Histol Histopathol (2016) 31: 339-348

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

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

Rodrigo O.D.S. Rossi[1], Ellen V. da Cunha[1], Antonia M.L.R. Portela[1], José R.S. Passos[1], José J.N. Costa[1], Anderson W.B. Silva[1], Márcia V.A. Saraiva[1], Christina A. Peixoto[2], Mariana A.M. Donato[2], Robert van den Hurk[3] and José R.V. Silva[1]

[1]Biotechnology Nucleus of Sobral - NUBIS, Federal University of Ceara, Sobral, CE, Brazil, [2]Laboratory of Ultrastructure, CPqAM/FIOCRUZ, Federal University of Pernambuco, Recife-PE, Brazil and [3]Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Summary. 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*.

Offprint requests to: José R.V. Silva, Biotechnology Nucleus of Sobral, NUBIS, Federal University of Ceara, Sobral, CE, Brazil. e-mail: jrvsilva@ufc.br or roberto_viana@yahoo.com
DOI: 10.14670/HH-11-674

Key words: Ovarian follicles, BMP-2, FSH, Cow, Culture

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*.

Material and methods

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 (3x3x1 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 x 100 and x 400 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 $P<0.05$.

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 (60x15 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°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 (Fig. 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 x 200 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 $P<0.05$.

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

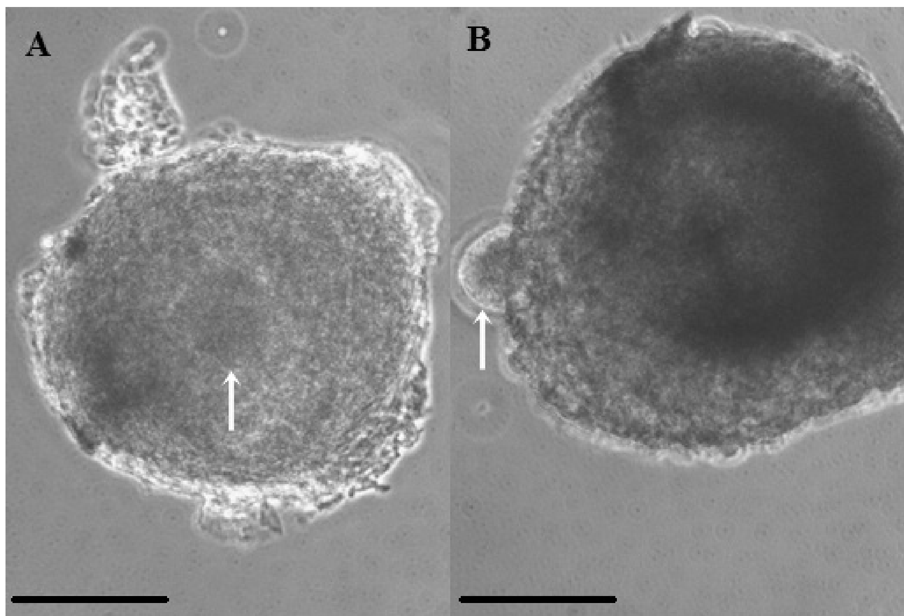

Fig. 1. Morphological normal **(A)** and degenerated follicles **(B)** after *in vitro* culture. Arrow: oocyte. Scale bars: 100 µm.

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°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 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 95°C, followed by 50 cycles of 15 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C. 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 72°C. 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 x 400 magnification. Ultra-thin sections (70 nm) were

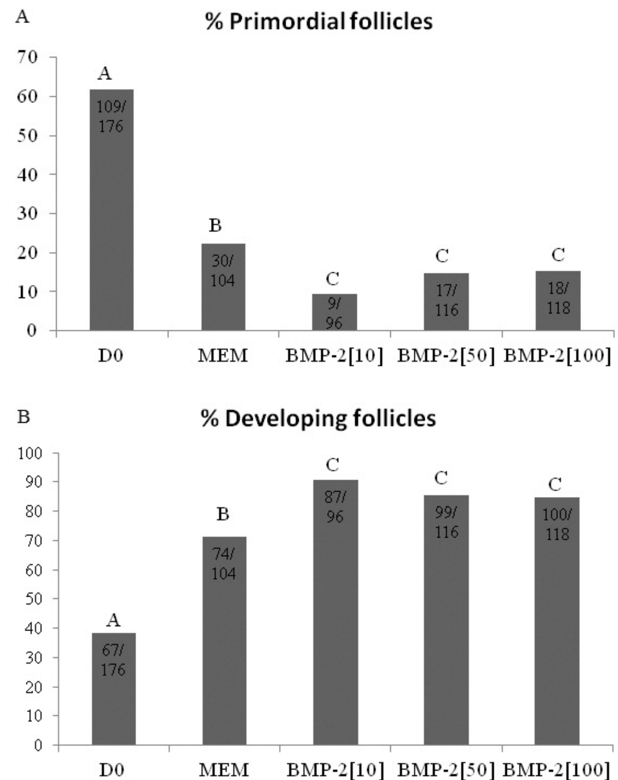

Fig. 2. Percentage of primordial **(A)** and developing follicles **(B)** in non-cultured tissues (control) and in tissues cultured for 6 days in α-MEM+ alone or with different concentrations of BMP-2. A,B - significant difference between treatments (P<0.05).

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

Target gene	Primer sequence (5' → 3')	Sense (s), anti-sense (as)	Position	GenBank accession no.
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	

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.

Results

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 (Fig. 2A) and increase of developing follicles (Fig. 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 (Fig. 3).

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

The initial diameter (day 0) of selected preantral follicles was approximately 200 μm (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.

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

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$^+$ + 10ng/mL BMP-2 (n=59)	α-MEM$^+$ + 50ng/mL BMP-2 (n=57)	α-MEM$^+$ + 100ng/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$^+$ + 10ng/mL BMP-2	α-MEM$^+$ + 50ng/mL BMP-2	α-MEM$^+$ + 100ng/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).

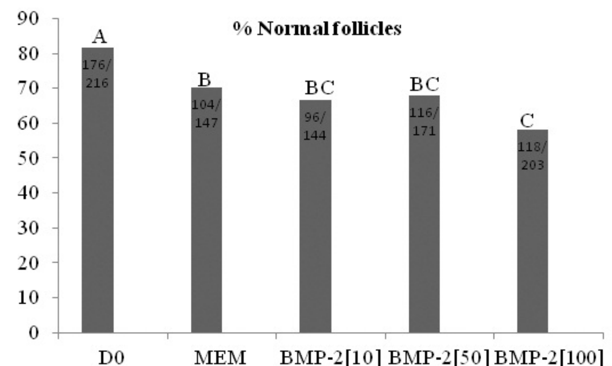

Fig. 3. Percentage of normal follicles in non-cultured tissues (control) and in tissues cultured for 6 days in α-MEM+ alone or with different concentrations of BMP-2. A,B,C - significant difference between treatments (P<0.05).

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 (Fig. 4A). In addition, FSH increased ($P<0.05$) the level of mRNA for *NLRP5* (Fig. 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 (Fig. 4C).

Table 4. Follicular diameters after 18-days culture of bovine preantral follicles in 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 Values within a row; values without a common superscript significantly differ ($P<0.05$).

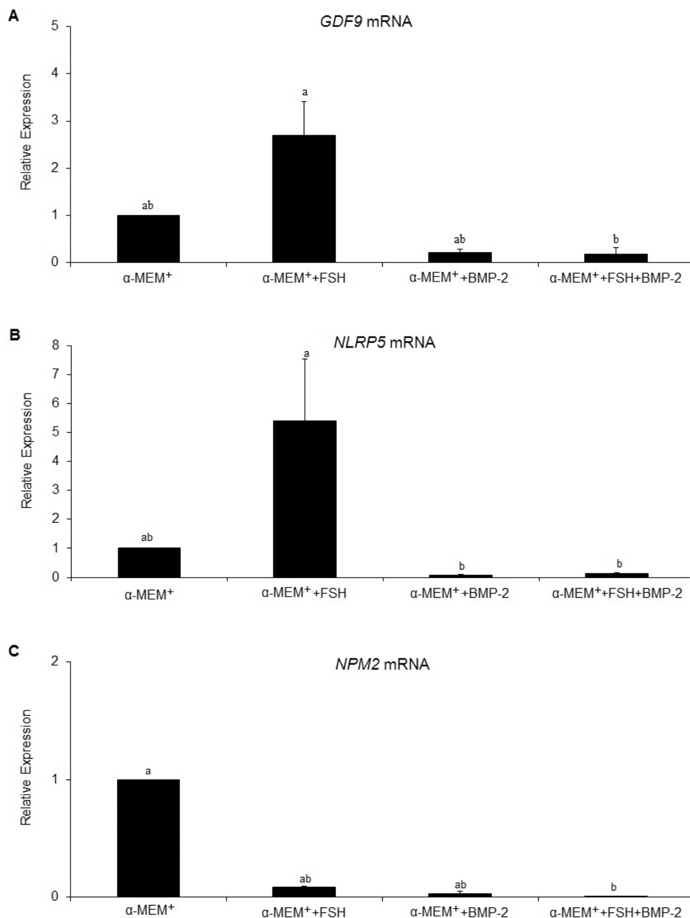

Fig. 4. Relative expression of mRNA for GDF9 **(A)**, NLRP5 **(B)** and, NPM2 **(C)** in oocytes cultured for 18 days in medium supplemented with BMP-2, FSH or both. a,b: values without a common superscript significantly differ ($P<0.05$).

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 (Fig. 5A). Follicles cultured in medium in which BMP-2 (Fig. 5B) or both FSH and BMP-2 (Fig. 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 (Fig. 5C) had ultrastructurally normal granulosa cells, but the oocyte was irregularly shaped and its ooplasm vacuolated.

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).

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 Yossefi, 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

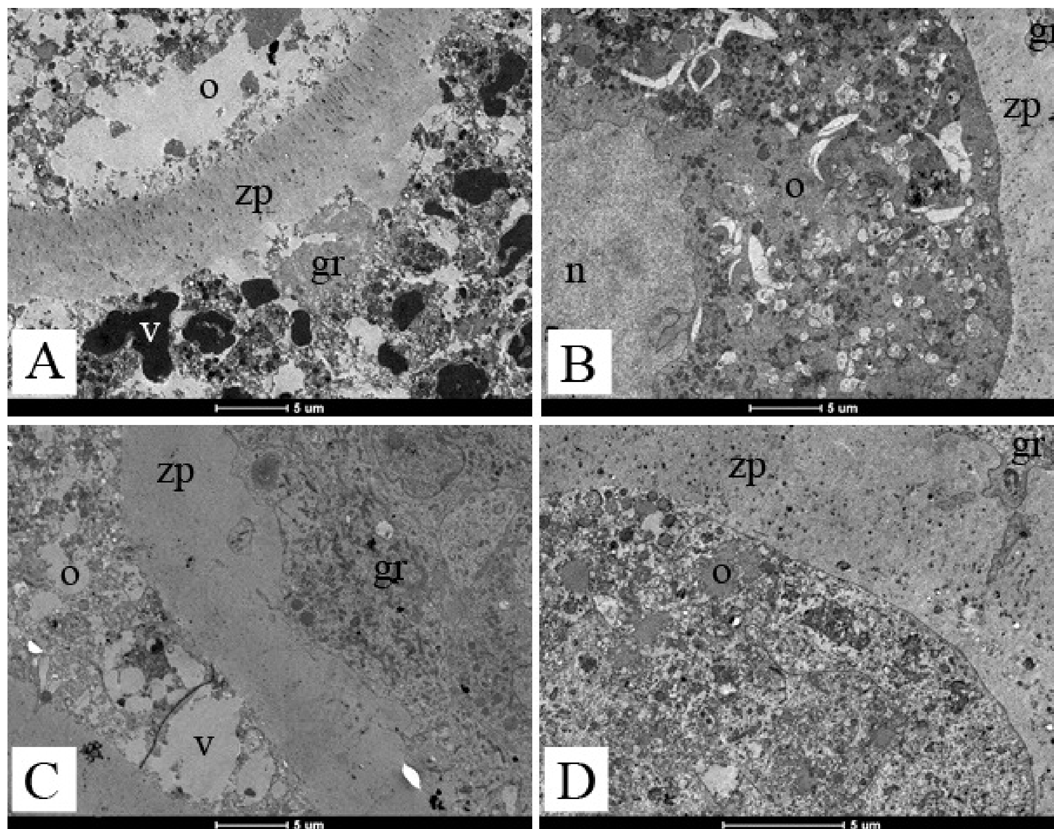

Fig. 5. Transmission electron microscopy micrographs of follicles after 18 days of culture in α-MEM$^+$ alone **(A)** or α-MEM$^+$, supplemented with BMP-2 **(B)**, FSH **(C)** or both BMP-2 and FSH **(D)**. n: nucleus, gr: granulosa cells, zp: zona pellucida, o: oocyte, v: vacuole. Scale bars: 5 μm.

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., 2005a,b; murine: Nakamura et al., 2010) but did not stimulate granulosa cell proliferation (Brankin et al., 2005a,b; 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 and Giudice, 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., 2010).

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*.

References

Alves A.M., Chaves R.N., Rocha R.M., Lima, L.F., Andrade P.M. Lopes C.A., Souza, C.E., Moura A.A., Campello C.C., Báo S.N., Smitz J. and Figueiredo J.R. (2013). Dynamic medium containing growth differentiation factor-9 and FSH maintains survival and promotes *in*

vitro growth of caprine preantral follicles after long-term *in vitro* culture. Reprod. Fertil. Dev. 25, 955-965.

Brankin V., Quinn R.L., Webb R. and Hunter M.G. (2005a). BMP-2 and -6 modulate porcine theca cell function alone and co-cultured with granulosa cells. Domest. Anim. Endocrinol. 29, 593-604.

Brankin V., Quinn R.L., Webb R. and Hunter, M.G. (2005b). Evidence for a functional bone morphogenetic protein (BMP) system in the porcine ovary. Domest. Anim. Endocrinol. 28, 367-379.

Braw-Tal R. and Yossefi S. (1997). Studies *in vivo* and *in vitro* on the initiation of follicle growth in the bovine ovary. J. Reprod. Fertil. 109, 165-171

Burkhart M.N., Juengel J.L., Smith P.R., Heath D.A., Perry G.A., Smith M.F. and Garverick H.A. (2010). Morphological development and characterization of aromatase and estrogen receptors alpha and beta in fetal ovaries of cattle from days 110 to 250. Anim. Reprod. Sci. 117, 43-54.

Burns K.H., Viveiros M.M., Ren Y., Wang P., Demayo F.J., Frail D.E., Eppig J.J. and Matzuk M.M. (2003). Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. Science 300, 633-636.

Campbell B.K., Souza C.J.H., Skinner A.J., Webb R. and Baird D.T. (2006). Enhanced response of granulosa and theca cells from sheep carriers of the FecB mutation *in vitro* to gonadotropins and bone morphogenic protein-2, -4, and -6. Endocrinology 147, 1608-1620.

Carabatsos M.J., Elvin J., Matzuk M.M. and Albertini D.F. (1998). Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev. Biol. 204, 373-384.

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

Cortvrindt R., Hu Y. and Smitz J. (1998). Recombinant luteinizing hormone as a survival and differentiation factor increases oocyte maturation in recombinant follicle stimulating hormone-supplemented mouse preantral follicle culture. Hum. Reprod. 13, 1292-1302.

Cushman R.A., Wahl C.M. and Fortune J.E. (2002). Bovine ovarian cortical pieces grafted to chick embryonic membranes: a model for studies on the activation of primordial follicles. Hum. Reprod. 17, 48-54.

de Bruin J.P., Dorland M., Spek, E.R. Posthuma G., van Haaften M., Looman C.W. and te Velde E.R. (2002). Ultrastructure of the resting ovarian follicle pool in healthy young women. Biol. Reprod. 66, 1151-1160.

Erickson G.F. and Shimasaki, S. (2003). The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod. Biol. Endocrinol. 1, 1-20.

Fatehi A.N., van den Hurk R., Colenbrander B., Daemen A.J., van Tol H.T. Monteiro R.M., Roelen B.A. and Bevers M.M. (2005). Expression of bone morphogenetic protein 2 (BMP-2), 4 (BMP-4) and BMP receptors in the bovine ovary but absence of effects of BMP-2 and BMP-4 during IVM on bovine oocyte nuclear maturation and subsequent embryo development. Theriogenology 63, 872-889.

Fortune J.E. (2003). The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Anim. Reprod. Sci. 78, 135-163.

Fuku E., Xia L. and Downey B.R. (1995). Ultrastructural changes in bovine oocytes cryopreserved by vitrification. Cryobiology 32, 139-156.

Hsueh A.J., Kawamura K., Cheng Y. and Fauser B.C. (2015). Intraovarian control of early folliculogenesis. Endocr. Rev. 36, 1-24.

Hulshof S.C., Figueiredo J.R., Beckers J.F., Bevers M.M., van der Donk J.A. and van den Hurk R. (1995). Effects of fetal bovine serum, FSH and 17beta-estradiol on the culture of bovine preantral follicles. Theriogenology 44, 217-226.

Inagaki K., Otsuka F., Miyoshi T., Yamashita M., Takahashi M., Goto J., Suzuki, J., and Makino H. (2009). p38-mitogen-activated protein kinase stimulated steroidogenesis in granulosa cell-oocyte cocultures: role of bone morphogenetic proteins 2 and 4. Endocrinology 150, 1921-1930.

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

Kawamura K., Cheng Y., Suzuki N., Deguchi M., Sato Y., Takae S., Ho C.H., Kawamura N., Tamura M., Hashimoto S., Sugishita Y., Morimoto Y., Hosoi Y., Yoshioka N., Ishizuka B. and Hsueh A.J. (2013). Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. Proc. Natl. Acad. Sci. USA 110, 17474-17479.

Li Y., Li R.Q., Ou, S.B., Zhang, N.F., Ren, L., Wei, L.N., Zhang, Q.X. and Yang, D.Z. (2014). Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. Reprod. Biol. Endocrinol. 12, 81.

Livak K.J. and Schmittgen T.D. (2001). Analysis of relative 535 gene expression data using real-time quantitative PCR and 536 the $2^{-\Delta\Delta CT}$. Method 25, 402-408.

Mao J., Wu G., Smith M.F., McCauley T.C., Cantley T.C., Prather R.S., Didion B.A. and Day B.N. (2002). Effects of culture medium, serum type, and various concentrations of follicle-stimulating hormone on porcine preantral follicular development and antrum formation *in vitro*. Biol. Reprod. 67, 1197-1203.

Mao J., Smith M.F., Rucker E.B., Wu G.M., McCauley T.C., Cantley T.C. Prather R.S., Didion B.A. and Day B.N. (2004). Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosal cell. Proliferation and suppression of apoptosis *in vitro*. J. Anim. Sci. 82, 1967-1975.

Matos M.H., Lima-Verde I.B., Bruno J.B., Lopes C.A., Martins F.S., Santos K.D., Rocha R.M., Silva J.R.V., Báo S.N. and Figueiredo. J.R. (2007). Follicle stimulating hormone and fibroblast growth factor-2 interact and promote goat primordial follicle development *in vitro*. Reprod. Fertil. Dev. 19, 677-684.

Nakamura E., Otsuka F., Inagaki K., Miyoshi T., Yamanaka R., Tsukamoto N. Suzuki J., Ogura T. and Makino H. (2010). A novel antagonistic effect of the bone morphogenetic protein system on prolactin actions in regulating steroidogenesis by granulosa cells. Endocrinology 151, 5506-5518.

Nath A., Sharma V., Dubey P.K., Pratheesh M.D., Gade N.E., Saikumar G. and Sharma G.T. (2013). Impact of gonadotropin supplementation on the expression of germ cell marker genes (NLRP5, ZAR1, GDF9, and BMP15) during *in vitro* maturation of buffalo (Bubalus bubalis) oocyte. *In vitro* Cell Dev. Biol. Anim. 49, 34-41.

Nuttinck F., Collette L., Massip A. and Dessy F. (1996). Histologic and autoradiographic study of the *in vitro* effects of FGF-2 and FSH on isolated bovine preantral follicles: preliminary investigation. Theriogenology 45, 1235-1245.

Pennetier S., Uzbekova S., Perreau C., Papillier P., Mermillod P. and

Dalbiès-Tran R. (2004). Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15, and VASA in adult bovine tissues, oocytes, and preimplantation embryos. Biol. Reprod. 71, 1359-1366.

Rebouças E.L., Costa J.J., Passos M.J., Silva A.W., Rossi R.O.D.S. van den Hurk R. and Silva J.R.V. (2014). Expression levels of mRNA for insulin-like growth factors 1 and 2, IGF receptors and IGF binding proteins in *in vivo* and *in vitro* grown bovine follicles. Zygote 22, 521-532.

Ribeiro R.P., Portela A.M., Silva A.W., Costa J.J., Passos J.R., Cunha E.V., Souza G.B., Saraiva M.V., Donato M.A., Peixoto C.A., van den Hurk R. and Silva J.R.V. (2014). Effects of jacalin and follicle-stimulating hormone on *in vitro* goat primordial follicle activation, survival and gene expression. Zygote 23, 537-549.

Richards J.S. (2001). The ovarian follicle - a perspective in 2001. Endocrinology 142, 1-10.

Saha S., Shimizu M., Geshi M. and Izaike Y. (2000). *In vitro* culture of bovine preantral follicles. Anim. Reprod. Sci. 63, 27-39.

Sánchez F., Adriaenssens T., Romero S. and Smitz J. (2009). Quantification of oocyte-specific transcripts in follicle-enclosed oocytes during antral development and maturation *in vitro*. Mol. Hum. Reprod. 15, 539-550.

Sánchez F., Romero S., Albuz F.K. and Smitz J. (2012). *In vitro* follicle growth under non-attachment conditions and decreased FSH levels reduces Lhcgr expression in cumulus cells and promotes oocyte developmental competence. Assist. Reprod. Genet. 29, 141-152.

Saraiva M.V.A., Rossetto R., Brito I.R., Celestino J.J.H., Silva C.M.G., Faustino L.R. Almeida A.P., Bruno J.B., Magalhães M.S., Matos M.H.T., Campello C.C. and Figueiredo J.R. (2010). Dynamic medium produces caprine embryo from preantral follicles grown *in vitro*. Reprod. Sci. 17, 1135-1143.

Selvaraju S., Folger J.K., Gupta P.S., Ireland J.J. and Smith G.W. (2013). Stage-specific expression and effect of bone morphogenetic protein 2 on bovine granulosa cell estradiol production: regulation by cocaine and amphetamine regulated transcript. Domest. Anim. Endocrinol. 44, 115-120.

Shi J., Yoshino O., Osuga Y., Koga K., Hirota Y. Nose, E. Nishii O., Yano T. and Taketani Y. (2011). Bone morphogenetic protein-2 (BMP-2) increases gene expression of FSH receptor and aromatase and decreases gene expression of LH receptor and STAR in human granulosa cells. Am. J. Reprod. Immunol. 65, 421-427.

Silva J.R.V., Lucci C.M., Carvalho F.C.A., Báo S.N., Costa S.H.F., Santos R.R. and Figueiredo J.R. (2000). Effect of coconut water and Braun-Collins solutions at different temperatures and incubation times on the morphology of goat preantral follicles preserved in situ. Theriogenology 54, 809-822.

Silva A.W.B., Bezerra F.T.G., Costa J.J.N., Rossi R.O.D.S., Passos M.J., Vasconcelos G.L. , Rossetto R., Donato M.A.M., Magalhães-Padilha D.M., Campello C.C., Saraiva M.V.A., Figueiredo J.R., Peixoto C.A., Van den Hurk R. and Silva J.R.V. (2014). Differential effects of activin-A and FSH on growth, viability and messenger RNA expression in cultured bovine preantral follicles. Livest. Sci. 160, 199-207.

Souza C.J., Campbell B.K., McNeilly A.S. and Baird D.T. (2002). Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. Reproduction 123, 363-369.

Spicer L.J. and Alpizar E. (1994). Effects of cytokines on FSH-induced estradiol production by bovine granulosa cells *in vitro*: dependence on size of follicle. Domest. Anim. Endocrinol. 11, 25-34.

Tassel R. and Kennedy J.P. (1980). Early follicular development and atretic changes in ovary of the lamb - fine structure and histochemistry. Aust. J. Biol. Sci. 33, 675-687.

Thomas F.H., Walters K.A. and Telfer E.E. (2003). How to make a good oocyte: an update on in-vitro models to study follicle regulation. Hum. Reprod. Update 9, 541-555.

Thomas F.H., Ethier J.F., Shimasaki S. and Vanderhyden B.C. (2005). Follicle stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. Endocrinology 146, 941-949.

Tong Z.B., Gold L., Pfeifer K.E., Dorward H., Lee E. Bondy C.A., Dean J. and Nelson LM. (2000). Mater, a maternal effect gene required for early embryonic development in mice. Nat. Genet. 26, 267-268.

Vasconcelos G.L., Saraiva M.V.A., Costa J.J.N., Passos M.J., Silva A.W.B., Rossi R.O.D.S., Portela A.M., Duarte A.B., Magalhães-Padilha D.M., Campelo C.C., Figueiredo J.R., van den Hurk R. and Silva J.R.V. (2013). Effects of growth differentiation factor-9 and FSH on *in vitro* development, viability and mRNA expression in bovine preantral follicles. Reprod. Fert. Develop. 25, 1194-1203.

Wandji S.A., Srsen V., Voss A.K., Eppig J.J. and Fortune J.E. (1996). Initiation *in vitro* of growth of bovine primordial follicles. Biol. Reprod. 55, 942-948.

Webb R., Nicholas B., Gong J.G., Campbell B.K., Gutierrez C.G., Garverick H.A. and Armstrong D.G. (2003). Mechanisms regulating follicular development and selection of the dominant follicle. Reproduction 61 (Suppl.), 71-90.

Wrana J.L., Attisano L., Wieser R., Ventura F. and Massagué J. (1994). Mechanism of activation of the TGF-β receptor. Nature 370, 341-347.

Wright C.S., Hovatta, O., Margara R., Trew G., Winston R.M.L., Franks S. and Hardy K. (1999). Effects of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles. Hum. Reprod. 14, 1555-1562.

Ying Y. and Zhao G.Q. (2001). Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. Dev. Biol. 15, 484-492.

Yuan W. and Giudice L.C. (1999). Insulin-like growth factor II mediates the steroidogenic and growth promoting actions of follicle stimulating hormone on human preantral follicles cultured *in vitro*. J. Clin. Endocrinol. Metab. 84, 1479-1482.

Zhu G., Cui Y., Wang Q., Kang Y, Yanzhi-Lv Y, Wang J., Song Y. and Cao B. (2013). Bone morphogenetic proteins (BMP) 2, 4, 6 and 7 affect ovarian follicular development through regulation of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) expression in goat granulosa cells. J. Cell Biol. Genet. 3, 14-21.

Accepted October 5, 2015