Study of GDF9 and BMP15 mRNA profiles in granulosa cells to predict oocyte quality in endometriosis patients undergoing in vitro fertilization

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Abstract. Growth differentiation factor-9 (GDF9) and bone morphogenetic 15 (BMP15) are oocyte-secreted factors (OSF) that are proteins and are members of the transforming growth factor β superfamily with roles in folliculogenesis to produce mature oocytes. GDF9 and BMP15 are candidates in the search for selective markers to predict oocyte quality. Women with endometriosis undergoing in vitro fertilization (IVF) usually present with reduced quality of their oocytes. The aim of this study is to examine GDF9 and BMP15 expressions in granulosa cells to evaluate oocyte quality in these patients. We collected 20 granulosa cell samples from patients suffering from endometriosis (test group) along with 12 samples of granulosa cells from those without endometriosis (control group) and measured GDF9 and BMP15 expressions. Absolute quantification real-time PCR was used to quantify gene expression levels, using pre-manufactured oligonucleotides as standards. Our results showed no significance differences in GDF9 or BMP15 expressions between patients with endometriosis and those without it (both p > 0.05). Hence, the expression levels of gdf9 and bmp15 should not be considered when predicting oocyte quality. Further investigations regarding other factors affecting oocyte quality should be conducted to obtain more information.

1. Introduction
Oocytes contain important components for the cellular mechanisms of fertilization, activation, genome remodeling, and epigenetic programing at an early stage of development [1]. The development of oocytes is influenced by endocrine, paracrine, and autocrine factors [2]. Granulosa cells are a component of the paracrine factors affecting the quality of oocytes. The development of follicles requires a reciprocal relationship between oocyte and granulosa cells for adequate development of both cell types [2].

Oocyte quality can be assessed by several methods such as observing their morphology, calculating their fertilization rate, and quantifying the gene expression profile of granulosa cells surrounding the
oocyte itself. The third method is most commonly used because it does not require direct contact with the oocyte [3]. The recurrent communication and interdependent association between granulosa cells and oocytes make granulosa cells a suitable candidate for reflecting the oocyte quality. Interactions between oocytes and granulosa cells are generally mediated by proteins of the transforming growth factor β (TGF-β) superfamily.

The growth differentiation factor-9 (GDF9) and bone morphogenetic 15 (BMP15) are members of the TGF-β superfamily with synergistic roles in the development, resistance, and function of oocytes. According to Li et al. (2014) [4], GDF9 and BMP15 are expressed by both oocytes and granulosa cells. Both genes are also found in the liquid follicles of patients undergoing in vitro fertilization and are associated with increased fertilization and embryonic development rates [5,6].

2. Methods
The study protocol had been approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital. Granulosa cells collected from patients confirmed as having endometriosis were used in this study. There were 20 granulosa cell samples obtained from women with endometriosis and 12 samples from those without endometriosis.

Total RNA from the samples was extracted using the QIAamp RNA Blood Mini Kit (QIAGEN; 52304), according to the manufacturer’s instructions. RNA was then reverse transcribed into cDNA using a Quantitect Reverse Transcription Kit (QIAGEN; 205311). The cDNA was used as a template for qRT PCR using the Quantitect SYBR Green PCR Kit (QIAGEN; 204143) and the primers represented in Table 1.

A standard curve was obtained using G-blocks oligonucleotide for each gene. Additionally, the quantification results of the GDF9 and BMP15 mRNA expressions was tested by t-test.

3. Results and Discussion
Overall, RNA from 40 granulosa cell samples was isolated, and the purity and quantity of each sample were verified (Table 2). Next, cDNA from each sample was synthesized at a final concentration of 5 ng/µl.

**Table 1. Forward and reverse primers for GDF9 and BMP15**

| Gene  | Primer   | Primer Sequence (5' to 3')          | Amplicon Size (bp) | Tm (°C) |
|-------|----------|------------------------------------|--------------------|---------|
| **GDF9** | Forward  | GAGTGTGAGCTCCATGACTTT             | 121                | 58      |
|       | Reverse  | CCCTTTACAGTATCGAGGTGTTG           |                    | 59      |
| **BMP15** | Forward  | GGAGTTCATGGAAAGGGAATCT            | 133                | 61      |
|       | Reverse  | GTGGAGGGAACACTGGTTATT             |                    | 58      |

A standard curve was obtained using G-blocks oligonucleotide for each gene. Additionally, the quantification results of the GDF9 and BMP15 mRNA expressions was tested by t-test.

**Table 2. RNA concentration and purity**

| No | CASE GROUP | CONTROL GROUP |
|----|------------|---------------|
|    | RNA concentration (ng/µl) | Purity (260/280) | RNA concentration (ng/µl) | Purity (260/280) |
|----|------------------|-----------------|------------------|-----------------|
| 1  | 13.5             | 2.09            | 47.9             | 1.83            |
| 2  | 12.4             | 2.09            | 16.2             | 2.16            |
| 3  | 28.4             | 2.11            | 25.4             | 1.65            |
| 4  | 15.5             | 0.5             | 46.8             | 2.07            |
| 5  | 17.3             | 2.11            | 24               | 1.75            |
| 6  | 10.1             | 1.99            | 12.1             | 0.35            |
| 7  | 8.5              | 1.98            | 22.1             | 1.35            |
Table 2. Continue

| No | CASE GROUP | CONTROL GROUP |
|----|------------|---------------|
|    | RNA concentration (ng/µl) | Purity (260/280) | RNA concentration (ng/µl) | Purity (260/280) |
| 8  | 15.4       | 0.69          | 22.3           | 1.56          |
| 9  | 15.6       | 1.31          | 28.4           | 2.23          |
| 10 | 12.1       | 0.37          | 21.1           | 1.45          |
| 11 | 62.6       | 2.07          | 43.4           | 2.07          |
| 12 | 12.1       | 2.28          | 66.2           | 2.08          |
| 13 | 24.0       | 2.08          | 83.9           | 2.13          |
| 14 | 12.6       | 0.35          | 145.4          | 2.11          |
| 15 | 10.5       | 1.57          | 134.4          | 2.11          |
| 16 | 13.3       | 2.00          | 83.8           | 2.11          |
| 17 | 44.8       | 2.13          | 190.0          | 2.08          |
| 18 | 303.8      | 2.09          | 53.9           | 2.1           |
| 19 | 10.7       | 2.08          | 92.2           | 2.13          |
| 20 | 11.7       | 2.05          | 38.5           | 2.16          |

The variability in the quantities of total RNA isolates may be caused by differences in the number of granulosa cells isolated. However, this study could not confirm the minimum number of granulosa cells due to the varieties of cells obtained from each patient. The low RNA concentration yields may also be due to the freezing and thawing of the samples.

The mRNA expression for GDF9 and BMP15 in 40 and 23 samples was quantified, respectively. The granulosa cells from the control group tended to show higher expression of GDF9 than those obtained from women with endometriosis. However, the difference was not significant.

Table 3. GDF9 and BMP15 concentration

| No | CASE GROUP | CONTROL GROUP |
|----|------------|---------------|
|    | GDF9 concentration (ng/µl) | BMP15 concentration (ng/µl) | GDF9 concentration (ng/µl) | BMP15 concentration (ng/µl) |
| 1  | 4.262E+00  | 4.427E+00     | 4.046E+00      | 2.317E+00      |
| 2  | 1.334E+00  | 3.571E+02     | 1.620E-04     | 3.090E+01     |
| 3  | 3.242E+03  | 2.868E+00     | 1.312E-04     | 2.189E+01     |
| 4  | 1.895E+04  | 3.019E+01     | 7.275E+00     | 1.455E+01     |
| 5  | 3.523E+00  | 1.206E+01     | 6.398E-01     | 1.256E+02     |
| 6  | 2.509E-05  | 1.455E+01     | 2.397E+00     | 1.865E+01     |
| 7  | 2.823E+01  | 4.122E+00     | 5.602E+00     | 6.353E+00     |
| 8  | 2.755E-05  | 7.388E+00     | 1.021E+01     | 1.951E+01     |
| 9  | 9.362E-01  | 3.734E+01     | 1.094E-03     | 3.523E+00     |
| 10 | 2.729E-03  | 1.033E+00     | 1.486E+00     | 2.868E+00     |
| 11 | 1.722E+00  | -             | 1.621E-02     | 2.868E-00     |
| 12 | 6.150E-05  | -             | 8.193E+01     | 1.722E+00     |
| 13 | 8.380E-01  | -             | 3.541E+02     | 1.206E+01     |
| 14 | 5.124E+00  | -             | 9.996E+02     | -             |
| 15 | 1.088E+04  | -             | 9.541E+00     | -             |
| 16 | 1.807E+01  | -             | 2.787E+02     | -             |
| 17 | 5.049E+00  | -             | 8.718E+02     | -             |
| 18 | 2.049E+01  | -             | 3.245E+05     | -             |
| 19 | 2.030E+02  | -             | 5.660E+02     | -             |
| 20 | 6.120E-04  | -             | 2.454E+02     | -             |
This study was only able to obtain BMP15 amplicons from 23 of the 40 samples (13 samples from the control group and 10 from the test samples). The results showed no significant differences.

Pearson correlation tests was also performed and it found no significant correlations in GDF9 and BMP15 expressions in granulosa cells with the level of maturity, fertilization rate, or division rate. It is possible this was due to the limited sample number and quantities. In addition, the low expressions of GDF9 and BMP15 may have been the causes of the weak correlations.

BMP15 and GDF9 are 2 well-described oocyte-derived growth factors that synergistically act to promote the ovulation of oocytes surrounded by granulosa cells. GDF9 protein plays an important role in controlling oocyte maturation during folliculogenesis. Al-Edani et al. showed that some members of the TGF-β superfamily are expressed in small quantities in granulosa cells of women who have passed their reproductive age. Other members of the TGF-β superfamily include anti-mullerian hormone, TGF-β1, inhibin, and activator receptors (ACVR2B) [7].

The GDF9 mRNA expression in women with endometriosis is lower than that in women without endometriosis (control group). We found the average GDF9 expression in patients with endometriosis was 1.76E+06, whereas in the control group, it was 1.64E+07. Higher GDF9 mRNA expression in women without endometriosis reflects better oocyte–granulosa communication compared with those with endometriosis.

BMP15 is a member of the TGFβ superfamily that also affects the physiology and growth of follicles. This protein is also a functional growth factor of oocytes regulating proliferation and differentiation of granulosa cells. BMP15 affects the development of the closest granulosa cell layer to oocytes, the cumulus cell layer [2].

Meanwhile, BMP15 mRNA expression in granulosa cells derived from women with endometriosis had values that were almost similar to those of women in the control group. Our statistical tests found no significant difference between the 2 groups. The average BMP15 expression level in endometriosis patients was 4.64E+04 and that in the control group was 2.02E+04.

4. Conclusion
This study found that GDF9 mRNA tended to be more highly expressed in women without endometriosis than in those with endometriosis; however, the difference was not significant. This result is supported by metadata showing that oocyte quality is better in women without endometriosis. BMP15 mRNA did not show any tendency to be higher or lower in our groups. Further research is needed to explore other biomarkers for assessing oocyte quality. Other studies should also include larger numbers of samples and repetitions to reduce data bias and to have more reliable statistical data.

References
[1] Desai S, Parihar M and Allahabadia G 2003 Infertility: Principles and practice. BI Publications Ltd, New Delhi: xv + 404 hlm
[2] Coticchio G, Albertini D F and Santis L D 2012 Oogenesis. Springer Science & Business Media, London: xii + 376 hlm
[3] Hamel et al 2008 Identification of differentially expressed markers in human follicular cells associated with competent oocytes Hum Reprod. 23 1118-27
[4] Li Y, Li R Q, Ou S B, Zhang N F, Ren L, Wei L N, Zhang Q X and Yang D Z 2014 Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans Reprod. Biol. Endocrinol. 9 81–90
[5] Wu Y, Tang L, Cai J, Lu X, Zhu X, Luo Q and Huang H 2007 High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development Hum. Reprod. 22 1526–31
[6] Strauss J F and Barbieri R L 2013 Yen and Jaffe’s reproductive endocrinology (Philadelphia: Elsevier Health Science) p 942
[7] Al-Edani T, Assou S, Ferrieres A, Deutsch S B, Gala A, Lecellier C, Ait-Ahmed O and Hamamah S 2014 Female aging alters expression of human cumulus cell genes that are essential for oocyte quality Biomed Research International 96 1–10.