. O conhecimento dos efeitos biológicos das BMPs 2 e 4 poderá contribuir para a produção de embriões a partir de oócitos de folículos imaturos cultivados *in vitro*, visto que os resultados alcançados até agora, a partir do cultivo de folículos pré-antrais, para a espécie bovina ainda são insatisfatórios.

A presente tese gerou informações relevantes no que diz respeitos as melhores concentrações de BMPs 2 e 4 para promover o crescimento e desenvolvimento de folículos pré-antrais bovinos. Entretanto, estudos complementares devem ser realizados testando essas concentrações de BMP-2 e 4 por um período de cultivo mais longo, podendo ser associadas a outros hormônios e/ou fatores de crescimento em meio de cultivo dinâmico, visando a obtenção de oócitos competentes derivados do cultivo de folículos pré-antrais.

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APÊNDICE A – LISTA DE FIGURAS

REVISÃO DE LITERATURA

INFLUÊNCIA DA BMP-2 NO DESENVOLVIMENTO FOLICULAR INICIAL E A EXPRESSÃO DE RNAM DE GENES ESPECÍFICOS DO OÓCITO EM FOLÍCULOS PRÉ-ANTRAIS BOVINOS CULTIVADOS *IN VITRO*

EFEITOS DA PROTEÍNA MORFOGENÉTICA ÓSSEA 4 (BMP-4) NO DESENVOLVIMENTO E SOBREVIVÊNCIA *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS BOVINOS INCLUSOS EM

FRAGMENTOS DE TECIDO OVARIANO

EFEITO DAS PROTEÍNAS MORFOGENÉTICAS ÓSSEAS 2 E 4 NA SOBREVIVÊNCIA E DESENVOLVIMENTO DE FOLÍCULOS SECUNDÁRIOS BOVINOS CULTIVADOS *IN VITRO*

APÊNDICE B – LISTA DE TABELAS

REVISÃO DE LITERATURA

INFLUÊNCIA DA BMP-2 NO DESENVOLVIMENTO FOLICULAR INICIAL E A EXPRESSÃO DE RNAM DE GENES ESPECÍFICOS DO OÓCITO EM FOLÍCULOS PRÉ-ANTRAIS BOVINOS CULTIVADOS *IN VITRO*

EFEITOS DA PROTEÍNA MORFOGENÉTICA ÓSSEA 4 (BMP-4) NO DESENVOLVIMENTO E SOBREVIVÊNCIA *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS BOVINOS INCLUSOS EM FRAGMENTOS DE TECIDO OVARIANO