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In Vitro Culture of Mouse Preantral Follicle in Supplemented Medium with Bone Morphogenetic Protein 15 (BMP15)

Amene Shekarian[1], Zahra Nadia Sharifi[1], Mohammad Mahdi Nazarnejad[2], Amirhossein Saeinia[3] Shabnam Abdi[1*]

1. Department of Anatomical Sciences, School of Medicine, Islamic Azad University Tehran Medical Sciences Branch, Tehran, Iran.
2. Department of Medical Science, Faculty of Medicine, Tehran Medical Science, Islamic Azad University, Tehran, Iran.
3. Department of Laboratory Science, Faculty of Paramedical, Tehran Medical Science, Islamic Azad University, Tehran, Iran.

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Corresponding Author:
Dr. Shabnam Abdi

Email:
sh.abdi@iautmu.ac.ir

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Abstract

Background: BMD-15 is a member of the superfamily of transforming growth factor-beta (TGF-β), which has a determinant role in fertility. This protein is essential for the development of ovarian follicles and acts mainly by binding to its receptor on the surface of granulosa cells.

Aim: The aim of this study was to evaluate the effect of BMP-15 on in vitro ovarian follicle growth and embryo development.

Methods: In this study, preantral follicles were isolated mechanically from 12 days NMRI mouse ovaries, then the follicles were cultured in basic growth medium enriched by FBS, FSH, and ITS and BMP-15-enriched for 12 days. During the culture, survival rate and follicular maturation, follicular diameter, level of estrogen, and progesterone secretion and embryo developmental rate were evaluated.

Results: The results of this study showed that the percentage of antral follicles, maturation rate and hormone levels and the diameter of follicles was significantly higher in BMP-15 supplemented media in comparison to basic media. Embryo development was also higher at BMP-15 enriched group in comparison to the group of follicles, which were grown in the basic culture media.

Conclusion: The present study demonstrates that supplemented media with BMP-15 to the ovarian preantral follicle culture enhances the in vitro growth of follicle embryo development.

Conflicts of Interest: The Authors declare no conflicts of interest.
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Introduction

The culture systems of preantral follicles have been used to evaluate the effect of environmental chemicals, mutagens, drugs and natural and artificial factors on folliculogenesis and the quality and genetic structure of oocyte. It is of great importance in clinical issues, researches and animal sciences (1-3). In a two-dimensional follicular culture system, which is developed by various researchers, the goal is to support ovarian growth, so that the follicle is out of spherical shape and the nutrients, hormones and gases are easily absorbed in this open structure, which increases the life rate of the oocyte (4-6). Ovarian follicle contains a stopped oocyte in the diplotene stage, which begins to grow in response to a number of symptoms (7-9).

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As it turns out, follicle growth is not only dependent on the morphological and functional changes of somatic granulosa cells and theca cells, but also dependent on positional factors that affect their development. Therefore, disruption of any of these mechanisms affects follicle formation, so that ovulation requires the proper correlation of all these processes at each stage of follicle development (9-12). Some of these factors include Kit Ligand (KL), epidermal growth factors (EGF), Activin, KGF, Insulin growth factors (IGFs), Leukemia Inhibitory Factor (LIF) (13-18).

BMP is the morphological protein of bone No. 15 and a member of the beta-transforming growth factor (TGF-β) family. This oocyte growth factor is critical for the early stages of follicle formation and subsequent development of the follicle. This secreted factor plays an important role in differentiating granulosa and regulating the key functions of granulosa cells, so oocytes control the differentiation and action of granulosa cells and its effect on gene expression patterns in follicular somatic cells is a mechanism as a result of this BMP (19-22).

Several studies have demonstrated that BMP-15 involved in the activation of primordial follicle, proliferation of granulosa cells, and cytoplasmic maturation of oocyte.

One of the major problems regarding to in vitro growth of ovarian preantral is low rates of oocytes maturation and embryo development than in vivo achieved samples. Considering the mentioned issues and considering that different researchers have focused their efforts on increasing the evolution rate of follicles in different culture systems using different factors, the present study was designed in order to measure the growth and development of preantral follicles of mouse ovarian tissue due to the influence of BMP-15.

Methods

Animals

In this study an immature 12-day-old female mouse from NMRI race was used. Adult male mouse were also used to extract sperm. The animals were kept in suitable conditions with 12 hours of light and 12 hours of darkness, 20-24℃ temperature and -40-50% humidity. Water and food were available for them in adequate portions. The mice were killed by amputation of the cervical spinal cord; their ovaries were removed from the body and placed in drops of 100 µL of α-MEM culture containing 10% FBS serum.

Follicle isolation

The ovaries were placed in 50 µL droplets of α-MEM culture containing 10% FBS. In order to separate the follicles in this study, a mechanical method was used in which the structure of the follicles remained intact and all types of cells and follicle receptor systems were preserved. This was done using the tip of a G29 needle attached to a 1-milliliter insulin syringe under the magnification of 25 stereo microscopes (Olympus, Japan). After isolation, preantral follicles with a diameter of 140 to 150 micrometers were selected, which had a central oocyte with 2-3 granulosa cell layers around it. Isolated preantral follicles, which were isolated from ovaries were cultured in a two-dimensional culture system, and each was divided into two subgroups according to the use of BMP-15 factor. A group which contained follicles which were cultured in a culture media and another group which contained cultured follicles in a culture media containing 50 ng of BMP-15.

Two dimensional in vitro culture of isolated preantral follicles

In order to cultivate preantral follicles, culture plate with 96 wells was used. First, 40 µL of culture medium was poured into the wells, then covered with 5 ml of paraffin. Preantral follicles with healthy morphology were individually transferred to 96 wells and cultured for 12 days in 37℃ incubator, 5% CO2 and humidity. Every other day, half of media was replaced with fresh culture media and the collected

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media were separately frozen at -80ºC until hormonal assay.

In vitro ovulation induction

To induce ovulation on the twelfth day, the culture medium was replaced with an IU/mL of 1.5 hCG (hCG; Organon, Griekenweg, Netherlands), so that 18 hours later, the mature oocytes (MII) were collected and counted.

Evaluation of ovarian follicle growth

At the end of this stage, diameter of the follicles, survival rate of follicles, the formation of antrum cavity the number and percentage of oocyte formation were evaluated under the reverse microscope and its percentage and the secretion of 17-beta-estradiol, progesterone and dehydroepiandrosterone hormones in the environment of follicles grown in each group at the end of the culture period was calculated and compared with live follicles.

Hormonal assay

Concentrations of 17 beta-estradiol using EIA method (Monobind, USA, sensitivity=6.5 pg/mL) and progesterone hormone level using ELISA technique (DiaPlus, USA, sensitivity=0.1 ng/mL) were investigated.

Fertilization and embryo development

The MII oocytes were collected from each subgroup and subjected to in vitro fertilization. After spinal cord injury through the cervical vertebrae, the epididymis tail of the NMRI adult male mouse was isolated for 7 to 8 weeks and transferred to droplets from the global culture medium containing 5 mg/ml BSA and stored for 1 hour in incubator conditions so that capacitation of the sperm gets accomplished. The collected active and healthy sperm were transferred to global drops containing 15 mg/ml BSA at a rate of 1-2× 10^6/ml. The oocytes from the studied groups were then transferred to droplets containing sperm. The rates of fertilization, 2cell, 4cell, 8 cell, morula and blastocyst embryos were assessed.

Statistical analysis

After collecting the information, the results were examined using Spss software (version 13). Statistically, $p < 0.05$ was significant in the accomplished tests. The percentage of mature and immature follicles and the number of embryos with ANOVA and Tukey's tests were measured within the group and between groups.

Results

The diameter of cultured isolated preantral follicles

Preantral follicles were cultured for 12 days in the absent and presence of 50 ng/mL BMP-15. On the second day of follicle culture in the two-dimensional culture system, the follicles were immobilized due to the growth of theca and granulosa cells and the adhesion of these cells to the bottom of the culture vessel. From the fourth day onwards, after duplication and rupture of the basement membrane, granulosa cells spread around the follicle and gave the follicle an irregular ridge shape. On the sixth to eighth day of cultivation, antrum was formed around the oocyte. On the tenth day of culture, a large antral cavity was observed (Figure 1).

As the results show, on the second day, the average diameter of the ovarian follicles isolated in the basic medium and mediums enriched with BMP-15 was 177.4±3.9 and 232.11± 2.8 µm, respectively. On the fourth day of culture, the average diameter of ovarian follicles cultured in the basic medium and enriched mediums with BMP-15 was 260.75±4.6 and 298.08±2.5 µm so that the increase in diameter was significantly higher in the cultivated follicles in the base medium enriched with BMP-15 compared with the control group ($p < 0.001$).

The growth and development of groups is compared in Table 2.

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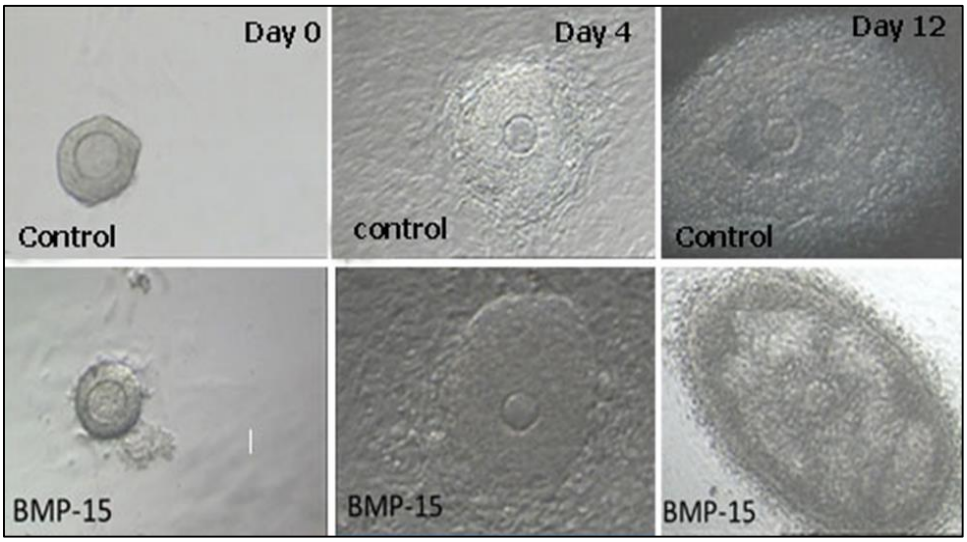

Figure 1. Morphology of preantral follicles cultured in a two-dimensional culture system for 12 days. Preantral follicles on the first day of culturing. On the fourth day of culture, the follicle attaches to the floor of the plate and the growth of granulosa and theca cells is obvious. On the twelfth day, the antrum hole is clearly visible (arrow).

Table 1. Changes in the mean diameter of isolated preantral follicles (µm) and cultured in two-dimensional culture.

Examined groups		Diameter of follicle (micrometer)		
		Day 0 Mean±SD	Day 2 Mean±SD	Day 4 Mean±SD
Preantral follicle	Basic medium	146±2.2	188.49±3.8	255.75±3.3
	Basic medium +BMP-15	150.58±1.8	240.11±2.3	278.12±2.9*

Day 0 is the first day that the follicles were cultured. *It had a significant difference with the basic medium group.

Follicular developmental rate

The survival rate of isolated ovarian follicles cultured in the basic medium and basic medium enriched with BMP-15 was 56% and 80%, respectively Table 2. There was a significant difference in the survival rate of cultivated follicles in the basic medium enriched with BMP-15 compared to other groups ($p < 0.001$). The percentage of antrum formation of follicles that were isolated from ovaries cultured in the basic medium and medium enriched with BMP-15 was 44.64% and 66.25%, respectively.

In the two-dimensional culture system, the percentage of MII oocytes in isolated follicles from ovaries cultured in the basic medium and medium enriched with BMP-15 was 21.42% and 47.5%, respectively.

Hormonal assay

The secretion rate of hormones in different groups is compared in Table 3. The secretion of 17-β estradiol hormone in the isolated follicles from the ovaries cultured in the basic medium and medium enriched with BMP-15 was 1564.5 and 3110.1 pg/mL, respectively. Also, the secretion of progesterone hormone in isolated ovarian follicles cultured in the basic medium and medium enriched with BMP-15 was 36.3 and 50.6 ng/mL, respectively. Also, the rate of secretion of dehydroepiandrosterone hormone in isolated follicles from ovaries cultured in the basic medium and medium enriched with BMP-15 was 14.3 and 19 µg/ml, respectively. Hormone secretion in follicles cultured in the BMP-15 enriched medium was significantly higher than other groups.

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Table 2. Comparison of growth of cultivated follicles for 12 days in different study groups.

Groups	Total Number of follicles	Alive follicles Number (percentage)	Degenerated follicles Number (percentage)	Antrum formation Number (percentage)	MII oocyte Number (percentage)
Basic medium	100	56	44	25(44.64)	12(21.42)
BMP-15 enriched	100	80	20	53(66.25)*	38(47.5)*

*There was a significant difference between control group and cultured group in BMP-15 enriched medium.

Table 3. Changes in hormone secretion in the follicles at the end of the 12th day of culture in all groups studied.

Groups	17-β estradiol(pg/mL) Mean±SD	Progesterone(ng/mL) Mean±SD	Dehydroepiandrosterone(µg/mL) Mean±SD
Basic medium	2028/5±19.5	41.3±1.3	12.3±1.56
KL enriched	*2810.1±28	*61.6±2.7	23±2

*There was a significant difference between control group and cultured group in BMP-15 enriched medium.

Embryo development

After insemination MII oocytes, which were extracted from cultivated follicles in different groups, the percentage of fertilization, two-celled embryos, morula and blastocyst stage is given in Table 4.

Table 4. Fertilization rate and embryo development of isolated follicles in different groups

Groups	MII oocyte number	Fertilization Number(percentage)	2cell Number (percentage)	Morula Number (percentage)	Blastocyst Number (percentage)
Basic medium	35	28(80)	20(57.14)	10(28.57)	5(14.28)
BMP-15 enriched	35	30(85.7)	25(71.42)	16(45.71)	10(28.57)*

*There was a significant difference between control group and cultured group in BMP-15 enriched medium.

The percentage of two-celled, morula and blastocysts of the hatching stage in mature oocytes resulting from isolated follicles from ovaries grown in the basic medium and medium enriched with BMP-15 was (80% And 85.7%), (57.14% and 71.42%), (28.57% and 45.71%), 14.28% and 28.57%), respectively. It can be realized that BMP-15 has an important and beneficial role in fertilization and embryo development.

Discussion

The major findings from the present study we achieved the highest developmental capacity of follicles, MII oocytes rate, fertilization rate, embryo developmental rate and level of hormone production in the basic medium enriched with BMP-15 compared with the control group. These data support the idea that BMP-15 factor could improve the growth and development of ovarian follicle. BMP-15 stimulates the growth of preantral follicles to become antral follicles, although it does not have a significant effect on the growth of follicles in the early stages. BMP15 stimulated the growth of granulosa and theca cells. The higher level of steroid hormone production grown in the percent of BMP-15 culture system may be due to high level of androgen provided by theca cells and accelerated increased

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aromatase activity and increased granulosa cell numbers.

The positive effect of BMP-15 on follicular growth and development, which was observed in the present study, is in agreement with similar previous reports. In a study conducted by Salehnia et al., the percentage of antral follicles, ovarian size and estradiol and progesterone levels, as well as expression of PCNA and ZP3 genes in BMP-15 culture medium was higher than the control group and the difference they had was significant (23, 26). Another study, which was conducted by Sheena Regan et al. showed that BMP signaling systems are present in many species, including humans. It has an important internal role in the production of transcription factors such as permeability, steroidogenesis, cell differentiation, maturation before ovulation, and the formation of a corpus luteum after ovulation (24). BMP also helps in regulating gonadotropin production in anterior hypophysis. A study conducted by Kona et al. found that BMP-15 and GDF9 followed a specific pattern of expression during the evolutionary development of ovarian follicles in sheep. The highest expression rates were GDF9 and BMP15 (3.38 ± 3.02 and 2.69 ± 0.6; $p \leq 0.05$) in primary follicles compared with preantral stage, primary antral stage, antral stage, and large antral stage follicles. Similarly, the expression of GDF9 and BMP15 in cumulus cells (0.16 ± 0.6 and 0.07 ± 0.07) and oocytes (07.07 ± 1.47 and 1.32 ± 0.03) were lowest in the antral stage follicles grown in vivo ($P \leq 0.05$) (25). Fumio Otsaka et al., discovered that the two key factors BMP-15 and GDF-9 were involved in ovarian function, and that identifying mutations in the two genes was shown to be an important factor in the fertility or infertility of several sheep breeds. Similarly, a large number of mutations in the BMP-15 and GDF-9 genes were identified in women with premature ovarian failure.

Conclusion

In summary, these studies provide evidence that BMP-15 supplementation during in vitro culture appeared to significantly increase follicular function and development.

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Not declared.

Conflicts of Interest

The authors declare no conflicts of interest.

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Not declared.

Ethics

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