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ASSISTED REPRODUCTION TECHNOLOGIES

# Inhibitory effects of controlled ovarian stimulation on the expression of GDF9 and BMP15 in oocytes from women with PCOS

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**Abstract**
*Purpose* To explore the effects of controlled ovarian stimulation (COS) on the expression of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) in oocytes and granulosa cells from patients with or without polycystic ovary syndrome (PCOS).
*Methods* This case–control study was conducted in the university affiliated hospital. The study comprised four groups of patients: eighteen PCOS patients with COS (stimulated-PCOS) and twenty-two PCOS patients without COS (unstimulated-PCOS), twenty-nine normal ovulatory women with COS (stimulated-control) and twenty-eight normal ovulatory women without COS (unstimulated-control). The oocytes and granulosa cells were collected and the abundance of GDF9 and BMP15 mRNA in the cells were detected by nested quantitative real-time PCR.
*Results* The abundance of GDF9 and BMP15 mRNA was significantly higher both in oocytes ($P<0.01$, $P<0.001$, respectively) and GCs ($P<0.01$, $P<0.05$, respectively) from stimulated-control group than in unstimulated-control group. However, there was no significant difference for GDF9 or BMP15 mRNA in oocytes from stimulated-PCOS goup compared with unstimulated-PCOS group ($P>0.05$, $P>0.05$, respectively). The abundance of GDF9 mRNA was significantly lower ($P<0.01$) while the abundance of BMP15 mRNA was significantly higher ($P<0.001$) in GCs from stimulated-PCOS group than in unstimulated-PCOS group.
*Conclusions* The controlled ovarian stimulation can promote the expression of GDF9 and BMP15 both in oocytes and GCs from normal ovulatory women. However, the stimulating effects may be inhibited in oocytes from PCOS patients, which subsequently impair cytoplasm maturation and lead to poor oocyte quality.

**Keywords** Growth differentiation factor 9 · Bone morphogenetic protein 15 · Polycystic ovary syndrome · Controlled ovarian stimulation · Oocyte quality

*Capsule* Inhibited expression of OSFs in PCOS oocytes.

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## Introduction

As one of the most common endocrine disorders, polycystic ovary syndrome (PCOS) affects up to one-fifth of reproductive women and accounts for 90 %–95 % of patients with anovulatory infertility [1–3]. Although a variety of controlled ovarian stimulation (COS) protocols are used for ovulation induction in women with PCOS, the pregnancy outcomes of PCOS patients are still unsatisfactory. The data from a meta-analysis has demonstrated a higher cancellation rate and lower fertilization rate in women with PCOS [4], which indicates impaired oocyte developmental competence.

More and more surveys have shown that oocyte-secreted factors (OSFs) are closely associated with oocyte quality and developmental potential [5], such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). Belonging to the transforming growth factor β (TGFβ) superfamily, GDF9 and BMP15 play crucial roles in follicular development, ovulation, oocyte maturation, and embryo development [6–9]. It has been documented that the ultrastructure of oocytes is abnormal in *gdf9*-deficient

female mice, and ovulation and the fertilization rates are decreased in the *bmp15* knock-out model [10]. Furthermore, the addition of OSFs to culture media can promote blastocyst formation and enhance fetal viability in different animal models [11, 12].

Since all of the oocytes were collected from stimulated cycles in that study, it is still unknown whether the oocyte response to ovarian stimulation is different between PCOS and normal ovulatory women. Due to close relationship between oocytes and granulosa cells, the effects of ovarian stimulation on granulosa cells are also worthy to be studied. This study was designed to determine the effects of COS on the expression of GDF9 and BMP15 in oocytes and granulosa cells from PCOS and normal ovulatory women, and to explore the difference of the effects between the two groups.

## Materials and methods

### Subjects

The present study was approved by the Institutional Review Board of Sun Yat-sen University and informed patient consents were obtained before the initiation of the study. This study included four groups of patients from May 27, 2010 to May 31, 2012: eighteen PCOS patients with COS (stimulated-PCOS) and twenty-two PCOS patients without COS (unstimulated-PCOS), twenty-nine normal ovulatory women with COS (stimulated-control) and twenty-eight normal ovulatory women without COS (unstimulated-control).

PCOS was diagnosed according to the Rotterdam criteria [13]. The selection criteria for normal ovulatory women were as follows: regular menstrual cycles (21–35 days); regular ovulations (confirmed by basal body temperature testing); basal follicle stimulating hormone (FSH) <10 IU/L; and normal body mass index (BMI, 18.5–23.0 kg/m$^2$). The exclusion criteria in all cases were as follows: premature ovarian failure, endometriosis, thyroid dysfunction, ovulation induction or sexual hormone medications within 3 months.

### Controlled ovarian stimulation

A standard long protocol was used in all cases. Briefly, gonadotropin-releasing hormone agonist (Triptorelin; Ipsen, Paris, France) was used during the mid-luteal phase for desensitization, followed by rhFSH (Gonal-F; Serono, Geneva, Switzerland) from the second or third day of menstruation. The initial dose of gonadotropin was between 75 and 300 IU, depending on age, antral follicle count, basal FSH level, and ovarian response in previous treatment cycles. Transvaginal ultrasonography was performed every 3–4 days to monitor follicular development and to adjust the dose of gonadotropins. When the dominant follicle was ≥18 mm in diameter or at least 3 follicles were ≥16 mm in diameter, rhFSH was stopped and a single injection of 10,000 IU of hCG (Serono, Geneva, Switzerland) was administered. Oocyte retrieval was performed 36–40 h later under transvaginal ultrasound guidance.

### Collection of oocytes and GCs

In stimulated-PCOS and stimulated-control groups, ooctyes and GCs were collected by egg retrieving surgery during Intracytoplasmic sperm injection (ICSI) therapeutic cycle. In unstimulated-PCOS group, the samples were collected by puncturing ovaries of PCOS patients with 20G needle under ultrasound monitoring. In unstimulated-control group, the patients accepted laparoscopy for extra-ovarian factors but still have normal cycle. Oocytes were isolated from ovarian cortexes with fine needle from antral follicles under dissecting microscope, and GCs were collected by centrifugation from the follicular fluid. Only one oocyte and one case of GCs were obtained from each patient. All of the surrounding cells were mechanically stripped so the denuded oocytes could be identified as a germinal vesicle (GV), or metaphase I (MI) or metaphase II (MII) oocyte according to the presence of a GV or the first polar body. As the oocytes were all at the GV stage in unstimulated groups, only GV oocytes were investigated in the present study. The nude oocytes and GCs were washed in PBS and transferred into RNase-free microcentrifuge tubes individually, followed by an addition of 50 μl of Trizol (Invitrogen, Carlsbad, CA, USA). All samples were stored in a −80 °C freezer until analysis.

### Nested quantitative real-time PCR

The nested quantitative real-time PCR was used to detect the abundance of GDF9 and BMP15 mRNA in each oocyte and each case of GCs. Briefly, total RNA was retracted and reversely transcribed into cDNA, then amplified with Taqman probes for quantitative analysis. The protocol for amplification of cDNA used an initial denaturizing step at 93 °C for 5 min followed by 30 cycles of 30 s at 93 °C, 45 s at 55 °C, 45 s at 72 °C, and finally 7 min at 72 °C. The protocol for quantitative real time PCR used an initial denaturizing step at 93 °C for 3 min followed by 40 cycles of 30 s at 93 °C and 45 s at 55 °C. Three replicates for each sample were performed and the means were used for statistical analysis. Relative gene expression was calculated as the abundance ratio of each target gene to $\beta$-actin. The specific primer sequences were summarized in Table 1.

### Statistical analyses

All statistical procedures were run on SPSS 11.5 (SPSS Inc., Chicago, Il, USA). General conditions were compared by

**Table 1** Information of specific primers used in nested quantitative real-time PCR analysis

| Gene | Primer sequence (5′→3′) | Size (bp) | Accession no. |
|---|---|---|---|
| GDF9 | | | |
| First Pair | Forward CGTCCCAACAAATTCCTCCTT<br>Reverse AGGCCAGCTCTGTCTCTCTCAT | 176 | NM_005260 |
| Second Pair | Forward CTGCTTTGCCTGGCTGTGT<br>Reverse CAAGGCATAGCCCCAGATTC | 105 | |
| BMP15 | | | |
| First Pair | Forward ACCATGGTGAGGCTGGTGAA<br>Reverse ACATGGCAGGAGAGATTGAAGC | 179 | NM_005448 |
| Second Pair | Forward GGCAAGGCCTCACAGAGGTA<br>Reverse CGGTAAACCACAGTGGCTCTAAC | 102 | |
| $\beta$-actin | | | |
| First Pair | Forward CTTACAGATCATGTTTGAGACCTTCAA<br>Reverse CTCAGGGCAGCGGAACC | 417 | NM_001101 |
| Second Pair | Forward GCGCGGCTACAGCTTCA<br>Reverse TCTCCTTAATGTCACGCACGA T | 59 | |

one-way ANOVA (LSD test). Since the values of OSFs mRNA expression were not normally distributed, the comparisons between unstimulated and stimulated groups were performed using Mann–Whitney test. $P<0.05$ was considered significant.

## Results

### General conditions

There were no significant differences in the general conditions among the four groups of patients (Table 2).

### Effects of COS on the expression of GDF9 and BMP15 mRNA in oocytes and granulosa cells from normal ovulatory women

In unstimulated oocytes from normal ovulatory women, the level of GDF9 mRNA ranged from 2.96 (25th percentile) to 109.73 (75th percentile) with a median of 24.79. The results in stimulated oocytes ranged from 55.38 (25th percentile) to 387.93 (75th percentile) with a median of 149.94. There was a significant increase in stimulated oocytes for GDF9 mRNA ($P<0.01$).

Also in these unstimulated oocytes, the level of BMP15 mRNA ranged from 0.05 (25th percentile) to 3.65 (75th percentile) with a median of 0.93. The results in stimulated oocytes ranged from 6.50 (25th percentile) to 96.11 (75th percentile) with a median of 41.65. Similarly, there was a significant increase in stimulated oocytes for BMP15 mRNA ($P<0.001$).

In unstimulated GCs from normal ovulatory women, the level of GDF9 mRNA ranged from 0.009 (25th percentile) to 0.21(75th percentile) with a median of 0.02. The results in stimulated GCs ranged from 0.06 (25th percentile) to 0.18 (75th percentile) with a median of 0.10. There was also a significant increase in stimulated GCs for GDF9 mRNA ($P<0.01$).

**Table 2** Comparisons of general conditions among four groups

| General indicators | Normal ovulatory women | | PCOS patients | | *P* value |
|---|---|---|---|---|---|
| | Unstimulated-control | Stimulated-control | Unstimulated-PCOS | Stimulated-PCOS | |
| No. of cases | 28 | 29 | 22 | 18 | |
| Age (y) | 30.79±2.61 | 30.62±3.00 | 30.55±1.69 | 28.67±2.45 | >0.05 |
| BMI(kg/m$^2$) | 21.28±1.10 | 20.40±1.50 | 20.96±1.53 | 19.35±1.55 | >0.05 |
| FSH (IU/L) | 5.14±1.21 | 5.13±1.40 | 4.29±1.07 | 4.73±1.04 | >0.05 |
| LH (IU/L) | 3.78±2.15 | 3.91±2.88 | 6.32±4.18 | 6.80±3.33 | >0.05 |
| PRL (μg/L) | 18.11±9.40 | 16.46±6.58 | 17.04±5.72 | 21.26±6.29 | >0.05 |
| $E_2$ (ng/mL) | 35.57±17.83 | 37.79±19.13 | 39.69±20.92 | 54.33±20.49 | >0.05 |
| T (nmol/L) | 0.73±0.25 | 0.69±0.25 | 0.89±0.34 | 1.11±0.29 | >0.05 |
| Gn dosage (IU) | – | 2082.76±627.96 | – | 1766.67±195.26 | >0.05 |

The data were expressed as the mean ± SD, and one-way ANOVA (LSD test) was used for multiple comparisons among four groups

**Table 3** Effects of controlled ovarian stimulation on the expression of GDF9 and BMP15 mRNA in oocytes and graunlosa cells from normal ovulatory women

| Samples | Genes | Unstimulated-control | Stimulated-control | *P* value |
|---|---|---|---|---|
| Oocytes | GDF9 | 24.79 (2.96–109.73) | 149.94 (55.38–387.93) | <0.01 |
| | BMP15 | 0.93 (0.05–3.65) | 41.65 (6.50–96.11) | <0.001 |
| GCs | GDF9 | 0.02 (0.009, 0.21) | 0.10 (0.06, 0.18) | <0.01 |
| | BMP15 | 0.008(0.001–0.16) | 0.02(0.01–0.03) | <0.05 |

The data were expressed as the median with the 25th–75th percentile range in *parentheses*. Mann–Whitney test was used for comparisons between Unstimulated-control and Stimulated-control groups

In the unstimulated GCs, the level of BMP15 mRNA ranged from 0.001(25th percentile) to 0.16(75th percentile) with a median of 0.008. The results in stimulated GCs ranged from 0.01 (25th percentile) to 0.03 (75th percentile) with a median of 0.02. There was also a significant increase in stimulated GCs for BMP15 mRNA ($P<0.05$).

Above results were summarized in Table 3.

### Effects of COS on the expression of GDF9 and BMP15 mRNA in oocytes and granulosa cells from PCOS patients

In unstimulated oocytes from PCOS patients, the level of GDF9 mRNA ranged from 2.29 (25th percentile) to 65.72 (75th percentile) with a median of 23.83. The results in stimulated oocytes ranged from 5.93 (25th percentile) to 489.19 (75th percentile) with a median of 44.81. There was no significant difference between the two groups ($P>0.05$).

Also in these unstimulated oocytes, the level of BMP15 mRNA ranged from 0.05 (25th percentile) to 29.32 (75th percentile) with a median of 0.09. The results in stimulated oocytes ranged from 0.05 (25th percentile) to 11.44 (75th percentile) with a median of 0.10. There was no significant difference between the two groups ($P>0.05$).

In unstimulated GCs from PCOS patients, the level of GDF9 mRNA ranged from 0.06 (25th percentile) to 0.16 (75th percentile) with a median of 0.11. The results in stimulated GCs ranged from 0.03 (25th percentile) to 0.09 (75th percentile) with a median of 0.05. The level of GDF9 mRNA was significantly lower in GCs from stimulated-PCOS group ($P<0.01$).

Also in the unstimulated GCs, the level of BMP15 mRNA ranged from $0.48\times10^{-5}$ (25th percentile) to $0.9\times10^{-5}$ (75th percentile) with a median of $0.5\times10^{-5}$. The results in stimulated GCs ranged from 0.007 (25th percentile) to 0.03 (75th percentile) with a median of 0.02. The level of BMP15 mRNA was significantly higher in GCs from stimulated-PCOS group ($P<0.001$).

Above results were summarized in Table 4.

## Discussion

Because oocyte-secreted factors (OSFs) play crucial roles in follicular development and oocyte maturation, we wonder whether the expression level and pattern of OSFs in follicles of PCOS patients is abmormal, and the effects of the ovarian stimulation on the expression of OSFs.

The results showed that the ovarian stimulation could promote the expression of GDF9 and BMP15 in oocytes from normal ovulatory women, in according with the research in mice, which demonstrated high FSH could upregulate the expression of GDF9 and BMP15 in oocytes [14]. After an exhaustive literature search, it was determined that the present study examined, for the first time, the effects of ovarian stimulation on the expression of GDF9 and BMP15 in human oocytes. The results interestingly indicate that there are some cross-talks between endocrine and paracrine/autocrine factors within the ovary, which promote the development of follicles in a

**Table 4** Effects of controlled ovarian stimulation on the expression of GDF9 and BMP15 mRNA in oocytes and granulosa cells from patients with PCOS

| Samples | Genes | Unstimulated-PCOS | Stimulated-PCOS | *P* value |
|---|---|---|---|---|
| Oocytes | GDF9 | 23.83 (2.29–65.72) | 44.81 (5.93–489.19) | >0.05 |
| | BMP15 | 0.09 (0.05–29.32) | 0.10 (0.05–11.44) | >0.05 |
| GCs | GDF9 | 0.11 (0.06, 0.16) | 0.05 (0.03, 0.09) | <0.01 |
| | BMP15 | $0.5\times10^{-5}$ ($0.48\times10^{-5}$–$0.9\times10^{-5}$) | 0.02,0.007–0.03) | <0.001 |

The data were expressed as the median with the 25th–75th percentile range in *parentheses*. Mann–Whitney test was used for comparisons between Unstimulated-PCOS and Stimulated-PCOS groups

synergistic way. However, the exact mechanisms still need to be investigated further.

Differently, there were no significant effects of the ovarian stimulation on the expression of GDF9 and BMP15 in oocytes from PCOS patients, suggesting poor response to the gonadotropins. Some paracrine disorders complicated in PCOS patients may contribute to the pathogenesis. It is speculated that the excessive secretion of anti-Mullerian hormone (AMH) is the basis of this finding. As a member of the TGF-β superfamily, AMH primarily plays a role in inhibiting the recruitment and selection of follicles [15]. It has been documented that the level of AMH is increased significantly, while the secretion of FSH is relatively insufficient in the follicular fluid of PCOS patients [16]. Thus the stimulating effects of FSH may be depressed by the amplified effect of AMH which has been proved to inhibit FSH-induced aromatase expression and $E_2$ production [17, 18]. Besides that, other endocrine disorders such as hyperandrogenism and hyperinsulinism which cause aberrant folliculogenesis in PCOS patients may also play roles in depressing the stimulating effect of FSH and inhibiting the expression of OSFs in the ooctyes [19].

In granulosa cells from normal ovulatory women, the level of GDF9 and BMP15 mRNA is significantly higher in stimulated group than in unstimulated group, which is consistent with that in oocytes. The function status of granulosa cells are the mirror of oocyte quality [20–22]. The results showed that gonadotropins may be beneficial to oocyte quality in an indirect way by acting on granulosa cells. But in granulosa cells from PCOS patients, the level of GDF9 mRNA is significantly lower while the level of BMP15 mRNA is significantly higher in stimulated group than in unstimulated group, which is totally opposite to each other. The aberrant expression pattern in granulosa cells from PCOS patients may be correlated with abnormal follicular development in PCOS.

We also found that the expression of OSFs in granulosa cells was significantly lower than that in oocytes, since OSFs are mainly secreted by oocytes. Moreover, the results showed that the expression of GDF9 mRNA was significantly higher than BMP15 mRNA in the same oocyte and the same case of granulosa cells, which suggests the proper ratio of GDF9/BMP15 should be exploited in future culture system in order to harvest the best oocytes and embryos.

In summary, this study demonstrates that COS can facilitate the expression of GDF9 and BMP15 both in oocytes and granulosa cells from normal ovulatory women, which is not shown in patients with PCOS. Some endocrine disorders complicated in PCOS patients may contribute to the insufficient response to ovarian stimulation, but the exact mechanisms should be studied further. These results may enlighten a new way for improving ovarian stimulation protocols and optimizing the present culture system for human oocytes.

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