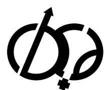



# Effects of BMP-7 and FSH on the development of goat preantral follicles and levels of mRNA for FSH-R, BMP-7 and BMP receptors after *in-vitro* culture

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## Abstract

This study aimed to evaluate the effects of FSH and BMP-7 on growth and on expression of FSH-R, BMP-7 and BMP receptors in cultured secondary follicles. Goat secondary follicles (~200 µm) were isolated and cultured *in vitro,* with 5% $CO_2$ in air at 39°C, for 6 days in the presence of BMP-7 (50 ng/ml) supplemented or not with FSH (50 ng/ml). Follicular diameter and the formation of the antrum were evaluated before and after culture. For each treatment, at the end of culture period, groups of 6 follicles were collected and, after extraction of total RNA and cDNA synthesis, the levels of mRNA for FSH-R, BMP-7 and BMP receptors in cultured secondary follicles were quantified by real time PCR. The results showed that addition of BMP-7 or FSH to culture medium stimulated growth of secondary follicles, while addition of both BMP-7 and FSH was needed to significantly increase the percentage of follicles forming an antrum and the follicular levels of mRNA for both BMP-7 and FSH-R. For BMP receptors, FSH reduced the levels of mRNA for BMPR-IA and BMP-RII in comparison with those follicles cultured in MEM alone and supplemented with BMP-7, respectively. In conclusion, like FSH, BMP-7 affects *in vitro* growth of cultured secondary follicles, but it stimulates antrum formation and expression of the mRNA's for BMP-7 and FSH-R only in presence of both BMP-7 and FSH. However, the levels of mRNA for BMP-RIA and BMP-RII are reduced in follicles cultured in medium supplemented with FSH.

**Keywords:** BMP receptors, goats, BMP-7, FSH, secondary follicles.

## Introduction

Folliculogenesis is the process by which primordial follicles grow and develop into the ovulatory follicle. Through this process, a healthy oocyte is usually selected for maturation. Evidence indicates that bone morphogenetic proteins (BMPs), members of the superfamily of transforming growth factor-β (TGF-β), play a key role in ovarian steroidogenesis and folliculogenesis in mammals (Shimasaki *et al*., 1999, 2003, 2004; Knight and Glister, 2006).

In regard to BMP-7 (also known as osteogenic protein-1), Lee *et al.* (2004) showed that this growth factor is produced by theca cells of mouse secondary and antral follicles and stimulates, in absence of FSH, the activation and growth of primordial follicles as well as the mRNA expression of receptors for FSH (FSH-R) in developing follicles during their 4-days culture within neonatal mouse ovaries. In addition, Miyoshi *et al*. (2006) found that, in cultured rat granulosa cells, BMP-7 increased FSH-induced estradiol synthesis, the response being further augmented when oocytes were co-cultured. In these latter experiments, BMP-7 and BMP-6 both inhibited FSH- and forskolin-induced progesterone biosynthesis and reduced cAMP formation independent of the presence or absence of oocytes. In ovine follicles, BMP-7 was detected in all cellular compartments (Juengel *et al.*, 2006) and, like BMP-2, -4 and -6, inhibited progesterone production from 6-days cultured granulosa cells and stimulated cellular granulosa cell proliferation. In human fetal follicles, the mRNA's of BMP-7 and their receptors BMPR-IA, BMPR-IB and BMPR-II were expressed in granulosa cells, oogonia and oocytes (Abir *et al.*, 2008). In follicles of adult women, however, mRNA expression for BMP-7 was restricted to granulosa cells, while BMPR-IA mRNA was detected in both granulosa cells and oocytes, BMPR-IB mRNA in oocytes, and BMPR-II mRNA expression was absent. Recently, Shi *et al.* (2010) demonstrated that BMP-7 increased expression of FSH-R in cultured human granulosa cells. Although the mRNA's of all three types of BMP receptors are expressed in all classes of caprine follicles (Silva *et al.*, 2004), and BMP-7 is found in theca cell of antral follicles (bovine: Glister *et al*., 2010, caprine: Frota *et al*., 2008), effects of BMP-7 associated or not with FSH on both ovarian follicle development and expression of FSH-R, BMP-7 and BMP receptors in goats have thus far not been explored. The present study aimed (1) to investigate, in presence and absence of FSH, a possible influence of BMP-7 on growth and antrum formation of *in vitro* cultured secondary follicles, and (2) to evaluate

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in these follicles the effects of FSH and BMP-7 on mRNA levels for FSH-R, BMP-7 and BMP receptors IA, IB and II.

## Materials and Methods

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

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

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

For *in vitro* studies, after selection, secondary follicles of approximately 200 µm were individually cultured in 100 µl drops of culture medium in petri dishes (60 x 15 mm, Corning, USA). Control culture medium consisted of α-MEM (pH 7.2 - 7.4) supplemented with 3 mg/ml bovine serum albumin (BSA), insulin 6.25 µg/ml, transferrin 6.25 µg/ml, selenium 6.25 ng/ml (ITS), 2 mM glutamine, 2 mM hypoxantine and 50 µg/ml of ascorbic acid under mineral oil. Incubation was conducted at 39°C for 6 days. For treatments, control culture medium was supplemented with 50 ng/ml of FSH (rFSH®, Nanocore, Brazil), 50 ng/ml of BMP-7 (R & D Systems Europe, Abingdon, Oxon, UK) or both. These concentrations of FSH and BMP-7 were those that promoted the highest *in vitro* growth rates of preantral follicles in previous studies (Glister *et al.*, 2004; Matos *et al.*, 2007). For culture, secondary follicles were randomly chosen and incubated for 6 days in the incubator with 5% $CO_2$ in air at 39°C. Every other day, 5 µl of the culture media were added to the drops. Cultures were replicated four times, and a mean number of 25 follicles were used per treatment. The morphology and follicular diameter were assessed at the beginning and end of cultivation with the aid of an inverted microscope. In addition, the percentage of secondary follicles that reached the antrum formation *in vitro* was determined.

To evaluate the effect of FSH and BMP-7 on expression of mRNA for FSH-R, BMP-7 and BMP receptors after a 6-day culture period, for each treatment, groups of 6 follicles were collected at the end of culture period and stored at -80°C until extraction of total RNA.

*Messenger RNA quantification for FSH-R, BMP-7 and BMP receptors in cultured follicles*

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

Prior to reverse transcription, the eluted RNA samples were incubated for 5 min at 70°C and chilled on ice. Reverse transcription was then performed in a total volume of 20 µl, which was comprised of 10 µl of sample RNA, 4 µl 5X reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNAseout, 150 units Superscript III reverse transcriptase, 0.036 U random primers (Invitrogen, São Paulo, Brazil), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 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 the mRNA for FSH-R, BMP-7 and BMP receptors 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 thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 45 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real time PCR Mastercycler (Eppendorf, Germany). Primers for FSH-R, BMP-7, BMP receptors and housekeeping genes (β-actin and ubiquitin) are shown in Table 1. The choice for the housekeeping genes was based on previous findings with fresh and cultured caprine follicles (Frota *et al.*, 2011). The delta-delta-CT method was used to transform CT values into normalized relative expression levels. The normalization of data was based on two reference genes (beta actin and

ubiquitin), and such genes were used to determine a normalization factor, that is basically the geometric mean of the transcription levels of these reference genes (Vandesompele *et al*., 2002).

Table 1. Primer pairs which have been used for housekeeping genes, FSH-R, BMP-7 and BMP receptors.

| Target gene | Primer sequence (5´→3´) | Sense (s)<br>Anti-sense (as) | Position | Genbank accession nº |
|---|---|---|---|---|
| β- ACTIN | ACCACTGGCATTGTCATGGACTCT | s | 188-211 | GI:28628620 |
| | TCCTTGATGTCACGGACGATTTCC | as | 363-386 | |
| UBQ | GAAGATGGCCGCACTCTTCTGAT | s | 607-631 | GI:57163956 |
| | ATCCTGGATCTTGGCCTTCACGTT | as | 756-780 | |
| BMP-7 | AGGCAGGCATGTAAGAAGCA | s | 78-98 | AF:508311 |
| | TTGGTGGCGTTCATGTAGGA | as | 203-223 | |
| BMPR-IA | ACGTTTGCGGCCAATTGTGT | s | 1664-1685 | GI:116003816 |
| | TTGTGAGCCCAGCATTCTGACA | as | 1753-1774 | |
| BMPR-IB | TTTGGATGGGAAAGTGGCGT | s | 653-672 | GI:297471905 |
| | TGCAGCAATGAAGCCCAAGA | as | 792-811 | |
| BMPR-II | TGTGCCAAAGATTGGCCCTT | s | 1602-1621 | GI:297471905 |
| | TGCTTGCTGCCGTTCATAGT | as | 1776-1795 | |
| FSH-R | AGGCAAATGTGTTCTCCAACCTGC | a | 250-274 | GI:95768228 |
| | TGGAAGGCATCAGGGTCGATGTAT | as | 316-340 | |

*Statistical analyses*

The nonparametric Kruskal-Wallis test was used to compare follicle diameters and mRNA levels for FSH-R, BMP-7 and BMP receptors, while the chi-square test was applied to compare percentages of follicles that reached antrum formation ($P < 0.05$).

## Results

Cultured secondary follicles had significantly larger diameters than uncultured (Day 0) follicles (Table 2). When follicle diameters were compared among treatments, the results showed that, compared to culture in MEM, addition of BMP-7, FSH or a blend of BMP-7 and FSH increased ($P < 0.05$) follicular growth. Besides, administration of the latter mixture resulted in an increase ($P < 0.05$) in the percentage of follicles that developed an antrum, whereas, no such effect was observed by the individual compounds (Table 2). Likewise, the levels for FSH-R mRNA (Fig. 1A) and BMP-7 mRNA (Fig. 1B) were increased ($P < 0.05$) in cultured follicles, when BMP-7 and FSH were both supplemented to the culture medium, but not when only BMP-7 or FSH was added. For BMP receptors, a reduction ($P < 0.05$) in the levels of mRNA for BMPR-II was observed in follicles cultured in the presence of FSH, when compared with those cultured in medium supplemented with BMP-7 (Fig. 1C). In addition, FSH reduced ($P < 0.05$) the levels of mRNA for BMPR-IA in comparison with those follicles cultured in MEM alone (Fig. 1D). No differences ($P > 0.05$) were observed in the levels of mRNA for BMPR-IB among follicles cultured in the different treatments (Fig. 1E).

Table 2. Diameters (mean ± SEM) of and antrum formation (%) in caprine secondary follicles, which have been cultured in α-MEM and α-MEM supplemented with BMP-7 and/or FSH.

| Treatments | Day 0 | Day 6 | Growth | Antrum formation |
|---|---|---|---|---|
| MEM | 200.2 ± 8.3 | 265.1 ± 15.6* | $64.9 \pm 8.7^{a}$ | $32.0\%$ (8/25)$^{a}$ |
| MEM + FSH | 227.2 ± 6.6 | 354.4 ± 14.0* | $127.2 \pm 9.5^{b}$ | $53.3\%$ (16/30)$^{ab}$ |
| BMP-7 | 258.2 ± 12.5 | 371.3 ± 20.3* | $109.0 \pm 12.8^{b}$ | $45.9\%$ (11/24)$^{ab}$ |
| BMP-7 + FSH | 265.7 ± 14.3 | 389.4 ± 20.5* | $123.7 \pm 10.7^{b}$ | $76.0\%$ (19/25)$^{b}$ |

*significant difference compared to day 0 ($P < 0.05$). [a,b]significant difference between treatments ($P < 0.05$).

Figure 1. Steady state level of mRNA for FSH-R (A), BMP-7 (B), BMPR-II (C), BMPR-IA (D), and BMPR-IB (E) in caprine secondary follicles cultured for 6 days in α-MEM supplemented with FSH, BMP-7 or both compounds. Geometric means of transcripts for β-actin and ubiquitin were used to normalize data. [a,b]Significant difference among treatments ($P < 0.05$).

## Discussion

This study shows for the first time that, when added for 6 days together with FSH, to *in vitro* cultures containing caprine secondary follicles, BMP-7 stimulates both their growth and antrum formation. Growth of these secondary follicles appears to be due to separate effects of BMP-7 and FSH, while antrum formation seems to be a result of an interaction between FSH and BMP-7. Upregulation of mRNA for FSH-R in

follicles cultured in the presence of both BMP-7 and FSH can be related to increased antrum formation. However, the mechanisms underlying the interactions between BMP-7 and FSH to stimulate antrum formation are not completely clear, since a reduction of mRNA for BMP-R was observed after FSH stimulation. Given that SMAD proteins and cyclic AMP, respectively, mediate BMP (Shimasaki *et al*., 2004) and FSH (Richards, 2001) signaling, it is likely that the differential regulation might involve crosstalk between these key pathways. Never before has BMP-7 been associated with antrum formation, but FSH was well related to this process in mouse (Hartshorne, 1997) and porcine (Mao *et al*., 2002) follicles. Furthermore, LH (mouse: Cortvrindt *et al*., 1998), epidermal growth factor (cow: Gutierrez *et al*., 2000), kit ligand (mouse: Driancourt *et al*., 2000) and activin (rat: Zhao *et al*., 2001) were found to stimulate antrum formation in mammals. Factors like vascular endothelial growth factor, insulin-like growth factor I, substance p, nitric oxide, prostacyclin, endothelin and histamine have been shown to affect permeability of capillaries (for review, see Rodgers and Irving-Rodgers, 2010) which, under *in vivo* circumstances, may influence antrum formation in follicles.

The presently demonstrated BMP-7-stimulated secondary follicle growth is probably due to granulosa cell proliferation, since this effect has been previously shown for rat (Lee *et al*., 2001; Juengel *et al*., 2006), bovine (Glister *et al*., 2004) and chicken (Onagbesan *et al*., 2003) cultured granulosa cells, which had been isolated from antral follicles. Possibly, granulosa cell proliferation is also a source of FSH-induced follicle growth. However, this gonadotropin is not widely known as an inducer of granulosa cell proliferation in early-staged follicles. FSH is more believed to act as a survival factor for early-staged follicles (Hulshof *et al*., 1995; van den Hurk and Zhao, 2005). Maybe, FSH-stimulated follicle growth is due to stimulated differentiation processes within granulosa cells that lead to their expansion. Similar results to ours were observed by Lee *et al*. (2004), which cultivated mouse ovaries with BMP-7 and maintained follicular viability up to day 4 of culture. In addition, Huang *et al*. (2001) showed that BMP-7 increased the secretion of FSH in the pituitary cells.

Apart from its effects on follicle growth, BMP-7 currently also increases the mRNA levels for BMP-7 and FSH-R in cultured secondary follicles, but only in the presence of FSH. Likewise, interaction between BMP-7 and FSH and a modulating effect of BMP-7 on FSH receptors were demonstrated in cultured rat granulosa cells. In these cells, BMP-7 enhanced and attenuated the stimulatory action of FSH on estradiol and progesterone production, respectively, and increased the sensitivity to FSH (Shimasaki *et al*., 1999). Also in cultured human granulosa cells (Shi *et al*., 2010), BMP-7 increased expression of FSH-R, when medium containing fetal bovine serum was used. Increased estradiol production is essential for appropriate development of follicles up to the preovulatory stage, while inhibited progesterone formation is important in blocking premature oocyte maturation and subsequent ovulation during this process (for review, see van den Hurk and Zhao, 2005). The presently obtained PCR and *in vitro* culture data point to a role of antral follicle-derived BMP-7 in the growth and differentiation of goat secondary and antral follicles. Recently, our group additionally found that BMP-7 facilitates the transition of cultured goat primordial follicles into primary follicles (Araújo *et al*., 2010).

The BMP-7 effects on caprine early follicles seem to be interfollicularly exerted, since only late secondary and antral follicles are able to produce this growth factor. The effects are brought about through binding of BMP-7 to their receptor complex, of which the components (BMPR-IA, BMPR-IB and BMPR-II) were previously found to be expressed in secondary follicles and all other classes of goat follicles (Silva *et al*., 2004). In comparison, in situ hybridization did identify mRNA labeling for BMP-R types IA, IB, and II in the granulosa cells and oocytes of most follicles in ovaries of normal cycling rats, which dynamically changed during follicular development (Shimasaki *et al*., 1999; Erickson and Shimasaki, 2003). BMP-R proteins were immunocytochemically demonstrated in ovine (Souza *et al*., 2002), bovine (Glister *et al*., 2004) and human (Abir *et al*., 2008) ovarian follicles, in a cell-specific manner. After culturing goat secondary follicles, FSH reduced the levels of mRNA for BMPR-II and BMPR-IA. In a recent study, a reduction of BMP-RIA was also observed after culture of goat secondary follicles for 18 days in medium containing increasing concentrations of FSH (Costa *et al*., 2011; Federal University of Ceara, Sobral, CE, Brazil; personal communication). Probably, the BMP-7-stimulated granulosa cell proliferation was reduced in the presence of FSH, which can explain why these compounds did not interact to stimulate secondary follicle growth, but only antrum formation. Wang and Roy (2009) demonstrated variation in the levels of BMPR-II and BMPR-IB in ovarian cells after FSH stimulus, showing that FSH differentially regulates the expression of BMP receptors in hamster postnatal ovary.

In conclusion, like FSH, BMP-7 promotes growth of goat cultured secondary follicles and, together with FSH, it stimulates antrum formation and increases the levels of mRNA for BMP-7 and FSH-R. Conversely, FSH alone reduces expression of mRNA for BMP-RIA and BMP-RII after 6 days of culture. The present findings indicate an interfollicular role of BMP-7 from antral follicles on growth and differentiation of secondary follicles, the latter effect requiring interaction with FSH.

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