

## Short Term Organ Culture of Mouse Ovary in the Medium Supplemented with Bone Morphogenetic Protein 15 and Follicle Stimulating Hormone: A Morphological, Hormonal and Molecular Study

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### Abstract

**Background:** Bone morphogenetic protein 15 (BMP15) is a growth factor derived from oocyte and is essential for *in vivo* ovarian follicular growth and in this study, its effects on the improvement of growth and development of follicles during *in vitro* culture of neonatal mouse ovaries was investigated.

**Methods:** Two week old mice were cultured for 7 days in the basic culture media with or without follicle stimulating hormone (FSH) and BMP15 as four experimental groups; FSH<sup>-</sup>/BMP15<sup>-</sup>, FSH<sup>+</sup>/BMP15<sup>-</sup>, FSH<sup>-</sup>/BMP15<sup>+</sup> and FSH<sup>+</sup>/BMP15<sup>+</sup>. The ovarian follicles at different developmental stages in paraffin embedding sections of cultured and non-cultured ovaries were counted and compared. The 17- $\beta$  estradiol (E2) and progesterone (P4) levels were analyzed in collected culture media. The expression ratio of developmental genes (PCNA, BMPR-IB, BMPR-II, FSH-R, CYP17 and ZP3) to housekeeping gene (GAPDH) was analyzed by real time PCR (RT-PCR) in comparison with non-cultured control ovaries. The data was compared by independent t-test and one-way ANOVA (with Tukey's Post Hoc test). The  $p < 0.05$  was considered significant.

**Results:** The percentage of antral follicles, ovarian size, concentration of E2 and P4 and the expression ratio of PCNA and ZP3 genes in the ovaries cultured in medium supplemented with BMP15 and FSH increased significantly in comparison with other cultured groups ( $p < 0.05$ ). The BMPR-IB, BMPR-II and FSH-R mRNA level was significantly lower ( $p < 0.05$ ) and CYP 17 mRNA level did not change in the FSH<sup>+</sup>/BMP15<sup>+</sup> group than other cultured groups.

**Conclusion:** This study demonstrated a favorable effect of BMP15 in combination with FSH on *in vitro* development of small size mouse follicles to antral stage.

**Keywords:** Bone morphogenetic protein 15, Follicle Stimulating Hormone, Gene expression, Organ culture, Ovary.

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### Introduction

Oocyte through the secretion of autocrine and paracrine factors plays an important role in the regulation of follicular development. Growth differentiation factor 9 and bone morphogenetic protein 15 (BMP15) are two oocyte-specific proteins and co-expressed by growing oocytes in rodents, sheep, and humans (1-3). These proteins

are two members of the TGF- $\beta$  superfamily and play fundamental roles in steroidogenesis of granulosa cells, oocyte maturation and ovulation, and embryo development in different species of mammals (4).

Several forms of BMP15 protein were secreted by the oocyte (1). BMP15 controls many aspects

of follicular development by binding to its two receptors including serine–threonine kinase type I (BMPR-IB) and type II (BMPR-II) on the surface of granulosa cells (5, 6). BMPR has been identified in primordial follicles of various mammalian species (6, 7). Moreover, the expression of BMPR is detected during *in vitro* culture of ovine cortical slices (8).

BMP15 may activate different signaling pathways during follicular development (9). It is considered as a proliferative factor in the early stages of follicular development and acts as mitogenic factors that stimulate granulosa cell growth and proliferation.

It also induces cumulus cell expansion from the primary stage to the follicle stimulating hormone (FSH) dependent stage by enhancing the expression of epidermal growth factor-like growth factor (3, 9, 10). From another point of view, BMP15 could act as a maturation factor in the later stages of follicular development even preovulatory stage (4, 11, 12).

BMP15 has been shown to regulate granulosa cell differentiation (13) and it is also involved in the selection of dominant follicle. Otsuka et al.'s (14) studies revealed that BMP15 stimulated proliferation of undifferentiated rat granulosa cells in an FSH-independent manner (14).

However, BMP15 is a survival factor and has roles in the regulation of cumulus cell apoptosis (15). Hussein et al. (16) showed the induction of cumulus cell apoptosis could be prevented by treatment with BMP15. Moreover, the BMP15 level in follicular fluid appears to be a good potential factor in predicting oocyte quality and subsequent embryo development (17, 18).

In spite of some investigations regarding the effect of BMP15 on *in vitro* development of follicles (19, 20), according to our knowledge, there was poor information about the effects of BMP15 alone or in combination with FSH on the expression of genes related to the maturation of oocytes and follicular cells including follicle stimulating hormone receptor (FSHR), cytochrome P450 family 17 (CYP17), proliferating cell nuclear antigen (PCNA), and zona pellucida glycoprotein 3 (Zp3) genes.

Thus, the objectives of the present study were to evaluate firstly the effects of BMP15 supplementation to the culture media of mouse ovaries alone or in combination with FSH on the growth and development of follicles. Secondly, to investigate their effects on the expression of developmental

genes including PCNA, BMPR-IB, BMPR-II, FSH-R, CYP17 and ZP3 by real-time RT-PCR.

### Methods

Reagents and materials of this research were obtained from Sigma-Aldrich (Germany) except mentioned otherwise.

**Animals:** 14 (n=35) and 21 (n=7) day old National Medical Research Institute (NMRI) female mice were cared according to the guide for the care and use of laboratory animals of Tarbiat Mo-dares University and housed under a 12 *hr* light: 12 *hr* dark at 20-25°C with enough humidity, water and food.

**Experimental Design:** The effects of BMP15 and FSH supplementation on the *in vitro* follicular development of two week old mouse ovaries were evaluated at the morphological and mRNA levels in comparison with non-cultured 14 and 21 day mouse ovaries. The function of cultured ovaries was evaluated by the level of 17-β estradiol (E2) and progesterone (P4).

The ovaries (n=70) from 14 day female mice (n=35) were collected in α- minimal essential medium (α-MEM; Gibco, UK) and cleaned from surrounding tissues. Some of these ovaries were considered for culturing in 4 experimental cultured groups as following (n=14 ovaries in each experimental group); the basic culture media without FSH and BMP15 (FSH<sup>-</sup>/BMP15<sup>-</sup>), the basic culture media with FSH and without BMP15 (FSH<sup>+</sup>/BMP15<sup>-</sup>), the basic culture media without FSH and with BMP15 (FSH<sup>-</sup>/BMP15<sup>+</sup>) and the basic culture media with FSH and BMP15 (FSH<sup>+</sup>/BMP15<sup>+</sup>).

Some of the two (n=14) and three (n=14) week old mouse ovaries were considered as non-cultured control group for light microscopic and molecular studies.

**Organ Culture:** The ovaries in each group (n=56 in total in at least 7 repeats) put individually on culture inserts (Millicell-CM, 0.4 μm pore size; Millipore Corp, Billerica, MA) in 24-well plates with basic culture medium consisted of α- MEM medium supplemented with 1% insulin, transferin, and selenium (ITS; Gibco, UK), 10% fetal bovine serum (FBS). In FSH supplemented groups, 100 *mIU/ml* recombinant follicle stimulating hormone (rFSH or Gonal-f; Serono, Switzerland) and in BMP15 treated groups, 10 *ng/ml* BMP15 were added to the basic culture media. Samples were cultured in a humidified incubator with 5% CO<sub>2</sub> at

37°C for 7 days. The culture media were replaced with fresh media every 2 days. At the end of culture period, some of the ovaries in each group (n=5 in each group) were fixed in Bouin's solution for light microscopic study and the others (n=9 in each group) were stored at -80°C for molecular studies.

**Light microscopy:** The fixed ovaries from cultured (n=5 in each group) and non-cultured groups (n=5 for 14 day mice and n=5 for 21 day mice) by Bouin's solution were dehydrated in a graded series of ethanol and cleared with xylol and finally embedded in paraffin wax. Tissue sections at 5  $\mu$ m thickness were serially sectioned and every 5th section of each ovary was mounted on a glass slide and stained with hematoxylin and eosin (H&E). The number of follicles within all stained sections was counted under light microscope at a magnification of  $\times$ 400.

The primordial follicles were defined as an oocyte encapsulated by squamose pregranulosa cells, primary follicles as those with one layer of cuboidal granulosa cells and preantral follicles as those with two and more layers of cuboidal granulosa cells. To avoid calculating a follicle more than once, only those with visible nucleus in the oocyte were counted.

**Ovarian surface area:** The ovarian surface area in all experimental groups on the beginning and end of culture period was estimated (n=5 in each group) using an inverted microscope and the photos of each ovary were prepared and imported into Image J software (National Institutes of Health, Bethesda). The surface area of each ovary was measured in units of pixels and converted to millimeters based on the conversion determined by measuring the image of the calibrated millimeter.

**Hormonal assay:** The concentration of E2 and P4 were measured in the collected media derived from cultured ovaries on days 2 and 7 of culture period (n=5 in each group). The media were stored at -20°C until hormonal assay. The concentration of hormones in medium was measured using enzyme-linked immunosorbent assay (ELISA) method (Diaplus, USA).

**Real-time RT-PCR:** Evaluation of PCNA, BMPR-IB, BMPR-II, FSH-R, CYP17 and ZP3 mRNA in two cultured groups including FSH<sup>+</sup>/BMP15<sup>+</sup> and FSH<sup>+</sup>/BMP15<sup>-</sup> (which has higher percentage of growing follicles) and non-cultured ovaries as control (14 and 21 day mouse ovaries) were done by real-time RT-PCR (at least 3 repeats). The ovaries from these groups were collected and pooled separately (3 ovaries for each replicate of experiments; n=9 total ovaries in each group).

**RNA isolation and reverse transcription reaction:** The RNA was extracted from the mouse ovaries using the RNX plus (Cinna Gen Co. Iran). Then, RNA samples were treated with DNase to eliminate any genomic DNA contamination just prior to proceeding with cDNA synthesis. The RNA concentration was determined by spectrophotometry, and the RNA samples were stored at -80°C until use. The cDNA was synthesized in a total volume of 20  $\mu$ l containing 5  $\mu$ g total RNA with reverse transcriptase using the cDNA kit (Fermentas, EU). All experiments were carried out in triplicate.

**Quantitative real-time RT-PCR assays:** The primers for real-time RT-PCR were designed using GenBank (<http://www.ncbi.nlm.nih.gov>) and Allele ID software (Table 1) and ordered and synthesized by CinnaGen Co. (Tehran, Iran). The house-keeping gene, GAPDH internal control. The PCR

**Table 1.** The characteristic of primers used for real-time RT-PCR assays

Target gene	Primer pair sequence (5'-3')	Accession number	Fragment size (bp)
GAPDH	GGAAAAGAGCCTAGGGCAT CTGCCTGACGGCCAGG	NM-007393	64
PCNA	AGGAGGCGGTAACCATAG ACTCTACAACAAGGGGCACATC	NM-011045	76
FSH-R	CCAGGCTGAGTCGTAGCATC GGCGGCAAACCTCTGAACT	NM-013523.3	79
CYP17	CGTCTGGGGAGAAACGGT CGTCAAAGACACCTGATGCCAAG	NM-007809.3	82
BMPR-IB	AAAGGTCGCTATGGGGAAGT GCAGCAATGAAACCCAAAAT	NM-007560.3	158
BMPR-II	GGCGACTATCAAGACACCTAAC CCAGACACAACACCACACAGT	NM-007561.3	102
ZP3	ACACGGTTAGTGCTGTGGATG GAGATGACGAAAGATGCCCTG	NM-011776.1	92

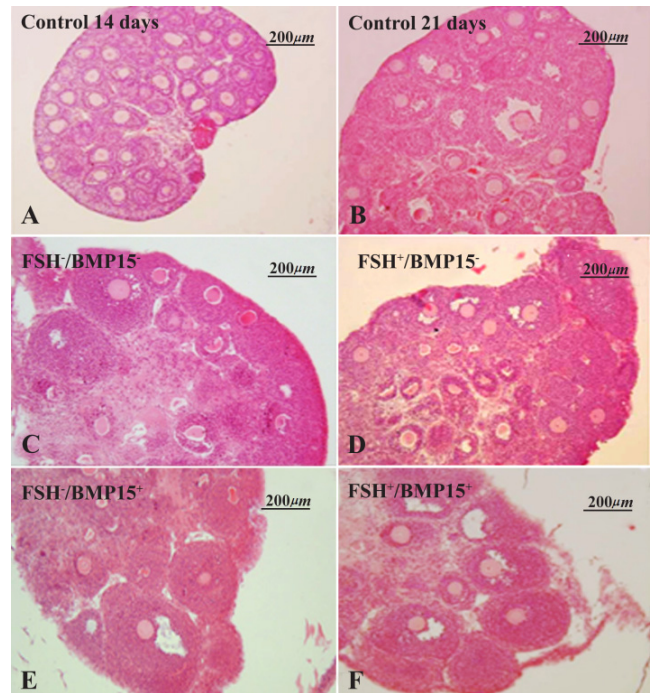
reactions were carried out in a 48-well plate with 20  $\mu$ l reaction volume consisting of 10  $\mu$ l 2 $\times$  SYBR Green RT-PCR Master Mix (Applied Biosystems, UK) with forward and reverse primers and diluted RT products. The mRNA level of PCNA, BMPR-IB, BMPR-II, FSH-R, CYP17 and ZP3 in ovaries was quantified using the ABI 7500 Sequence Detector (Applied Biosystems, UK) according to the manufacturer's instructions. The PCR protocol included an initial denaturation at 95°C for 5 min, followed by 40 cycles consisting of denaturation at 95°C for 15 s. Annealing was carried out at 60°C for target genes for 30 s, then extension at 72°C for 30 s. At the end of amplification cycles, melting temperature analysis was carried out at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Amplification was repeated on cDNA for at least three independent isolates of ovaries mRNA to insure reliability of the data. After completing the PCR run, melt curve analysis was used to confirm the amplified product. For each sample, the reference gene and the target genes were amplified in the same run. Then, relative quantization of target genes was determined using the Pfaffl method.

**Statistical analysis:** Statistical analysis was carried out with SPSS software. The normality and developmental rates of follicles in all groups of study were assessed by one way ANOVA and Tukey's HSD was used as post hoc tests. The results of E2 and P4 measurement were compared by independent t-test between the groups. The data of real-time RT-PCR between the groups were analyzed by one-way ANOVA and post-hoc Tukey's tests. A p-value of less than 0.05 was considered statistically significant.

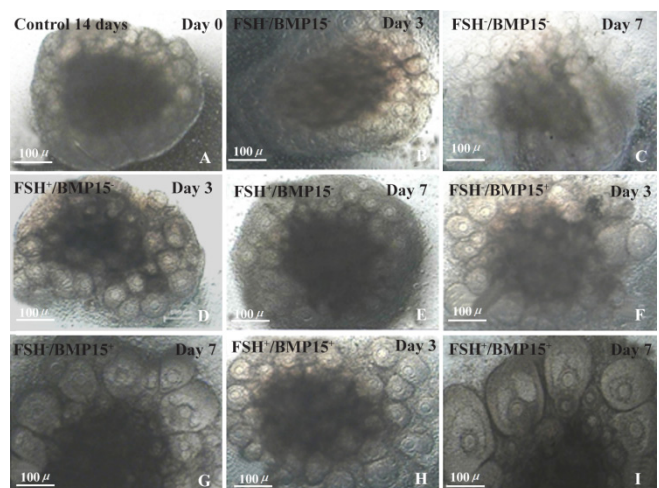
### Results

**Follicular Morphology in Cultured Ovaries:** The morphology of cultured mouse ovaries in the presence and absence of BMP15 and FSH and their non-cultured control groups (14 day and 21 day mouse ovaries) using H&E staining, is shown in figure 1. The light microscopic observations of ovarian sections showed the normal structure of follicles at different developmental stages.

The photomicrograph of cultured ovaries using an inverted microscope was shown in figure 2. The appearance of growing follicles became apparent during the culture period and could be observed as swellings on the surface of the cultured ovary.



**Figure 1.** Photomicrographs of cultured and non-cultured mouse ovaries sections stained with hematoxylin and eosin: Micrographs of non-cultured; A: 14 day mouse ovary; B: 21 day mouse ovary, C: 14 day mice ovaries cultured without FSH and BMP15 (FSH<sup>-</sup>/BMP15<sup>-</sup>), D: cultured with FSH and without BMP15 (FSH<sup>+</sup>/BMP15<sup>-</sup>), E: cultured without FSH and F: with BMP15 (FSH<sup>-</sup>/BMP15<sup>+</sup>) and cultured with FSH and BMP15 (FSH<sup>+</sup>/BMP15<sup>+</sup>)



**Figure 2.** Photomicrographs of mouse ovaries viewed under the invert microscope in A: 14 day mouse ovary on the beginning of culture; B, C: cultured ovaries without FSH and BMP15 (FSH<sup>-</sup>/BMP15<sup>-</sup>), D, E: cultured with FSH and without BMP15 (FSH<sup>+</sup>/BMP15<sup>-</sup>), F, G: cultured without FSH and with BMP15 (FSH<sup>-</sup>/BMP15<sup>+</sup>) and H, I: cultured with FSH and BMP15 (FSH<sup>+</sup>/BMP15<sup>+</sup>) on days 3 and 7, respectively

**The percentage of normal follicles in cultured ovaries:** The rates of follicles at various developmen-

**Table 2.** The mean percent of normal follicles at different developmental stages in cultured and non-cultured mouse ovaries

Groups	No. of Ovaries	Total No. of Follicles	Normal Follicle (Mean%±SE)	Atretic Follicle (Mean%±SE)	Primordial Follicle (Mean%±SE)	Primary Follicle (Mean%±SE)	Preantral Follicle (Mean%±SE)	Antral Follicle (Mean%±SE)
Non-cultured 14 days	5	384	369 (96±0.54)	15 (4±0.57)	237 (63.15±0.48)	32 (8.11±0.51) <sup>a</sup>	116 (30.72±0.60)	0
Non-cultured 21 days	5	308	295 (96±0.52)	12 (4±0.61)	164 (53.16±0.59)	41 (13.16±0.59)	59 (19.31±0.78)	45 (15.37±0.35)
Cultured-FSH/BMP15 <sup>-</sup>	5	206	145 (71±0.53)	61 (29±0.54)	105 (51.14±0.52) <sup>a</sup>	35 (17.25±0.46) <sup>a</sup>	55 (27.96±0.85) <sup>b</sup>	11 (5.1±0.51) <sup>ab</sup>
Cultured-FSH <sup>+</sup> /BMP15 <sup>-</sup>	5	223	168 (75±0.61)	55 (25±0.58)	108 (49.19±0.59) <sup>a</sup>	35 (15.18±0.61) <sup>a</sup>	41 (18.23±1.43) <sup>ac</sup>	38 (16.36±0.34) <sup>ac</sup>
Cultured-FSH/BMP15 <sup>+</sup>	5	286	211 (74±0.58)	74 (26±0.61)	148 (52.51±0.62) <sup>a</sup>	37 (13.62±0.34) <sup>a</sup>	271 (5.07±0.57) <sup>abcd</sup>	28 (10.11±0.21) <sup>abcd</sup>
Cultured-FSH <sup>+</sup> /BMP15 <sup>+</sup>	5	258	209 (81±0.55)	49 (19±0.59)	128 (50.14±2.48) <sup>a</sup>	36 (13.19±2.07) <sup>a</sup>	28 (11.69±1.65) <sup>abcde</sup>	67 (27.82±0.37) <sup>abcde</sup>

a: Significant differences with non-cultured 14 days group in the same column ( $p<0.05$ ), b: Significant differences with non-cultured 21 days group in the same column ( $p<0.05$ ), c: Significant differences with cultured-FSH/BMP15<sup>-</sup> group in the same column ( $p<0.05$ ), d: Significant differences with cultured-FSH<sup>+</sup>/BMP15<sup>-</sup> group in the same column ( $p<0.05$ ), e: Significant differences with cultured-FSH/BMP15<sup>+</sup> group in the same column ( $p<0.05$ )

tal stages in cultured and non-cultured mouse control ovaries are presented in table 2. Ovaries derived from 14 day old mice at the beginning of the culture contained mostly primordial follicles (63.15±0.48%), with a small proportion of primary (8.11±0.51%) and preantral follicles (30.7±0.60) and no antral follicles. After 7 days of *in vitro* organ culture, the rate of primordial follicles in all cultured groups decreased between 49.19 to 52.51% and there was no significant difference between cultured groups in this regard.

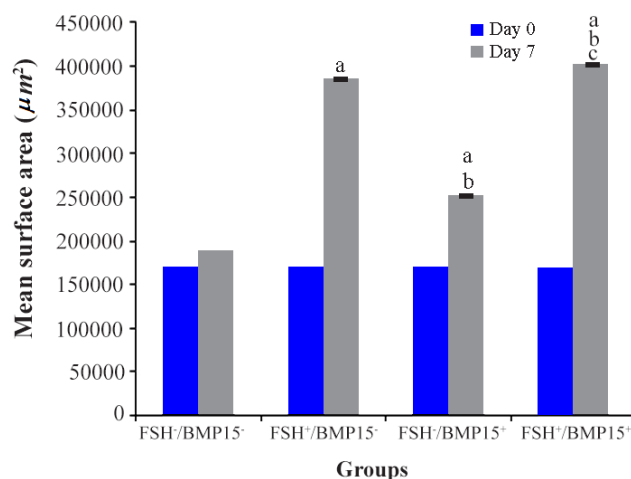
The percentage of primary follicles in all cultured groups was 13.19 to 17.25% and there was not any significant difference in the rate of primary follicles in all cultured groups.

The proportion of preantral follicles was 11.69 to 27.96% in all cultured groups and these rates were significantly different in comparison with non-cultured 14 day mouse ovaries ( $p<0.05$ ). Treatment with BMP15 was associated with significantly more developing follicles than non-cultured and cultured without BMP15 ( $p<0.05$ ).

The percentage of antral follicles was significantly higher (27.82±0.37%) in cultured-FSH<sup>+</sup>/BMP15<sup>+</sup> group than other cultured groups and non-cultured 21 day control group ( $p<0.05$ ).

**Surface area of cultured ovaries:** The area of cultured ovaries in all groups of study increased during the culturing period and their data are summarized in figure 3. At the end of the culture period, the mean surface area of ovaries in FSH<sup>+</sup>/BMP15<sup>+</sup> groups was significantly higher (401965.6±413.53  $\mu\text{m}^2$ ) than other groups and it was significantly lower in FSH/BMP15<sup>-</sup> groups (190221.39±57.46  $\mu\text{m}^2$ ;  $p<0.05$ ).

**Hormonal assay:** The levels of E2 and P4 on days 2 and 7 of culturing period in all groups of study



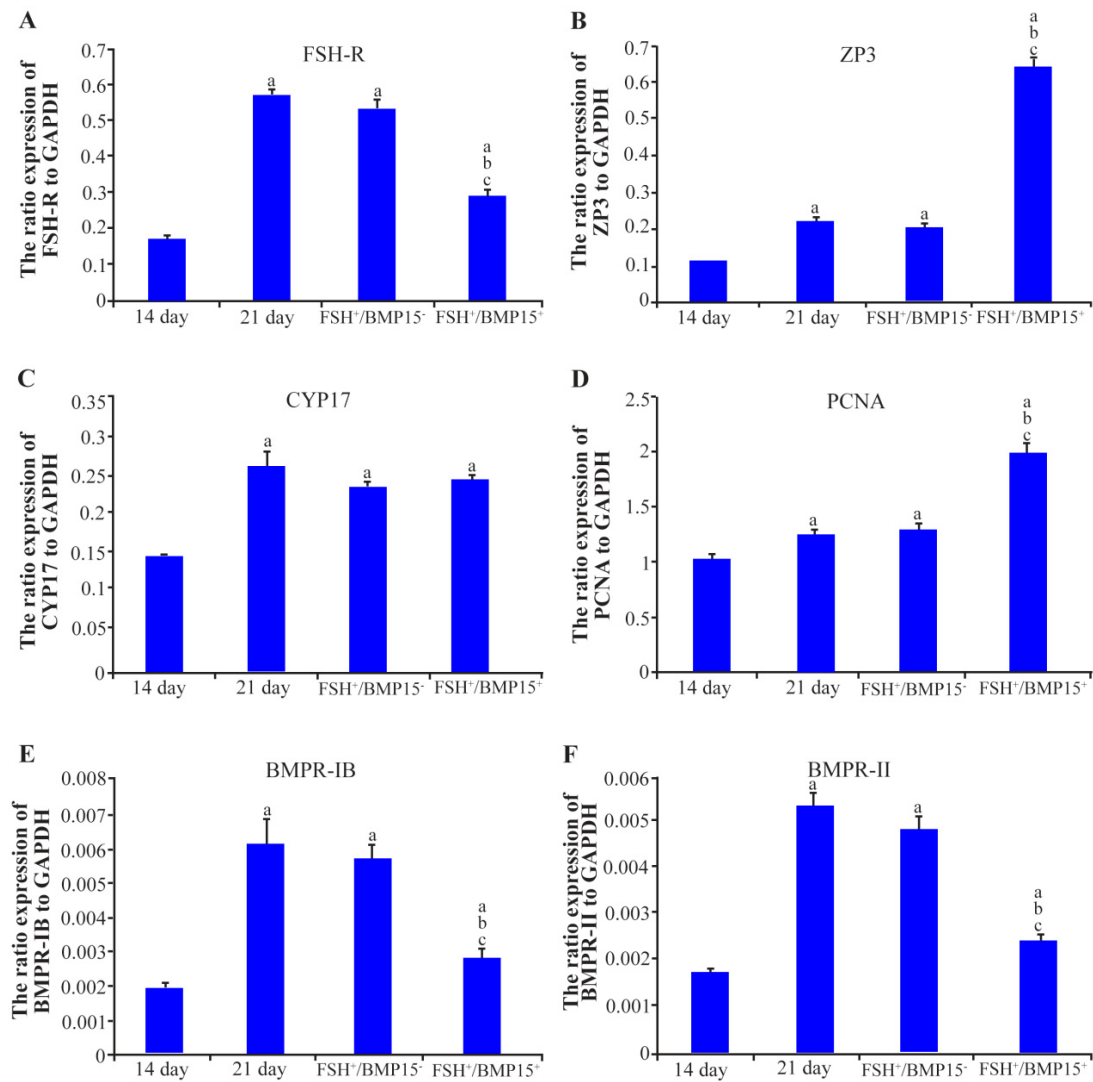
**Figure 3.** The mean surface area ( $\mu\text{m}^2$ ) of mouse ovaries on the beginning day 0 and day 7 of culture period. a: significant differences with FSH/BMP15<sup>-</sup> group; b: significant differences with FSH<sup>+</sup>/BMP15<sup>-</sup> group, c: significant differences with FSH/BMP15<sup>+</sup> group ( $p<0.05$ ). The surface area of cultured ovaries in all groups significantly increased on day 7 than the beginning of culture ( $p<0.05$ )

**Table 3.** The level of 17  $\beta$  estradiol (pg/ml) in collected media during culture period

Groups	Day 2 of culture (Mean±SE)	Day 7 of culture (Mean±SE)
Cultured-FSH/BMP15 <sup>-</sup>	2327±11.59	5424.66±29.56
Cultured-FSH <sup>+</sup> /BMP15 <sup>-</sup>	2323.66±16.37	12616±71.01 <sup>a</sup>
Cultured-FSH/BMP15 <sup>+</sup>	2344.33±10.68	6544.34±27.11 <sup>ab</sup>
Cultured-FSH <sup>+</sup> /BMP15 <sup>+</sup>	2318±9.45	16542±69.31 <sup>abc</sup>

a: Significant differences with cultured-FSH/BMP15<sup>-</sup> group in the same column ( $p<0.05$ ), b: Significant differences with cultured-FSH<sup>+</sup>/BMP15<sup>-</sup> group in the same column ( $p<0.05$ ), c: Significant differences with cultured-FSH/BMP15<sup>+</sup> group in the same column ( $p<0.05$ )

were presented and compared in tables 3 and 4, respectively. The levels of E2 and P4 in culture



**Figure 4.** The expression ratio of FSHR, ZP3, CYP17, PCNA, BMPR-IB and BMPR-II to GAPDH in cultured mouse ovaries in the presence and absence of BMP15 and non-cultured groups, respectively; A-F: (a) significant difference with 14 day mouse ovary ( $p < 0.05$ ), (b) significant difference with 1 day mouse ovary ( $p < 0.05$ ) and (c) significant difference with FSH<sup>+</sup>/BMP<sup>-</sup> group ( $p < 0.05$ )

**Table 4.** The level of progesterone (ng/ml) in collected media during culture period

Groups	Day 2 of culture (Mean±SE)	Day 7 of culture (Mean±SE)
Cultured-FSH/BMP15 <sup>-</sup>	118±2.08	211.66±4.97
Cultured-FSH <sup>+</sup> /BMP15 <sup>-</sup>	110.33±3.92	473±12.66 <sup>a</sup>
Cultured-FSH/BMP15 <sup>+</sup>	118±3.46	325.33±8.84 <sup>ab</sup>
Cultured-FSH <sup>+</sup> /BMP15 <sup>+</sup>	109.66±3.84	698.66±12.90 <sup>abc</sup>

a: Significant differences with cultured-FSH/BMP15<sup>-</sup> group in the same column ( $p < 0.05$ ), b: Significant differences with cultured-FSH<sup>+</sup>/BMP15<sup>-</sup> group in the same column ( $p < 0.05$ ), c: Significant differences with cultured-FSH/BMP15<sup>+</sup> group in the same column ( $p < 0.05$ )

media of all groups significantly increased on day 7 in comparison to day 2. These levels were sig-

nificantly higher in FSH<sup>+</sup>/BMP15<sup>+</sup> group and they were significantly lower in FSH<sup>-</sup>/BMP15<sup>-</sup> group than other groups ( $p < 0.05$ ).

**Real time RT-PCR analysis:** The relative expression of PCNA, BMPR-IB, BMPR-II, FSH-R, CYP17 and ZP3 genes compared with the house-keeping gene (GAPDH) in all groups is shown in figure 3. According to real-time RT-PCR results, greater levels of mRNA for PCNA and ZP3 were seen in FSH<sup>+</sup>/BMP15<sup>+</sup> group than BMP15<sup>-</sup>/FSH<sup>+</sup> or non-cultured control groups ( $p < 0.05$ ). The BMPR-IB, BMPR-II and FSH-R mRNA level was significantly lower in the FSH<sup>+</sup>/BMP15<sup>+</sup> group in comparison with controls ( $p < 0.05$ ). As the results dem-

onstrated, the relative expression of CYP 17 did not change significantly in all experimental groups.

### Discussion

The results of the present study showed that the supplementation of ovarian organ culture with BMP15 and FSH yields the higher percentages of follicular development to antral stage; moreover, it could cause an increase in size and function of ovaries by production of high levels of E2 and P4. From another point of view, in the group without any of these factors (FSH and BMP15) or the groups without FSH, the percentage of antral follicles and the mean surface area of ovaries were low. These results revealed the proliferative and maturation effects of BMP15 on the granulosa cells which lead to growth of follicles and an increase in size of ovaries. This conclusion is in agreement with others which demonstrated that, this factor stimulated the growth of primordial and primary follicles in humans and goat (11-13). Kedem et al. (13) also showed BMP15 promoted activation of human primordial follicles in a dose-dependent manner. It was shown that supplementation of culture media with BMP-15 maintained the ultrastructural integrity of isolated caprine pre-antral follicles and promoted the growth of pre-antral follicles cultured for seven and 18 days (13, 20, 21). However, *in vivo* condition showed that the higher GDF9 levels in the follicular fluid were significantly correlated with oocyte nuclear maturation and embryo quality (18).

Real-time RT-PCR was used to analyze the expression levels of some genes related to follicular development and our results showed that treatment with BMP15 and FSH up-regulated the expression of ZP3 and PCNA genes. Increased expression of ZP3 gene in ovaries cultured with BMP15 may be connected with oocyte quality. Zona pellucid is a glycoprotein which surrounds the mammalian oocyte and plays an important role in the fertilization and binding of sperm. The ZP of mouse is composed of three (ZP1-3) glycoproteins and in mice, ZP3 acts as the primary sperm receptor (22, 23).

In the present study, treatment with BMP15 led to increased expression of PCNA and this enhancement is due to proliferative effect of BMP15 on granulosa cells. Proliferating cell nuclear antigen protein (PCNA) is a 36 KDa protein and its expression has been reported in fetal and adult ovaries in several mammals (24, 25). Different expression of PCNA was reported with the devel-

opment of fetal and newborn rat and mouse ovaries (26). Oktay et al. reported that in rat ovaries, the expression of PCNA was not detected in granulosa cells or oocytes in primordial follicles, but increased with the initiation of follicle growth (27). The result of Tománek and Chronowska's study demonstrated that follicular growth and development in pig ovary may be effectively monitored by determining the granulosa cell expression of PCNA (28).

However, the present study showed that treatment with BMP15 had no effect on CYP17 gene expression. Cytochrome P450c17 $\alpha$  that is encoded by the CYP17 gene is an enzyme that produces androgen precursors which convert to estrogens during ovarian steroidogenesis (28, 29). The P450s enzyme (CYP11A1) is present in the internal membrane of the mitochondria converting cholesterol into pregnenolone, mainly in the theca interna cells (30).

Other parts of our molecular results showed BMP15 and FSH supplementation down-regulated the expression of BMPR-IB, BMPR-II and FSH-R in cultured mouse ovaries. The FSH is a glycoprotein and secreted from the pituitary gland and binds to a specific FSH receptor on the somatic cells of the ovary and it is essential for follicular growth (31). FSHR is a G protein-coupled, seven-transmembrane receptor linked to the adenylate cyclase or other pathways (31). The inhibitory effect of BMP15 on the expression of FSHR was shown before by Otsuka et al. (14). Their observations revealed that BMP15 can also inhibit FSH actions by suppressing rat granulosa cell FSHR expression (14). BMPR-IB and type II are detected on the surface of granulosa cells (33) and are more expressed in the largest follicles and were up-regulated in advanced atretic follicles (33). It was suggested that BMP15 could prevent the follicular regression and enhance follicular development by down-regulation of BMPR gene. In agreement with this suggestion, Bo Zhaia et al.'s (15) results suggest that BMP15 regulates several genes which are involved in the regulation of the gene balance in cumulus cell survival and apoptosis (15).

Our results also showed the beneficial effects of BMP15 on follicular development at the morphological and molecular levels are more prominent in the presence of FSH. In this regard, Guéripel et al. (10) showed that BMP15 levels increase during gonadotropin-induced follicular development, in parallel with oocyte maturation. However, ac-

ording to our observation, it seems that FSH was needed for improvement of *in vitro* follicular maturation and development. More studies are needed to reveal the involved mechanisms that regulate these effects.

In parallel with other assessments in this study, steroid hormones (E2 and P4) were used as a marker of granulosa and theca cell function and data showed the steroidogenic pathways were present and increased during the *in vitro* culture of ovarian tissue in the presence of BMP15 and FSH. Therefore, BMP15 coordinates with FSH to promote the development of cumulus cells and maintain their competence to undergo expansion and steroidogenesis (30). Similarly, Marcondes et al. (29) demonstrated that estrogen induced an increase in the ovarian theca-interstitial area, the secondary follicle population and expression of some developmental gene.

### Conclusion

This study demonstrated a favorable effect of BMP15 in combination with FSH on the *in vitro* development of small size mouse follicles to antral stage.

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### Conflict of Interest

Authors declare no conflict of interest.

### References

- Mester B, Ritter LJ, Pitman JL, Bibby AH, Gilchrist RB, McNatty KP, et al. Oocyte expression, secretion and somatic cell interaction of mouse bone morphogenetic protein 15 during the peri-ovulatory period. *Reprod Fertil Dev.* 2015;27(5):801-11.
- Fenwick MA, Mora JM, Mansour YT, Baithun C, Franks S, Hardy K. Investigations of TGF- $\beta$  signaling in preantral follicles of female mice reveal differential roles for bone morphogenetic protein 15. *Endocrinology.* 2013;154(9):3423-36.
- Paulini F, Melo EO. The role of oocyte-secreted factors GDF9 and BMP15 in follicular development and oogenesis. *Reprod Domest Anim.* 2011;46(2):354-61.
- Juengel JL, Bodensteiner KJ, Heath DA, Hudson N L, Moeller CL, Smith P, et al. Physiology of GDF9 and BMP15 signalling molecules. *Anim Reprod Sci.* 2004;82-83:447-60.
- Gasperin BG, Ferreira R, Rovani MT, Bordignon V, Duggavathi R, Buratini J. Expression of receptors for BMP15 is differentially regulated in dominant and subordinate follicles during follicle deviation in cattle. *Anim Reprod Sci.* 2014;144(3-4):72-8.
- McNatty KP, Juengel JL, Reader KL, Lun S, Myllymaa S, Lawrence SB, et al. Bone morphogenetic protein 15 and growth differentiation factor 9 cooperate to regulate granulosa cell function. *Reproduction.* 2005;129(4):473-80.
- de Resende LO, Vireque AA, Santana LF, Moreno DA, de Sa Rosa e Silva AC, Ferriani RA, et al. Single-cell expression analysis of BMP15 and GDF9 in mature oocytes and BMPR2 in cumulus cells of women with polycystic ovary syndrome undergoing controlled ovarian hyperstimulation. *J Assist Reprod Genet.* 2012;29(10):1057-65.
- Mery L, Lefevre A, Benchaib M, Demirci B, Salle B, Guerin JF, et al. Follicular growth in vitro: detection of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) during in vitro culture of ovine cortical slices. *Mol Reprod Dev.* 2007;74(6):767-74.
- Otsuka F, McTavish KJ, Shimasaki S. Integral role of GDF-9 and BMP-15 in ovarian function. *Mol Reprod Dev.* 2011;78(1):9-21.
- Guéripel X, Brun V, Gougeon A. Oocyte bone morphogenetic protein 15, but not growth differentiation factor 9, is increased during gonadotropin-induced follicular development in the immature mouse and is associated with cumulus oophorus expansion. *Biol Reprod.* 2006;75(6):836-43.
- Lima IM, Brito IR, Rossetto R, Duarte AB, Rodrigues GQ, Saraiva MV, et al. BMPRII and BMPRII mRNA expression levels in goat ovarian follicles and the in vitro effects of BMP-15 on preantral follicle development. *Cell Tissue Res.* 2012;348(1):225-38.
- Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ, Hovatta O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. *J Clin Endocrinol Metab.* 2002;87(1):316-21.
- Kedem A, Fisch B, Garor R, Ben-Zaken A, Gizunterman T, Felz C, et al. Growth differentiating factor 9 (GDF9) and bone morphogenetic protein 15 both activate development of human primordial follicles in vitro, with seemingly more beneficial effects of GDF9. *J Clin Endocrinol Metab.* 2011;96(8):E1246-54.
- Zhai B, Liu H, Li X, Dai L, Gao Y, Li C, et al. BMP15 prevents cumulus cell apoptosis through CCL2 and FBN1 in porcine ovaries. *Cell Physiol Biochem.* 2013;32(2):264-78.



15. Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15. Identification of target cells and biological functions. *J Biol Chem*. 2000;275(50):39523-8.
16. Hussein TS, Froiland DA, Amato F, Thompson JG, Gilchrist RB. Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *J Cell Sci*. 2005;118(Pt 22):5257-68.
17. Wu YT, Tang L, Cai J, Lu XE, Xu J, Zhu XM, et al. High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. *Hum Reprod*. 2007;22(6):1526-31.
18. Gode F, Gulekli B, Dogan E, Korhan P, Dogan S, Bige O, et al. Influence of follicular fluid GDF9 and BMP15 on embryo quality. *Fertil Steril*. 2011;95(7):2274-8.
19. Passos MJ, Vasconcelos GL, Silva AW, Brito IR, Saraiva MV, Magalhaes DM, et al. Accelerated growth of bovine preantral follicles in vitro after stimulation with both FSH and BMP-15 is accompanied by ultrastructural changes and increased atresia. *Theriogenology*. 2013;79(9):1269-77.
20. Celestino JJ, Lima-Verde IB, Bruno JB, Matos MH, Chaves RN, Saraiva MV, et al. Steady-state level of bone morphogenetic protein-15 in goat ovaries and its influence on in vitro development and survival of preantral follicles. *Mol Cell Endocrinol*. 2011;338(1-2):1-9.
21. Rossi RO, Costa JJ, Silva AW, Saraiva MV, Van den Hurk R, Silva JR. The bone morphogenetic protein system and the regulation of ovarian follicle development in mammals. *Zygote*. 2016;24(1):1-17.
22. Avella MA, Xiong B, Dean J. The molecular basis of gamete recognition in mice and humans. *Mol Hum Reprod*. 2013;19(5):279-89.
23. Gupta SK, Bhandari B, Shrestha A, Biswal BK, Palaniappan C, Malhotra SS, et al. Mammalian zona pellucida glycoproteins: structure and function during fertilization. *Cell Tissue Res*. 2012;349(3):665-78.
24. Picut CA, Swanson CL, Scully KL, Roseman VC, Parker RF, Remick AK. Ovarian follicle counts using proliferating cell nuclear antigen (PCNA) and semi-automated image analysis in rats. *Toxicol Pathol*. 2008;36(5):674-9.
25. Muskhelishvili L, Wingard SK, Latendresse JR. Proliferating cell nuclear antigen--a marker for ovarian follicle counts. *Toxicol Pathol*. 2005;33(3):365-8.
26. Zhang Z, Shen B, Wang Y, Chen Y, Wang G, Lin P, et al. Molecular cloning of proliferating cell nuclear antigen and its differential expression analysis in the developing ovary and testis of penaeid shrimp *Marsupenaeus japonicus*. *DNA Cell Biol*. 2010;29(4):163-70.
27. Oktay K, Schenken RS, Nelson JF. Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. *Biol Reprod*. 1995;53(2):295-301.
28. Rowe E, Van Horn A, Rockwell LC. CYP17 genotype modifies the impact of anthropometric variation on salivary estradiol in healthy women. *Am J Phys Anthropol*. 2015;156(4):665-70.
29. Marcondes RR, Carvalho KC, Duarte DC, Garcia N, Amaral VC, Simões MJ, et al. Differences in neonatal exposure to estradiol or testosterone on ovarian function and hormonal levels. *Gen Comp Endocrinol*. 2015;212:28-33.
30. Kempisty B, Ziółkowska A, Ciesiółka S, Piotrowska H, Antosik P, Bukowska D, et al. Association between the expression of LHR, FSHR and CYP19 genes, cellular distribution of encoded proteins and proliferation of porcine granulosa cells in real-time. *J Biol Regul Homeost Agents*. 2014;28(3):419-31.
31. Scarlet D, Walter I, Hlavaty J, Aurich C. Expression and immunolocalisation of follicle-stimulating hormone receptors in gonads of newborn and adult female horses. *Reprod Fertil Dev*. 2015. [Epub ahead of print].
32. Otsuka F. Multifunctional bone morphogenetic protein system in endocrinology. *Acta Med Okayama*. 2013;67(2):75-86.
33. Gasperin BG, Ferreira R, Rovani MT, Bordignon V, Duggavathi R, Buratini J, et al. Expression of receptors for BMP15 is differentially regulated in dominant and subordinate follicles during follicle deviation in cattle. *Anim Reprod Sci*. 2014;144(3-4):72-8.