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Effects of BMP-4 and FSH on growth, morphology and mRNA expression of oocyte-secreted factors in cultured bovine secondary follicles

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Abstract

This study evaluated the effect of different concentrations of bone morphogenetic protein-4 (BMP-4), as well as the interaction of BMP-4 and follicle stimulating hormone (FSH) on growth, ultrastructural integrity, and expression of mRNA for growth differentiation factor-9 (GDF-9), BMP-15, maternal antigen that the embryo requires (Mater) and nucleoplasmin-2 (Npm-2) in bovine secondary follicles cultured *in vitro* for 18 days. Follicles cultured in the presence of 50 ng/ml BMP-4 had a progressive increase in their diameters with the increase of culture period from 0 to 6 and 12 days, but no significant differences were observed among treatments. The presence of both FSH and BMP-4 in a culture medium did not stimulate follicle growth when compared to the control medium. After 12 days, the percentage of normal follicles was maintained similar to that of day 0 in the medium supplemented with both FSH and BMP-4, but no significant differences among treatments were observed after 18 days of culture. BMP-4 maintained the ultrastructural integrity of follicles after 18 days of culture, while follicles cultured in medium supplemented with FSH or both BMP-4 and FSH had oocyte with irregular zona pellucida, vesicular bodies, and an abundance of vacuoles. Follicles cultured in the presence of BMP-4 had an increase in the levels of BMP-15 mRNA, when compared to those cultured in medium supplemented with FSH alone. In conclusion, the addition of BMP-4 in culture medium contributes to preserve follicular ultrastructure, but BMP-4 did not interact positively with FSH. Regarding secondary follicles cultured in the presence of FSH, BMP-4 increases the expression of mRNA for BMP-15.

Keywords: BMP-4, BMP-15, GDF-9, cow, culture, secondary follicles.

Introduction

Oocyte growth and proliferation of granulosa cells are essential events during folliculogenesis. These processes are controlled by interactions between local growth factors and gonadotropins (Artini *et al*., 2007). Among growth factors locally produced in the ovaries of ruminants, BMPs play an important role in the control of primordial germ cell formation, oocyte growth and maturation. BMP family members belong to the transforming growth factor-β (TGF-β) superfamily and act as autocrine and/or paracrine factors that regulate the development of ovarian follicle (Shimasaki *et al*., 2004). In bovine antral follicles, the expression of BMP-4 has been demonstrated in granulosa (Glister *et al*., 2004) and theca cells (Glister *et al*., 2004; Fatehi *et al*., 2005) and oocytes (Fatehi *et al*., 2005), respectively. In mono-ovulatory species the *in vitro* culture of granulosa cells demonstrated that BMP-4 and other members of the BMP family have a major role in modulating proliferative and differentiative responses (Campbell *et al*., 2006). In murine gonadotrope cell lines, BMP-4 interacts with activin-A to modulate FSH secretion (Lee *et al*., 2007; Nicol *et al*., 2008), while in human granulosa-like tumor cell lines, an interrelationship between BMPs and FSH has been described, i.e., FSH stimulates endogenous BMP signaling, while BMPs inhibit FSH receptor expression (Miyoshi *et al*., 2006). This interaction is important for the maintenance and development of granulosa cells. However, the effects of FSH and BMP-4 on cultured bovine secondary follicles, as well as their interplay during their respective actions are still unknown.

During follicle development, expression of oocyte-secreted factors, such as GDF-9 (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). It is well known that preimplantation embryo development is largely dependent on maternal transcripts and proteins synthesized during oogenesis. The maternal antigen that the embryo requires (Mater) is one such oocyte-specific maternal gene and was first identified in mouse (Tong *et al*., 2000). *Mater*-null female mice present normal folliculogenesis, ovulation and fertilization, but their embryos do not develop beyond the 2-cell stage coincident with the maternal-to-embryo transition. The precise Mater function remains to be elucidated, although the global transcription decrease described in two-cell embryos lacking Mater may

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suggest a role in embryonic genome activation (Tong *et al*., 2000). Mater protein expression and intracellular localization throughout folliculogenesis and preimplantation embryo development was already described in the bovine species (Pennetier *et al*., 2006). After fertilization, to ensure formation of a diploid genome, maternal and paternal DNA must undergo remodeling and nucleoplasmin 2 (Npm-2), another oocyte-specific nuclear factor that plays an important role in this process (Burns *et al*., 2003). Despite these factors being considered important markers for oocyte competence, it is unknown if their expression is influenced by BMP-4, FSH or both in the bovine species.

Thus, the aim of this study was to evaluate the effect of different concentrations of BMP-4 and the possible interaction between FSH and BMP-4 on growth, ultrastructural integrity, and mRNA expression of GDF-9, BMP-15, Mater and Npm-2 in cultured bovine secondary follicles.

Materials and Methods

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

Experiment 1: Effect of different concentrations of BMP-4 on in vitro *culture of secondary follicles*

Ovaries (20 pairs) from adult cows 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). Subsequently, ovaries were transported to the laboratory within an hour at 4°C (Chaves *et al*., 2008).

In the laboratory, the ovaries were stripped of surrounding fat tissue and ligaments, and 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 culture (Fig. 1).

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, called α-MEM$^+$, consisted of α-MEM (pH 7.2 - 7.4) supplemented with 3.0 mg/ml bovine serum albumin (BSA), 10 µg/ml insulin, 5.5 µg/ml transferrin and 5 ng/ml selenium (ITS), 2 mm glutamine, 2 mm hypoxanthine and 50 µg/ml of ascorbic acid. The secondary follicles were randomly distributed over the following treatments: α-MEM$^+$ alone (culture control) or supplemented with 10, 50 or 100 ng/ml of recombinant human BMP-4 (Sigma-Aldrich, USA). The follicles were incubated at 38.5°C, with 5% CO_2 in air, for 12 days. On days 2, 4, 6, 8 and 10 of culture, 60 µl of medium was replaced with fresh medium. To evaluate follicle growth, on days 0, 6 and 12, two perpendicular measurements were performed in the normal follicles using an inverted microscope with 200X magnification and Motic Images Plus 2.0 ML software (Motic, Causeway Bay, Hong Kong). In addition, the percentages of follicles that reached antrum formation *in vitro* were determined, and once a translucent cavity was visible between the granulosa cells, the antrum was considered to be formed.

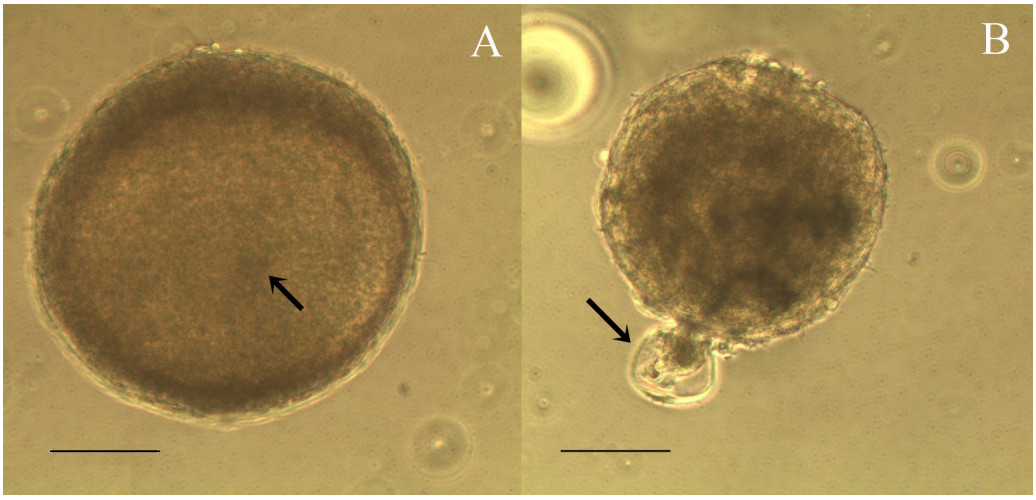

Figure 1. Morphological normal (A) and degenerated follicles (B) after *in vitro* culture. Bar = 100 µm. Arrows point to rupture of basement membrane.

Experiment 2: Assessment of interaction between BMP-4 and FSH on cultured secondary follicles

To investigate a possible interaction between BMP-4 and FSH, the concentration of BMP-4 that had the best results in experiment 1 was used to culture secondary follicles for a longer culture period (18 days) in experiment 2. Ovaries from 20 cows were used to isolate the follicles and, after selection, they 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 the same used in experiment 1. The secondary follicles were randomly distributed over the following treatments: α-MEM^{+} alone (control) or supplemented with 50 ng/ml of recombinant human BMP-4 (Sigma-Aldrich, USA), sequential FSH (FSH from sheep pituitary, Sigma, St. Louis, USA) or both BMP-4 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 (Silva *et al*., 2014). The follicles were incubated at 38.5^{o}C, with 5% CO_2 in air for 18 days. On days 2, 4, 8, 10, 14 and 16 of culture, 60 µl of the medium was replaced with fresh medium, whereas on days 6 and 12, the medium (100 µl) was totally replaced in order to change the concentration of FSH. On days 0, 6, 12 and 18, the normal follicles were measured as described above for experiment 1, and the percentage of follicles that reached antrum formation was determined. At the end of the culture period, for each treatment, 6-8 cultured follicles were collected for real-time PCR, while another 8 follicles were fixed for transmission electron microscopy (TEM), both from four different replicates.

Expression of mRNA in oocytes from secondary follicles grown in vitro

To evaluate the effects of BMP-4, FSH and their combination on mRNA expression of GDF-9, BMP-15, Mater and Npm-2, at the end of the culture period, secondary follicles that showed increase in diameter and normal morphology were mechanically dissociated, and three groups of 6 to 8 denuded oocytes from each treatment were collected and then stored at -80°C until extraction of total RNA. Total RNA extraction was performed using the Trizol purification kit (Invitrogen, Sao Paulo, Brazil). According to the manufacturer's instructions, 800 µl of Trizol solution was added to each frozen sample and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini column. After the binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz units/ml) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 µl RNAse-free water. Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70^{o}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 5X reverse transcriptase buffer (Invitrogen, Sao Paulo, Brazil), 8 units of RNAse out, 150 units of Superscript III reverse transcriptase, 0.036U random primers (Invitrogen, Sao 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 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), 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 GDF-9, BMP-15, Mater, Npm-2 and housekeeping gene GAPDH (Table 1). This housekeeping gene has shown highest stability in bovine follicles (Rebouças *et al*., 2013) and, thus, was used to normalize the 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^{o}C, followed by 50 cycles of 15 sec at 95^{o}C, 30 sec at 58^{o}C, and 30 sec at 72^{o}C. Primer efficiency was determined by using serial dilutions of the target cDNA. The final extension lasted 10 min at 72^{o}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).

Table 1. Primer pairs used in 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	
GDF-9	ACAACACTGTTCGGCTCTTCACCC	S	332 – 356	Gi:51702523
	CCACAACAGTAACACGATCCAGGTT	As	426 – 451	
BMP-15	AAGTGGACACCCTAGGGAAA	S	237 – 257	Gi: 8925958
	TTGGTATGCTACCCGGTTTGGT	AS	362 – 384	
NMP-2	TCTGGACCTGTGTTCCTCTGT	S	374 – 395	Gi:280967451
	ATCGTCGTCGTCATCATCTTC	AS	461 – 481	
MATER	AATGACGACGCTGTGTTCTG	S	3107 – 3127	NM_001007814
	GCGGTTCTCAGGTTCTTCAG	AS	3294 – 3313	

Ultrastructural features of secondary follicles

In order to better examine follicular morphology, TEM was performed to analyze the ultrastructure of bovine secondary follicles that showed an increase in diameter and normal morphology under light microscopy from day 18 of *in vitro* culture. Isolated follicles (n = 5, 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 4 h 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 1 h 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). Afterwards, 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 follicles classified as morphologically normal 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.

Statistical analysis

Data from follicular diameters were submitted to the Kolmogorov-Smirnov test to confirm normal distribution. The data did not show homogeneity of variance and were analyzed by the Kruskal-Wallis non-parametric test. ANOVA and Dunn's multiple comparisons (GraphPad Instat) were used to compare follicle diameters and the levels of messenger RNA for BMP-15, GDF-9, Mater and Npm-2 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-4 on secondary follicles in vitro

The secondary follicles selected for *in vitro* culture had an approximate diameter of 199.3 µm on day 0. After 6 days of *in vitro* culture, different from follicles from control medium alone or from medium with 10 ng/ml of BMP-4, follicles cultured in medium supplemented with 50 ng/ml or 100 ng/ml of BMP-4 had a significant increase ($P < 0.05$) in their diameters when compared to day 0 (Table 2). After 12 days of culture, in all media tested, a significant increase in follicular diameter was observed when compared to day 0. When compared to day 6, follicles cultured in medium containing 50 ng/ml of BMP-4 had a significant increase in diameter ($P < 0.05$) on day 12. However, follicles cultured in control medium alone or supplemented with 10 or 100 ng/ml of BMP-4 had no significant increase in their diameters with the increase of culture period from 6 to 12 days.

Table 2. Follicular diameters of bovine preantral follicles cultured for 0, 6 or 12 days in α-MEM$^+$ alone or supplemented with 0, 10, 50 or 100 ng/ml BMP-4 (means ± SD).

Day of culture	α-MEM$^+$	α-MEM$^+$ + 10ng/ml BMP-4	α-MEM$^+$ + 50ng/ml BMP-4	α-MEM$^+$ + 100ng/ml BMP-4
D0	202.9 ± 4.1^a	203.7 ± 3.9^a	198.6 ± 4.3^a	192.2 ± 4.9^a
D6	215.9 ± 4.4^{ab}	216.8 ± 5.2^{ab}	217.0 ± 5.2^b	209.3 ± 4.6^b
D12	237.6 ± 7.9^b	231.0 ± 5.5^b	238.2 ± 6.8^c	225.0 ± 6.0^b

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

As shown in Table 3, except for follicles cultured in medium containing 100 ng/ml of BMP-4, other treatments maintained the percentages of normal follicles (Fig. 1) after 6 days of culture when compared to day 0. However, when compared to day 0, after 12 days of culture, a reduction in the percentages of normal follicles was observed in all treatments ($P < 0.05$). At the end of the culture period, a few number of follicles had antrum formation (α-MEM$^+$: 0/31, α-MEM$^+$ + 10 ng/ml BMP-4: 2/30, α-MEM$^+$ + 50 ng/ml BMP-4: 1/30, α-MEM$^+$ + 100 ng/ml BMP-4: 0/31) and no significant differences were observed among treatments.

Experiment 2: Effect of BMP-4 and FSH on cultured secondary follicles

A significant increase in follicular diameter was observed in follicles cultured in α-MEM$^+$ alone or supplemented with FSH for 6 days, and for all treatments, a significant increase in follicle diameter from day 6 to day 18 of culture was observed (Table 4). Compared to day 0, after 6 days of culture, a reduction in the percentages of morphologically normal follicles was observed in the control group, but the presence of FSH, BMP-4 or both in culture medium

maintained the percentage of normal follicles similar to day 0. After culturing follicles in medium supplemented with both FSH and BMP-4 for 6 and 12 days, the percentages of normal follicles were significantly higher than those seen in the control medium. After 12 days of culture, the percentage of normal follicles was maintained similar to that of day 0 in medium supplemented with both FSH and BMP-4 (Table 5). No significant differences among treatments were observed after 18 days of culture.

Table 3. Percentages of morphologically normal follicles after culture of bovine preantral follicles in α-MEM$^+$ alone or supplemented with 0, 10, 50 or 100 ng/ml BMP-4 (means ± SD).

Day of culture	α-MEM$^+$	α-MEM$^+$ + 10ng/ml BMP-4	α-MEM$^+$ + 50ng/ml BMP-4	α-MEM$^+$ + 100ng/ml BMP-4
D0	100.0% ^{a} (40/40)	100.0% ^{a} (38/38)	100.0% ^{a} (37/37)	100.0% ^{a} (39/39)
D6	95.0% ^{a} (38/40)	92.1% ^{ab} (35/38)	89.2% ^{ab} (33/37)	82.5% ^{b} (32/39)
D12	77.5% ^{b} (31/40)	78.9% ^{b} (30/38)	81.1% ^{b} (30/37)	79.5% ^{b} (31/39)

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

Table 4. Follicular diameters of bovine preantral follicles after 18-days of culture in α-MEM$^+$ alone or supplemented with 50ng/mL BMP-4, FSH or a mixture of FSH and BMP-4 (means ± SD).

Days of culture	α-MEM$^+$	FSH	BMP-4	BMP-4 + FSH
D0	204.6 ± 23.2^{a}	194.1 ± 14.6^{a}	194.9 ± 20.9^{a}	195.4 ± 19.0^{a}
D6	223.2 ± 23.6^{b}	221.8 ± 37.1^{b}	212.6 ± 24.0^{a}	216.2 ± 30.0^{a}
D12	243.3 ± 27.3^{bc}	268.8 ± 82.3^{bc}	254.9 ± 47.9^{b}	275.4 ± 84.8^{b}
D18	277.3 ± 54.1^{c}	303.2 ± 92.7^{c}	280.7 ± 69.4^{b}	293.9 ± 94.2^{b}

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

Table 5. Percentages of morphologically normal follicles after culture of bovine preantral follicles in α-MEM+ alone or supplemented with 50ng/ml BMP-4, FSH or a mixture of FSH and 50ng/ml BMP-4.

Day of culture	α-MEM$^+$	FSH	BMP-4	BMP-4 + FSH
D0	100%^{Aa} (50/50)	100%^{Aa} (44/44)	100%^{Aa} (48/48)	100%^{Aa} (43/43)
D6	82.0%^{Ab} (41/50)	90.9%^{ABa} (40/44)	93.8%^{ABa} (45/48)	97.7%^{Ba} (42/43)
D12	66.0%^{Ab} (33/50)	72.7%^{ABb} (32/44)	77.1%^{ABb} (37/48)	88.4%^{Ba} (38/43)
D18	60.0%^{Ab} (30/50)	68.2%^{Ab} (30/44)	64.6%^{Ab} (31/48)	69.8%^{Ab} (30/43)

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

Figure 2 shows that the presence of BMP-4, FSH, or both in culture medium did not significantly influence the expression of mRNA for GDF-9 (Fig. 2A), Mater (Fig. 2B) and Npm-2 (Fig. 2D) in oocytes from cultured bovine follicles. In contrast, follicles cultured in the presence of BMP-4 had an increase in the levels of BMP-15 mRNA (Fig. 2C), compared to those cultured in medium supplemented with FSH alone (P < 0.05). At the end of the culture period, a few number of follicles had antrum formation (α-MEM$^+$: 1/30, α-MEM$^+$ + FSH: 3/30, α-MEM$^+$ + BMP-4: 3/31, α-MEM$^+$ + FSH + BMP-4: 4/30) and no significant differences were observed among treatments.

Ultrastructural analysis showed that oocytes from follicles cultured for 18 days in α-MEM$^+$ exhibited vacuolated cytoplasm and no recognizable organelles (Fig. 3A). On the other hand, follicles cultured in medium supplemented with BMP-4 had a well-preserved oocyte, with visible organelles, and normal granulosa cells surrounding the oocyte (Fig. 3B). However, follicles cultured in medium supplemented with FSH (Fig. 3C) or both BMP-4 and FSH (Fig. 3D) had oocyte with irregular zona pellucida, vesicular bodies, and an abundance of vacuoles with an electron dense content. Granulosa cells of follicle culture in the presence of both BMP-4 and FSH had condensed chromatin, but those from follicle culture in the presence of only FSH were well preserved.

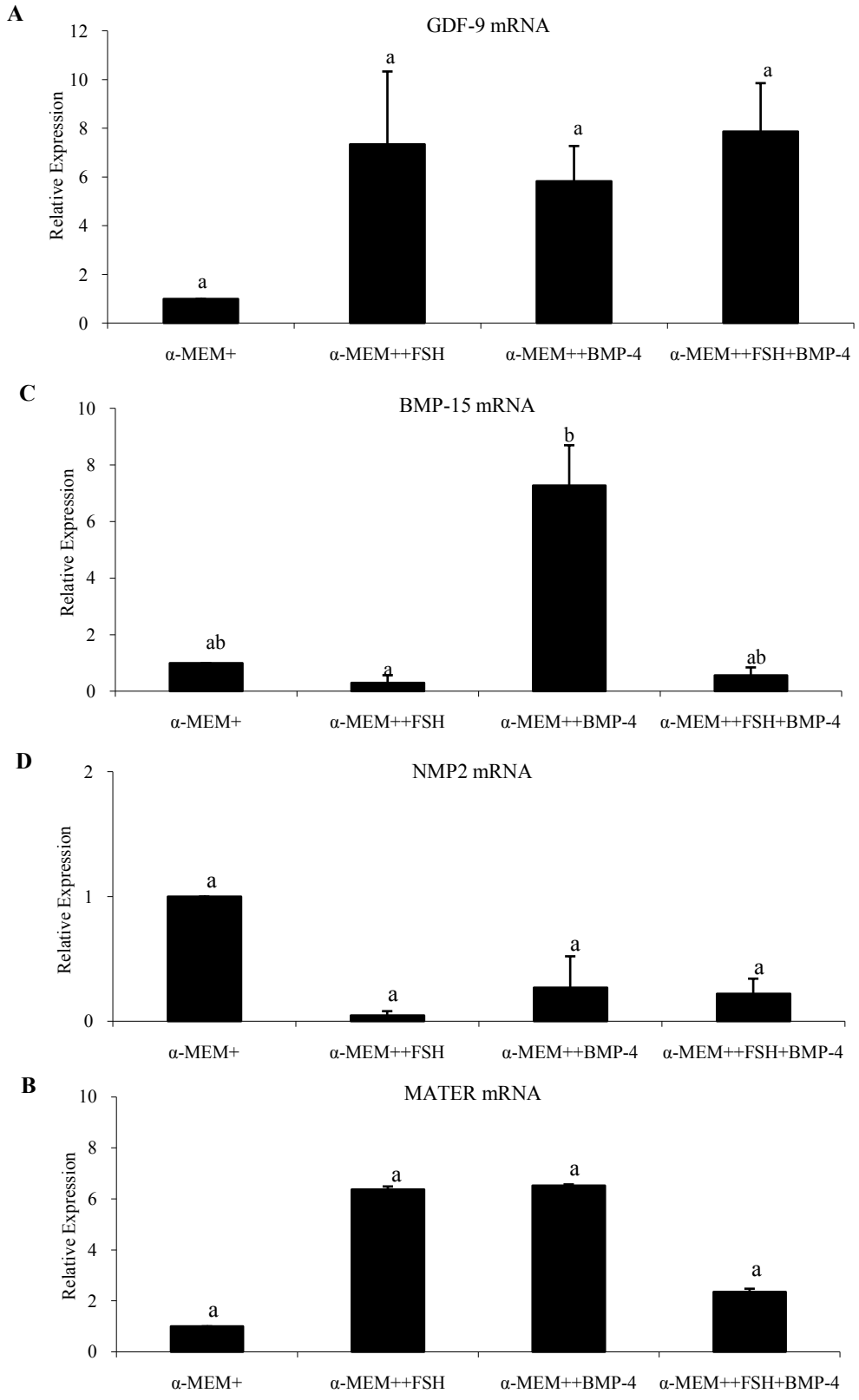

Figure 2. Relative expression of mRNA for GDF-9 (A), Mater (B), BMP-15 (c) and Npm-2 (D) in oocytes cultured for 18 days in medium supplemented with BMP-4, FSH or both. a,b: values without a common superscript significantly differ ($P < 0.05$). α-MEM^{+}: α-MEM (pH 7.2 - 7.4) supplemented with 3.0 mg/ml bovine serum albumin (BSA), 10 µg/ml insulin, 5.5 µg/ml transferrin and 5 ng/ml selenium (ITS), 2 mM glutamine, 2 mM hypoxanthine and 50 µg/ml of ascorbic acid.

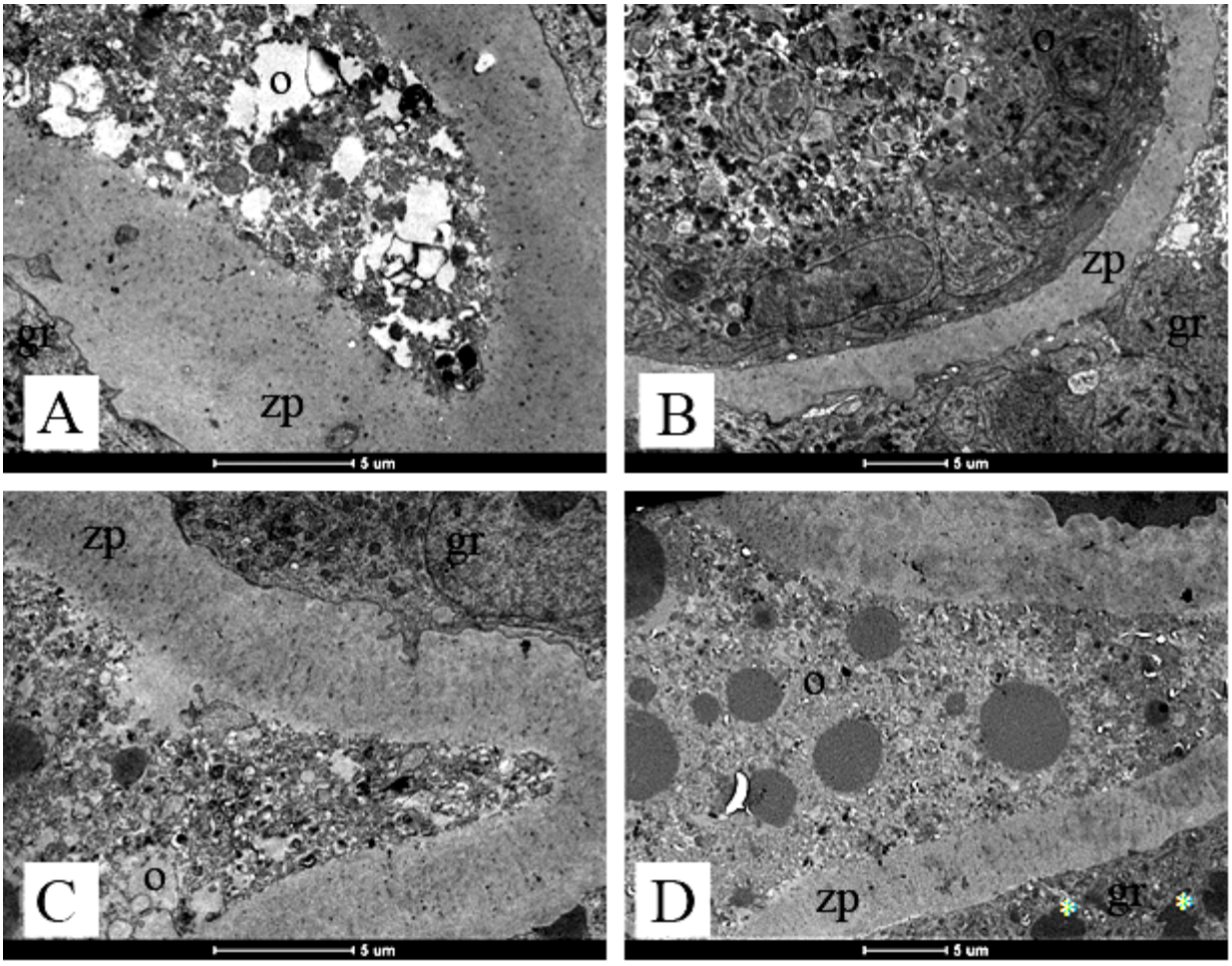

Figure 3. Transmission electron microscopy micrographs of follicles after 18 days of cultured in MEM alone (A) or supplemented with BMP-4 (B), FSH (C) or both BMP-4 and FSH (D). Legend: gr: granulosa cells, zp: zona pelucida, o: oocyte.*: condensed chromatin. Bars: 5 µm

Discussion

This study demonstrates that the presence of BMP-4 at a concentration of 50 ng/ml promotes continuous growth of bovine secondary follicles with the increase of culture period from zero to six and 12 days of culture. It is known that granulosa cell proliferation and antrum formation are important events that take place during the growth of early follicles. Previous studies have reported that BMP-4 stimulates proliferation of granulosa cells *in vitro* (cow: Glister *et al*., 2004, rat: Juengel *et al*., 2006) which can explain the influence of BMP-4 on secondary follicles during culture. During *in vitro* culture, the formation of the antral cavity has a large impact on bovine follicle growth (Vasconcelos *et al*., 2013), but BMP-4 had no effect on antrum formation, which can explain the absence of differences among treatments at the end of culture. The presence of hormones and other growth factors in the culture medium, such as GDF-9 (Vasconcelos *et al*., 2013) is probably required to potentiate antrum formation in cultured bovine secondary follicles. The presence of other substances can also be important for the maintainance of follicle morphology.

After adding either FSH or BMP-4 in culture medium a tendency to improve follicle morphology was seen after 6 and 12 days *in vitro*, but their presence significantly increased the percentages of normal follicles. FSH is considered a critical hormone for the survival of secondary follicles (Xu *et al*., 2010) and its receptor has been observed in granulosa cells of bovine secondary follicles (Wandji *et al*., 1992). In addition, McLaughlin *et al*. (2010) demonstrated that FSH maintained normal oocyte morphology and interactions between the oocyte and granulosa cells after *in vitro* culture of bovine secondary follicles. Regarding BMP-4, previous studies reported that it reduces the levels of apoptosis in granulosa cells cultured *in vitro* (Childs *et al*., 2010) and stimulates the growth of primordial follicles (rat: Nilsson and Skinner, 2003). Kayamori *et al.* (2009) demonstrated that BMP-4 increases the expression of survivin mRNA in cultured granulosa cells and results in the inhibition of cell apoptosis.

Furthermore, BMP-4 is associated with granulosa cell survival via non-Smad specific pathways, such as PI3K/PDK-1/PKC (Shimizu *et al*., 2012), while it inhibits the nuclear transfer of caspase-activated DNase (Kayamori *et al*., 2009).

Evaluation of follicles cultured for 18 days by light microscopy revealed no efects of FSH, BMP-4 or both on follicle morphology, but ultrastructural analysis showed that BMP-4 has a protective effect on bovine secondary follicles cultured *in vitro*. The well preserved ultrastruture of follicles cultured in the presence of BMP-4 was not correlated with increased follicle growth. Previous studies have shown that high rates of follicle growth *in vitro* cannot always be linked with follicle quality, since accelerated growth of bovine preantral follicles after stimulation with both FSH and BMP-15 was associated with ultrastructural changes and increased atresia (Passos *et al*., 2013).

In bovine species, the results obtained with the culture of secondary follicles are extremely discreet, the follicular growth rate is low and antrum formation *in vitro* is a rare event, especially if compared to the results obtained in other domestic ruminants such as sheep and goats (Rossetto *et al*., 2013). It is important to consider that the rate of preantral follicle development is slow, requiring about 300 days for a primordial follicle to grow to antrum formation (Gougeon, 1986). Therefore, the results presented in this study are quite relevant since it shows the involvement of BMP-4 and FSH at specific stages of the early follicular growth *in vitro*, which emphasizes the importance of developing a sequential medium to support the follicular needs according to the stage of development.

Increasing the culture period from 12 to 18 days was not followed by follicle growth, even in the presence of FSH, BMP-4 or both. Thus, the effect of BMP-4 can be associated with initial steps of bovine secondary follicles growth *in vitro*. In addition, antrum formation was not stimulated by FSH and BMP-4 in bovine follicles cultured *in vitro*. Since FSH (Gutierrez *et al*., 1997) and BMP-4 (Kim *et al*., 2013) can also stimulate differentiation of granulosa cells, the addition of these substances for periods longer than 12 days of culture may have contributed to differentiate granulosa cells with consequent reduction in follicular growth via granulosa cell proliferation. Differentiated granulosa cells become more dependent on hormones and growth factors (Khalaf *et al*., 2013). Furthermore, Juengel *et al*. (2006) showed that media composition or length of the culture period may influence the responsiveness of granulosa cells to BMPs.

In the current study, compared to follicles cultured in the presence of FSH, BMP-4 increased the expression of BMP-15 mRNA in cultured bovine secondary follicles, but not that of GDF-9, Mater and Npm-2. It is known that BMP-15 can regulate granulosa cell functions like proliferation, differentiation and cumulus expansion (Eppig, 2001; Richards *et al*., 2002; Gilchrist *et al*., 2004; Hussein *et al*., 2006; Su *et al.,* 2008). In goats, BMP-15 is expressed in the oocyte from the primary follicular stage onwards (Silva *et al.,* 2004). After being synthesized, BMP-15 binds to type I (BMPRIB) and II (BMPRII) receptors on the cell surface (Moore *et al*., 2003). Sanchez *et al*. (2009) have shown a significant down-regulation of BMP-15 and coincident stimulation of differentiation of rat granulosa cells during the transition from secondary to antral follicles (Wu and Matzuk, 2002). BMP-15 specifically interacts with BMP-4, suggesting inhibition upstream of receptor binding (Di Pasquale and Brivanlou, 2009). It has been proposed that the addition of BMP-4 to culture medium promotes a balance between various factors involved in the mechanisms of folliculogenesis (Pierre *et al*., 2004).

In conclusion, the addition of BMP-4 in culture medium contributes to preserve follicular ultrastructure, but BMP-4 did not interact positively with FSH at the end of the culture period. Regarding secondary follicles cultured in presence of FSH, BMP-4 increases the expression of mRNA for BMP-15.

Declaration of interest

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

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