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Bone Morphogenetic Proteins are Significantly Reduced in the Follicular Fluid of Han Chinese Polycystic Ovary Syndrome Patients

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting up to 10% of women of childbearing age. Traditionally, androgen elevation has been considered the main pathological cause for PCOS. However, it is increasingly recognized that bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β) family, play important roles in follicle recruitment, follicle selection, and follicle-stimulating hormone (FSH) responsiveness.

Methods: Twenty-eight PCOS patients and fourteen tubular infertility patients were recruited from patients seeking reproductive assistance at The Center of Reproductive Medicine in Taiyuan, China. Each patient went through an *in vitro* fertilization and embryo transplantation (IVF-ET) procedure. The mRNA and protein levels of hormones, hormone receptors, and BMPs were evaluated using real-time PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively.

Results: Hormone levels were comparable in PCOS and control patients. In contrast, significant differences were observed in BMP levels in these two groups. BMP-5, -6, -7, and -8A levels were all markedly lower in follicular fluid in PCOS patients ($p<0.05$), with BMP-7 being the most significantly down regulated biomarker ($p=0.0004$). The levels of all BMPs were positively correlated with follicle-stimulating hormone receptor (FSHR).

Conclusions: Expression levels of multiple BMPs were significantly down-regulated in follicular fluid from PCOS patients. Their low abundance as well as their correlation with FSHR confirmed BMPs as key regulators of PCOS pathogenesis.

Keywords: Polycystic ovary syndrome; Bone morphogenetic protein; Biomarker; Follicular fluid; Follicle-stimulating hormone; Luteinizing hormone

Abbreviations:

PCOS: Polycystic Ovary Syndrome; BMP: Bone Morphogenetic Protein; TGF-β: Transforming Growth Factor-β; IVF-ET: *In Vitro* Fertilization and Embryo Transplantation; FSH: Follicle-Stimulating Hormone; LH: Luteinizing Hormone; E2: Estradiol; P: Progesterone; PRL: Prolactin; T: Testosterone

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects up to 10% of women of reproductive age [1]. PCOS is a leading cause of female infertility, being present in 20-30% of total infertile patients. Based on the Rotterdam criteria, PCOS symptoms include irregular periods, excess body and facial hair, high levels of androgen, and difficulty getting pregnant [2]. The causes of PCOS remain elusive but a combination of genetic and environmental factors is likely involved, including susceptibility single-nucleotide polymorphism [3,4], obesity, diet and lifestyle, etc. A key feature of PCOS is the imbalance of hormones, such as pituitary-secreted follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and ovary-produced estradiol (E2). FSH is a key hormone in the regulation of folliculogenesis and female fertility [5]. In the ovary, FSH triggers differentiation and proliferation of granulosa cells, and leads to the development of preovulatory follicles. It is widely observed that FSH is less expressed in PCOS patients. In clinical practice, an LH/FSH ratio greater than two is been considered the "gold standard" of PCOS diagnosis [1].

Obesity is also a crucial factor contributing to PCOS pathogenesis. Moran et al. found that more than one third of PCOS pateints in their study/survey were overweight with a body mass index greater than 25 kg/m^2 [6]. Obesity was significantly associated with an increased risk of hirsutism and menstrual cycle disturbance. Furthermore, loss of body weight induced by diet or lifestyle change reduced blood androgen levels and improved ovulation [6].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily of extracellular signaling molecules [7]. BMPs were first identified in bone and cartilage [8-10], and later in other tissues including the ovary [11,12]. BMPs have been shown to regulate several key biological processes in the ovary including cell proliferation, differentiation, apoptosis, and steroidogenesis [7,11,13]. BMPs are expressed in a cell-specific manner in the ovary, and display spatial and temporal changes in expression depending on the stage of follicular development. For example, in rodent species, GDF-9 and BMP-15 are secreted from oocytes during

folliculogenesis [14-16]; BMP-4 and -7 are expressed by theca cells in rats [17], and BMP-6 is expressed by mouse oocytes [18]. Consistent with their ovary-specific expression, BMP-4, -6, and -7 have been shown to enhance basal and insulin-like growth factor (IGF)-stimulated secretion of estradiol, inhibin-A, activin-A, and follistatin [19]. In PCOS patients, Khalaf et al. reported an over expression of BMP-6 and BMPR1A in granulosa cells. They showed that BMP-6 and BMP-7 exerted a stimulatory effect on basal E2 production while BMP-4 and BMP-6 inhibited FSH-induced E2 production [20]. It is now well recognized that BMPs play an important regulatory role in the mammalian ovary, central to follicular recruitment and selection mechanisms.

Most of these previous BMP studies were performed with granulosa cells, while little is known regarding BMP expression levels and the interplay with hormones in the follicular fluid, a unique product of both the transfer of blood plasma constituents and the secretory activity of the oocyte and granulosa-theca cells [21]. The expression levels and effects of BMPs and hormones on ovulation in PCOS patients have not been investigated in the context of *in vitro* fertilization and embryo transplantation (IVF-ET). The aim of the present study is to determine the level of multiple BMP factors, hormones, and hormone receptors in the follicular fluid from PCOS patients. In addition, potential mechanisms of interaction between BMPs and FSHR were also investigated.

Materials and Methods

Subjects

Between Oct. 1, 2014 and Jan. 16, 2015, patients visiting The Center of Reproductive Medicine, Women Health Center in Shanxi, China were evaluated and separated into groups (PCOS and non-PCOS). Diagnosis of PCOS was based on Rotterdam Criteria [22], including oligo-ovulation and/or anovulation, excess androgen activity, and polycystic ovaries confirmed by transvaginal ultrasound. The control group consisted of tubal infertility patients. Compared to patients with PCOS, the control group has normal menstrual periods, normal endocrine levels, no abnormities of the uterus or ovaries by ultrasound evaluations, no family history of diabetes, and have not previously been exposed to hormone treatments. In total, 28 PCOS patients and 14 control patients were recruited for this comparative test. The study was IRB approved and all patients signed consent forms allowing their samples to be analyzed.

Ovarian stimulation and follicular fluid collection

Both PCOS patients and the control patients received *in vitro* fertilization and embryo transfer (IVF-ET) treatments, following either the standard long protocol or short protocol. Results of the IVF-ET treatments and the viability of fetuses will be reported separately. For the long protocol, starting at the luteal phase of the first menstrual cycle, gonadotropin-releasing hormone agonist (GnRH-a, Triptorelin Acetate, Chengdu Tiantaishan Pharmaceutical Co., Chengdu, China) was subcutaneously injected. Fourteen days after the GnRH-a injection, 150-300 U of gonadotropin FSH was administrated daily to promote follicle development (Gonal-F, Serono, Switzerland). Based on follicle development, human urinary gonadotropin (Menotrophins, Lizhu Pharma., Zhuhai, China) was administered to qualified patients during the late follicular phase. Recombinant human choriogonadotropin-alfa solution (hCG, Merck Serono S.p.A, Italy) was injected at the 250 μg dosing level when at least two follicles with ≥18 mm average diameter were detected. Transvaginal ultrasound-guided follicular aspiration was performed about 36 hours after the hCG injection and oocytes were retrieved. Human granulosa cells (GCs) were obtained from follicular fluid at the same time. For the short protocol, GnRH was injected on the second day of the menstrual cycle, and FSH was given on the third day. Three to five days later, follicle development was evaluated. If two or more follicles with ≥18 mm average diameter were detected, HCG was given and granulosa cells and oocytes were harvested 36 hours later. Samples of light-yellow, transparent follicular fluid free of blood contamination were centrifuged at 1,500xg for 10 minutes and the supernatants were stored at -80°C for FSHR and BMPs analysis.

Determining sex hormone levels in patients serum

Peripheral blood samples were collected from all subjects during the early follicular phase (days 2-5) of the menstrual cycle and on the day before HCG injection. After centrifugation, serum was collected and stored at -80°C until further analysis. Quantitative determination of sex hormone levels in serum were performed for prolactin (PRL), FSH, LH, testosterone (TES), progesterone (PROG) and E2 using chemiluminescent enzyme immunoassays via an Automated Enzyme Immunoassay Analyzer (AIA-2000ST, TOSOH CORPORATION), following the manufacturer's guidance.

Determining FSHR and BMPs levels in follicular fluid

Protein levels of FSHR, BMP-2, -4, -5, -6, -7, and -8A in follicular fluid were determined using enzyme-linked immunosorbent assay (ELISA) kits (Jianglai Biomart, Inc., Shanghai, China) following the manufacturer's instructions.

Real-time semi-quantitative PCR to test mRNA levels in GCs

Total RNA was extracted from GCs using the E.Z.N.A tissue RNA kit (OMEGA). One microgram of RNA was reverse-transcribed using the Quanti Tect Reverse Transcription kit (Qiagen). Real-time PCR (RT-PCR) was conducted using CFX Connect real-time system (Bio-Rad Laboratories, Inc.) The PCR was initiated by incubation at 95°C for 3 minutes, then denatured at 95°C for 15 seconds, annealed at 60°C for 1 minute, and extended at 72°C for 40 cycles. All analyses were performed in triplicate. Results were analyzed using CFX Manager Software. Each mRNA was normalized to GAPDH. For each sample, the value of $2^{-\Delta\Delta Ct}$ was calculated and data are graphically represented as relative expression.

The primer sequences were as follows.

FSHR [forward primer (F): AGTGTCATGGTGATGGGCTGGAT,

Reverse primer (R): GGGTTCCGCACTGTGAGGTAGAT],

LHR (F: ATTCCCAAACCAAGGGCCAGTAC,

R: GACACCGACAAGGGGCAACATAG),

BMP2(F:GGGAGAAGGAGGAGGCAAAGAAA, R:GGAAGCAGCAACGCTAGAAGACA),

BMP4 (F: ACCGAATGCTGATGGTCGTTTTA,

R: ACCGAATGCTGATGGTCGTTTTA),

BMP5 (F: ATATGGTAGTACGCTCATGTGGC,

R: TTCCCCGTTTGTCTGAAAGTATG),

BMP6 (F: AGCAAGCTGAGTTTGGATGTCTG,

R: CCCACTTCCCCGATTTCTGTTCT),

BMP7(F:CTCCAAGACGCCCAAGAACCAGG,

R: GCTGTCATCGAAGTAGAGGACGGA),

BMP8A(F:GCCAGACTTCTAGCAACTTTAGCC,

R: GACCACCCTTATTTATGCTCCTGAT),

GAPDH (F: ATGGGCAGCCGTTAGGAAAGC,

R: CCTGGAAGATGGTGATGGGATT).

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc. Chicago, IL, USA). Data were presented as mean ± standard deviation followed by the range. The Shapiro-Wilk test and one-way ANOVA was used to check data distribution. Spearman correlation coefficients were calculated to evaluate any correlations between BMPs and hormones. A p-value of less than 0.05 was considered statistically significant, while a P-value over 0.05, but less than 0.15 was considered a trend.

Results

Patient characteristics

In total, 28 cases of PCOS patients were recruited from all patients seeking reproductive assistance at The Center of Reproductive Medicine the Women's Health Center in Shanxi, China. Each PCOS case was confirmed clinically. Fourteen cases of tubal infertility were included as controls for this study. There is no significant difference between the PCOS and control groups regarding age, waist hip ratio, and the age of initial period. However, PCOS patients exhibited significantly higher body mass index than the controls (p=0.0073) (Table 1).

Characteristic	PCOS Patients (n=28)	Controls (n=14)	P value
Age	29.3 (23-35)	28.4 (24-31)	0.342
BMI (kg/m^2)	25.2 (18.7-34)	21.7 (18-29.2)	0.0073
WHR	0.8 (0.75-0.95)	0.8 (0.76-0.89)	0.4515
Age of first period	14.1 (11-23)	13.4 (11-15)	0.25
BMI: body mass index; WHR: waist/hip ratio.			

Table 1: Characteristics of PCOS patients and the controls. Data represents mean (range).

Comparison of hormone levels before Gn and hCG stimulation

Prior to oocyte stimulation, all PCOS patients took oral contraceptives (Ethinylestradiol and Cyproterone Acetate Tablets, Bayer Weimar GmbH und Co. KG, Germany) for one to three months to reduce their testosterone levels, participated in weight-control programs, and maintained healthy diets. After their testosterone level fell into the normal range (9.8-82.1 ng/dl) and was deemed compatible with pregnancy, patients received IVF-ET procedures. HCG was administrated when at least two follicles reached a mean diameter of 18 mm. Serum samples were collected first during the early follicular phase (days 2-5) of the menstrual cycle before Gn stimulation to represent baseline levels. Serum was collected again on the day before hCG injection to reflect hormone level changes. As shown in Table 2, before Gn stimulation, PCOS patients had higher LH and lower FSH levels than those in the control group, consistent with the reported higher LH/FSH ratio characteristic of PCOS. Most hormone levels such as E2, P, PRL, and T were comparable between PCOS and the controls, with the exception of FSH being significantly lower in PCOS patients (p=0.0441). All patients received Gn treatment, with similar dosing and time span. On the day before HCG administration, LH was lowered while E2 and P were markedly increased. Such a change pattern was similar for both the PCOS and the control groups.

	Hormone (unit)	PCOS Patients (n=28)	Controls (n=14)	P value
Pre-Gn stimulation level	LH (mIU/ml)	6.9 ± 3.3 (1.7-13.7)	4.7 ± 1.3 (2.6-6.2)	0.1288
	FSH (mIU/ml)	6.7 ± 1.7 (2.6-9.1)	8.1 ± 1.1 (6.4-9.7)	0.0441
	E2 (pg/ml)	54.3±23.0 (28.5-115.1)	47.3±22.0 (29.2-93.4)	0.4802
	P (ng/ml)	0.5 ± 0.2 (0.2-1.0)	0.4 ± 0.2 (0.2-0.6)	0.4985
	PRL (ng/ml)	14.0 ± 8.9 (5.3-40.6)	14.9 ± 4.3 (8.0-20.3)	0.7905
	T (ng/dl)	53.0±25.9 (18.0-114.3)	35.8±11.5 (19.7-48.0)	0.1051
Gn treatment	Gn Dose(IU)	2652±1020 (1275-5850)	3117±745.5 (1900-4278)	0.2353
	Duration (Day)	11.0 ± 2.1 (7-15)	10.4 ± 1.3 (8-12)	0.1022
Pre-HCG stimulation level	LH (mIU/ml)	2.8 ± 6.6 (0.2-26.8)	2.2 ± 1.8 (0.3-6.7)	0.7546
	E2 (pg/ml)	3180±1258 (531.1-4800)	3462±1185 (1149-4800)	0.4886
	P (ng/ml)	1.4 ± 1.4 (0.2-5.8)	1.2 ± 0.7 (0.3-2.4)	0.5529
LH: luteinizing hormone; FSH: follicle-stimulating hormone; E2: estradiol; P: progesterone; PRL: prolactin; T: testosterone.				

Table 2: Serum hormone levels before Gn stimulation and before HCG stimulation in PCOS and the control patients. Data are presented as mean±STDEV followed by range.

Hormone receptors and BMP levels

After HCG stimulation, oocytes were harvested for *in vitro* fertilization; follicular fluid was collected to evaluate protein levels of hormone receptors and BMPs, and granulosa cells collected to evaluate mRNA levels of the same hormone receptors and BMPs. As shown in Figure 1, mRNA levels of FSHR, LHR, and all BMP factors were

comparable and no statistically significant differences were detected for these factors between PCOS patients and the controls.

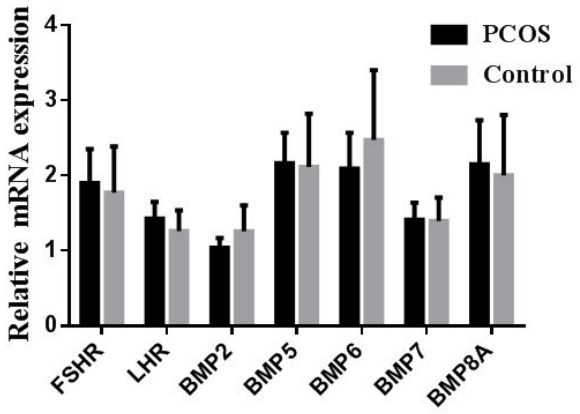

Figure 1: Comparison of FSHR and BMP mRNA levels in ovarian granulosa cells between PCOS and the control patients.

In contrast, ELISA tests revealed that the protein level of FSHR was lower in PCOS patients (Table 3), although the difference didn't reach statistical significance.

	PCOS Patients (n=28)	Controls (n=14)	P value
FSHR (IU/L)	8.4 ± 9.7 (0.8-51.8)	18.6 ± 11.7 (9.9-57.5)	0.3958
BMP-2 (ng/L)	11.0 ± 5.6 (5.4-32.2)	13.2 ± 7.6 (8.5-38.8)	0.1712
BMP-4 (pg/ml)	97.8 ± 43.5 (72.1-255.2)	129.5 ± 59.9 (89.4-328.3)	0.1558
BMP-5 (ng/L)	119.1 ± 31.8 (96-222.5)	144.5 ± 52.1 (116.2-321.4)	0.0296
BMP-6 (ng/L)	34.6 ± 5.8 (30.8-58.5)	44.8 ± 11.0 (36-78)	0.0047
BMP-7 (pg/ml)	348.6 ± 319.1 (200.3-1517)	672.8 ± 718 (279.2-3098)	0.0004
BMP-8A (ng/L)	21.2 ± 5.4 (17.1-39.8)	26.3 ± 8.6 (19.1-54)	0.0355

FSHR: follicle-stimulating hormone receptor; BMP: bone morphogenetic protein.

Table 3: Comparison of FSHR and BMP protein levels in follicular fluid between PCOS patients and the controls. Data are presented as mean±STDEV followed by range.

All BMPs protein levels were lower in PCOS patients. While the difference of BMP-2 and -4 between PCOS and the control didn't reach statistical significance, BMP-5, -6, -7, and -8A were significantly lower in PCOS patients than in the controls ($p<0.05$). Among them, BMP-7 was the most significantly reduced factor in follicular fluid in PCOS patients ($p=0.0004$).

BMPs correlated with FSHR

To understand the interplay between BMPs and ovary hormones, we evaluated the correlation between BMPs and FSHR. All BMPs positively correlated with FSHR ($p<0.0001$) (Table 4), indicating that patients with high BMPs tended to have high levels of FSHR.

Correlation with FSHR	r-value	p-value
BMP-2	0.7676	<0.0001
BMP-4	0.8808	<0.0001
BMP-5	0.8511	<0.0001
BMP-6	0.8934	<0.0001
BMP-7	0.7366	<0.0001
BMP-8A	0.7075	<0.0001

Table 4: BMPs positively correlate with FSHR.

Discussion

One feature of PCOS patients is oligo-ovulation or anovulation, thus making it hard for affected patients to conceive [1]. The process of follicle maturation and ovulation is a complex process regulated by a number of hormones and growth factors via endocrine and paracrine mechanisms [23,24]. Understanding expression levels of these factors as well as their mutual interplays will undoubtedly further our knowledge of this disease, offer insight into the mechanism of PCOS pathogenesis, and help identify novel targets for clinical intervention.

In this small study, we found that PCOS patients had an average BMI of 25.2 kg/m^2, significantly higher than what was observed in the control group. This is consistent with the knowledge that being overweight is a common feature of women with PCOS [1,25,26]. Additionally, we also observed that in serum, PCOS patients had higher LH and lower FSH levels than the control patients prior to hormone stimulation. Upon Gn treatment, accompanied by lifestyle changes and healthier diets, by the time of HCG stimulation, all patients exhibited lowered LH levels (Table 2). This suggests that successful weight loss is an effective method of restoring ovulation and menstruation that should be used as a major treatment tool in obese PCOS patients.

In recent years, the importance of BMP family members in ovulation has been increasingly appreciated [7,12,19]. In this study, protein levels of BMP-2, -4, -5, -6, -7, and -8A were measured in follicular fluid from 28 PCOS and 14 control patients. Compared to plasma, follicular fluid represents an ovary-specific microenvironment and will provide more relevant information [21]. Despite BMP mRNA levels being comparable (Figure 1), most BMP protein levels differed significantly between the PCOS and the control patients (Table 3).

BMP-2, -5, -6 are produced by GCs [7]. They can regulate GC differentiation and function as well as oocyte and theca cells [7]. Our results indicate that at the mRNA level BMP-2, -5, -6 are similarly expressed in PCOS and control patients (Figure 1); yet at the protein level, all three factors were less abundant in PCOS patients (Table 3).

The mechanism is not clear, and may involve a defect in the translational machinery or enhanced degradation of these factors. Nevertheless, lower protein levels of these markers may indicate an overall weakened BMP signaling pathway.

BMP-4 and -7 are secreted by theca cells [12,27]. Intrabursal administration of BMP-7 can reduce primordial follicle number while increasing the number of primary, preantral and antral follicles, suggesting positive paracrine action of theca-derived BMP-7 on GCs of growing preantral follicles. Similarly, BMP-4 has been shown to increase the number of developing preantral follicles in cultured neonatal rat ovaries. Our data demonstrated that BMP-7 was the most significantly reduced factor in PCOS follicular fluid ($p=0.0004$), suggesting that weakened BMP-7 signaling may play a crucial role in affected oocyte maturation in PCOS patients.

BMP-8A is expressed primarily in developing skeletal tissues [28]. It is also involved in the maintenance of spermatogenesis and the integrity of the epididymis [29]. Although its role in oocyte maturation remains largely unknown, our data did reveal a significant reduction in its level in PCOS patients. Further studies will be required to fully clarify the function of BMP-8A.

Lastly, we found that all BMPs positively correlated with FSHR (Table 4). FSHR expression on target cells is essential for modulation of ovarian function by FSH. FSHR is not expressed until midway through follicle development, and in mature follicles, maintenance of FSHR expression is required to avoid death by atresia [30]. Our data confirmed strong positive correlations between BMPs and FSHR. Since PCOS is a well-known endocrine-metabolic disorder, BMPs may play important roles in endocrine homeostasis in PCOS.

To our knowledge, this is the first report of multiple BMP levels in follicular fluid in PCOS patients. Several of our data should be highlighted. First, BMP-7 is the most significantly down-regulated protein in PCOS patients. Second, all BMPs significantly correlated with FSHR. Accumulating evidence indicates that BMP-7 is a cytokine with pleiotropic functions. Its potential application as a therapeutic target has been suggested in treatment of cardiovascular [31], fibrotic [32], metabolic [33], and neurodegenerative diseases [34,35]. Additionally, Townsend et al. reported that systemic treatment of obese mice with BMP-7 resulted in increased energy expenditure and decreased food intake, leading to a significant reduction in body weight and improvement of metabolic syndrome [36]. It is likely that lowered BMP-7 levels in follicular fluid, higher BMI in PCOS patients, hormone imbalance are all related. Our work suggests that intrabursal administration of BMP-7 may prove beneficial to facilitate oocyte maturation, and could potentially help PCOS patients to restore balanced endocrine/paracrine regulation. The clinical application of this finding awaits further investigation.

Trial Registration

Department of Health of Shanxi Province, # 201201014.

Competing Interests

The authors claim no conflict of interest.

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