Expressions of growth differentiation factor-9 on granulosa cells of infertile women with endometriosis undergoing in vitro fertilization

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Abstract. Endometriosis may cause ovarian physiology disturbances and one of them, by affecting folliculogenesis that leads to a decreased oocyte quality. The oocyte plays an important role in regulating and promoting follicle growth by oocyte growth factors production. Several growth factors have been identified in human oocytes, including growth differentiation factor-9 (GDF-9). However, studies on GDF-9 expression in the granulosa cells of infertile women with endometriosis are sparse. To investigate the expression of GDF-9 mRNA in the granulosa cells of endometriosis patients undergoing in vitro fertilization (IVF) and identify the correlation between GDF-9 expression and oocyte quality. This cross-sectional study was conducted at the Yasmin IVF Clinic and Dr. Sander B Clinic, Jakarta from July 2014 to July 2017. In total, 50 granulosa cell samples were collected from 25 women with endometriosis and 25 controls who were healthy. The granulosa cell samples were collected at the time of oocyte retrieval. GDF-9 mRNA expression was investigated by real-time PCR. The numbers of retrieved oocytes, mature oocytes, and the oocyte morphology score were lower in patients with endometriosis than in controls; the difference was statistically significant. GDF-9 mRNA expression levels were quantitatively lower in the endometriosis groups than in the control group (5.05 [0.00002–3523.0] ng/mL vs. 81.93 [1.47–32450] ng/μL; p = 0.01). However, no correlation was obtained between GDF-9 expression levels and oocyte quality (oocyte morphology score and fertilization rate). The GDF9 mRNA level was lower in the endometriosis group than in the control group. However, no direct relationship was noted between individual GDF-9 level and oocyte quality. Hence, large-scale studies are needed to confirm whether GDF-9 expression is correlated with the oocyte quality and to ascertain whether GDF-9 has the potential for use as a new molecular marker to predict oocyte developmental competency.

1. Introduction

Endometriosis is a chronic gynecological condition with a prevalence rate of 5%–12% in women of reproductive age and causes dysmenorrhea, dyspareunia, pelvic pain, and infertility [1]. A study suggested that 25%–50% of women with infertility have endometriosis and vice versa, whereas 30%–
50% of women with endometriosis face infertility issues. To date, the concept of ideal therapy for infertility in women with endometriosis remains debatable [2]. However, among all known approaches, assisted reproductive therapy (ART) has been reported to have the highest success rate [3,4].

The conclusions reached by studies on the outcomes of in vitro fertilization (IVF) in endometriosis patients are diverse. A meta-analysis study of the effects of endometriosis on the outcomes of IVF concluded that endometriosis interferes with all the aspects of the reproductive process; therefore, the IVF success rate in women with endometriosis has only reached approximately half of the treated population with other IVF indications [5]. Harb et al. [6] reported that severe endometriosis affects implantation and clinical pregnancy ratios. In addition, a systematic review of the literature has indicated no significant difference in the clinical pregnancy outcomes and live births between women with or without endometriosis in fresh cycles. However, the same study mentioned that patients with endometriosis have reduced ovarian reserve, that is, they have fewer oocytes. The presence of an association between ovarian reserves and poor reproductive outcomes may explain the diverse nature of these conclusions. A recent meta-analysis suggested that women with endometriosis have a lower ART success rate than women without endometriosis. The number of oocytes obtained per cycle and the rate of fertilization were also influenced. This finding supports the hypothesis that endometriosis affects ovarian reserve and oocyte quality [7].

The relationship between endometriosis and infertility has been widely reported in the literature, albeit with no clear causal relationships. One of the hypothesis on this is that endometriosis may be one of the cause of ovarian physiology disturbances that results in folliculogenesis and contributes to decreased oocyte quality [8].

One of the most interesting findings relevant to this study in recent years is that the process of folliculogenesis is controlled by the growth factors produced by oocytes. To date, several GFs have been identified in mammalian oocytes, including growth differentiation factor-9 (GDF-9) [9]. GDF-9 belongs to the family of transforming growth factor-beta (TGF-β), which is a paracrine factor that plays a role in the intracellular communication between oocytes and somatic cells (such as the granulosa cells) produced by oocytes from early primary follicle stage to the time of ovulation. The roles of GDF-9 include proliferating and differentiating the granulosa cells, preventing apoptosis, and supporting cholesterol biosynthesis in the cumulus cells10-12. The research on GDF-9 expression and its association with infertility in patients with endometriosis is limited. Hendarto et al. [13] found that GDF-9 expression was lower in women with endometriosis [13]. Recent studies on the granulosa cells of women with endometriosis who had undergone controlled ovarian stimulation revealed that moderate–severe endometriosis can significantly decrease the expression of mRNA GDF-9 in comparison with women without endometriosis (p = 0.034), and this in turn leads to poor oocyte maturation and embryo quality [14].

There has been no research conducted in Indonesia regarding GDF-9 expression in the granulosa cells of endometriosis patients undergoing ART. Owing to the fact that GDF-9 is an important factor in normal folliculogenesis, the dysregulation of the GDF-9 expression may contribute to the deviation of folliculogenesis in patients with endometriosis, which may affect fertility. The results of this study are expected to provide an overview of GDF-9 expression in the granulosa cells of endometriosis patients and their correlation with the resultant oocyte quality.

2. Materials and Methods
This was a cross-sectional study with a comparative analytic design to determine the differences in GDF-9 expression in infertility patients caused by endometriosis and sperm factor (in the control group). In addition, this study also analyzed the correlations between GDF-9 expression and oocyte qualities assessed from the oocyte morphology and fertilization rate. GDF-9 expression was assessed in the form of quantitative data obtained by performing RT-PCR on the granulosa cell samples.

In total, 25 patients with endometriosis and 25 control subjects with male factor were involved in this study. All patients underwent IVF from July 2014 to July 2017 at the Yasmin IVF Clinic and Smart
IVF Clinic Dr. Sander B. All patients underwent a short protocol for controlled ovarian hyperstimulation.

The granulosa cells were collected by the embryologists at the time of follicular aspiration. Generally, the granulosa cells separate automatically from the oocytes, whereas the cumulus cells remain attached to the oocytes. The granulosa cells were then placed in a tube containing 500 μL of RNA and stored at −80º until further use.

RNA isolation of the granulosa cells was performed using the Qiagen Rneasy Mini Kit. The measurement of RNA quantity was performed using the Nanodrop 2000 Spectrophotometer at a wavelength of 260/280 nm. After the measurement, the sample was transferred to a 0.2-mL tube, wherein each tube contained 10–15 μL of RNA extract liquid, followed by storage at −80 ºC or processing for cDNA synthesis.

The cDNA synthesis method was performed using the Qiagen Quantitect Reverse Transcription Kit. The procedure then continued by the RT-PCR method of the granulosa cells using the Qiagen Quantitect SYBR Green PCR Kit. The primers used occur in a concentrated form; therefore, each primer was diluted with 80 μL of free-water RNase to reach the desired concentration of 100 μM for each primer. Temperature melting (Tm) was determined by calculation using the formula: Tm = 2 ºC (A + T) + 4 ºC (G + C), wherein A is Adenine, T is Thymine, G is Guanin, and C is Cytosine. The final Tm of each gene was determined by taking the middle number of the reverse and forward primers. The sequences and primary characteristics of GDF-9 are given in the Table 1.

![Table 1. Sequence and primer characteristics for RT-PCR](image)

| Gen  | Primer name | Primer sequence (5′ to 3′) | Amplicon size | Temp (ºC) |
|------|-------------|----------------------------|---------------|-----------|
| GDF-9| Forward     | CTGAAGTGGGACA               | 107           | 2         |
|      |             | ACTGGATT                   |               |           |
|      | Reverse     | GTGTGAACCTGGAG              |               | 2         |
|      |             | AGCCATA C                  |               |           |

The determination of the standard curve was started with the determination of the standard sample with the greatest RNA concentration and the highest purity. The next step was to determine the dilution serial concentration in multiples, according to the diversity of the sample RNA concentration such that the last dilution series can still reach the lowest RNA concentration in the sample to be compared. The sample was then diluted 4 times before entering the cDNA stage with the same amount of volume in each dilution. The sample was then subjected to RT-PCR with the same procedure. When the machine was running, the samples were categorized as “standard” on the computer program, and then the concentration column was filled according to the initial dilution concentration while still in the form of RNA extracts. After the RT-PCR run was complete, the standard curve construction results were used for comparison with those of other sample.

Data analysis was performed using the qPCR result via a quantitative method based on the standard curve (ng/μL). Data normality was assessed by the Shapiro–Wilk test. GDF-9 expression in patients with endometriosis who underwent IVF is presented as mean ± standard deviation of the distribution of normal or median data (minimum–maximum) if the distribution of data is not normal. The unpaired t-test (if data distribution is normal) or the Mann–Whitney U-test (if data distribution is abnormal) was performed to compare the differences in GDF-9 expressions between patients with endometriosis and control subjects. Furthermore, the Pearson test, or the Spearman’s test, was performed to determine the correlation between GDF-9 expression in the subject and the oocyte quality (based on the fertilization rate and oocyte morphology score). A P value of <0.05 was considered to be statistically significant. Statistical analyses were performed using IBM SPSS (Statistical Package for Social Sciences) version 22.
A modified scoring system was employed to assess the oocyte morphology based on the Xia criteria (Table 2) with a maximum score of 6.

**Table 2. Xia-modified oocyte morphology scoring**

| Criteria               | Score      |
|------------------------|------------|
|                        | 0          |
|                        | 1          |
|                        | 2          |
| First polar body       | -          |
| Periviteline space     | Wide       |
| Granul cytoplasmic     | Present    |
|                        | Fragmented |
|                        | Intact     |
|                        | Normal     |
|                        | Not present|

3. Results

The mean age of the subjects was 33.32 ± 4.24 years, and the mean duration of infertility of the subjects was 4.76 ± 4.23 years. The characteristics of the patients with endometriosis and control subjects are given in Table 3. It can be seen from the table that the data on the numbers of retrieved oocytes and mature oocytes as well as the oocyte morphology score were significantly lower in the endometriosis group than those in the control group.

**Table 3. Patients characteristics.**

| Characteristic          | Endometriosis | Male factor | p     |
|-------------------------|---------------|-------------|-------|
| Age (year)              | 33.76 ± 4.28  | 32.88 ± 4.27| 0.47  |
| Duration of infertility (year) | 5.04 ± 4.59   | 4.48 ± 3.92  | 0.93  |
| Number of oocyte retrieved | 10.08 ± 5.67  | 14.20 ± 7.97 | 0.04  |
| Mature oocyte           | 8.28 ± 4.91   | 12.20 ± 7.44 | 0.03  |
| Fertilization rate (%)  | 63.26 ± 16.22 | 68.95 ± 26.34 | 0.38  |
| Oocyte morphology score | 4.37 ± 0.45   | 4.87 ± 0.34  | <0.001|
| rFSH                    | 3081.6 ± 852.03| 2654.7 ± 639.28 | 0.12  |

The GDF-9 expressions were abnormally distributed (Kolmogorov–Smirnov test for normality, p < 0.001). The median GDF-9 level of all subjects was 15.66 (0.00002–32450) ng/μL. A significant difference was noted between GDF-9 subjects, in which patients with endometriosis showed lower values than those without endometriosis (5.05 (0.00002–3523.0) ng/mL vs. 81.93 (1.47–32450) ng/μL; P = 0.01). The graph of comparison of the GDF-9 levels in both the groups is shown in Figure 1.

No significant correlation was obtained between the GDF-9 expression levels and oocyte quality in the endometriosis group and in the control group.
Figure 1. GDF-9 expressions in both the tested groups

Table 4. Correlation between GDF-9 expression and oocyte morphology score and fertilization rate.

| GDF-9       | Oocyte morphology score | Fertilization rate |
|-------------|-------------------------|--------------------|
|             | Correlation (r)         | p                  | Correlation (r) | p       |
| All subjects| 0.213                   | 0.14               | 0.087           | 0.55    |
| Endometriosis| 0.107                   | 0.61               | 0.207           | 0.32    |
| Control     | 0.205                   | 0.32               | 0.188           | 0.37    |

*Spearman correlation coefficient

4. Discussion
The hypothesis that endometriosis causes infertility remains controversial because the causal relationship has not been clearly established yet. Several hypotheses have been proposed to explain the relationship between endometriosis and infertility and one of which states that endometriosis causes an ovarian physiological disorder by affecting folliculogenesis, resulting in a decreased oocyte quality. Several studies have revealed a decrease in the oocyte quality in mild, moderate, and severe endometriosis [15,16]. As we know, oocytes gradually reach their competence through the process of folliculogenesis, wherein the oocyte grows and its somatic cells differentiate. During oocyte growth, a close interaction occurs between the granulosa cells and oocyte via a bi-directional communication. The granulosa cells provide the nutrients and growth regulators required for oocyte development. In addition, oocytes facilitate the growth and differentiation of the granulosa cells through the production of growth factors by oocytes [17]. In recent years, the process of folliculogenesis is controlled by growth factors, known as oocyte-secreted factors, such as GDF-9 [11,18,19]. Although produced by oocytes, a paracrine factor, GDF-9 is also known to occur in the follicular fluid and granulosa cells [12].

In this study, we noted that the number of oocyte retrieved and the number of mature oocytes were lower in the endometriosis group than those in the control group. This result agrees with that of a similar study conducted by Neto et al. [20] on ovarian reserve and reproductive outcomes in women with endometriosis who had undergone IVF. The present study revealed a significant difference in the number of oocyte retrieved in endometriosis compared no non-endometriosis (4 [2–8] vs. 6 [3–10]; P < 0.001). This result is lower than that found in our study (endometriosis group, 10.08 ± 5.67 vs. control group, 14.20 ± 7.97; P = 0.04). Some other studies have reported different results. A population-based retrospective cohort study with a total of 347,185 (fresh and frozen) cycles found that endometriosis was associated with lower oocyte count, lower implantation rate, and pregnancy rates, although this association was confounded by other etiology of infertility, which accompanies endometriosis [20].
Some studies have reported that the fertilization rate and live birth rate were unaffected by endometriosis despite fewer oocyte production [21].

The GDF-9 expression level in this study was lower in the endometriosis group than in the control group (5.05 [0.00002–3523.0] ng/mL vs. 81.93 [1.47–32450] ng/μL; p = 0.01), which was statistically significant. A few studies have been conducted on GDF-9 expression in the granulosa cells of patients with endometriosis. Kawabe et al. [14] are the only ones who studied the effects of moderate to severe endometriosis on GDF-9 mRNA expression in the granulosa cells of patients under controlled ovarian stimulation. They found that the ratio of GDF-9 mRNA expression in the endometriosis group was lower than that in the control group (p = 0.034); this is similar to our result. Hendarto et al. [22] evaluated GDF-9 concentrations in the follicular fluid of patients with endometriosis using ELISA. In their study, the GDF-9 concentrations in women without endometriosis was 191.52 + 102.9 pg/mL, with mild endometriosis was 139.67 + 94.31 pg/mL, and with severe endometriosis was 105.98 + 50.42 pg/mL; these differences were statistically significant. All the aspirated follicles in this study were dominant follicles (>17 mm), as determined by ultrasonography. Compared with the results obtained by Hendarto et al. [13], it can be concluded that GDF-9 mRNA expression was lower in the granulosa cells than in the follicular fluid [22].

The concentration of GDF-9 mRNA in the endometriosis group appears to have a wide range with the lowest concentration of 6.05 × 10−5 ng/μL and the highest concentration of 3.26 × 103 ng/μL. This condition can be attributed to the granulosa cells collected in the process of ovum pick-up derived from all the follicles with varying follicle diameter. The number of the granulosa cells from smaller follicles is lower than that from larger follicles. Extremely low concentrations of GDF-9 mRNA may be derived from fewer granulosa cells, whereas higher mRNA concentrations may be derived from larger follicles with more granulosa cells. This study could have benefited from the aspiration of the granulosa cells from the follicles of the same size (size-matched).

The assessment of oocyte quality has become one of the major embryological concerns in IVF. Although oocyte morphology remains the most popular method of oocyte selection, there are now several cellular and molecular markers that have been proposed as predictors of oocyte quality. Li et al. [22] studied 2426 cumulus cells on the day of oocyte retrieval for GDF9 and BMP15 mRNA expression and their correlation with oocyte maturation, fertilization, and embryo quality. Their research concluded that the expression levels of GDF9 and BMP15 mRNAs are closely related to oocyte maturity, fertilization rate, embryo quality, and pregnancy outcomes. Therefore, GDF9 and BMP15 mRNAs in the granulosa cumulus cells may be considered as new molecular markers for the prediction of oocyte development potential [23].

Despite GDF-9 being involved in folliculogenesis, steroid production, and oocyte maturation, the GDF-9 concentration in the granulosa cells in this study did not show any significant correlation with the oocyte quality. However, our results indicate that endometriosis affects ovarian reserve and folliculogenesis considering the lower number of retrieved oocytes, the number of mature oocytes, and lower oocyte morphology scores in the endometriosis group, which were statistically significant.

This study has some limitations. First, the sample size of the study was insufficient, as seen from a fairly wide 95% confidence interval. Therefore, studies with a large sample size are warranted to confirm whether the expression of the GDF-9 is important in infertility in patients with endometriosis and their correlation with oocyte quality and to ascertain whether GDF-9 can be used as a new molecular marker to predict the oocyte development potential. Furthermore, it is important to cluster the degree of endometriosis in the future to prove whether the degree of endometriosis is also related to oocyte quality.

5. Conclusion

The GDF9 mRNA level was found to be lower in the endometriosis group than that in the control group. However, we did not find any direct relationship between individual GDF-9 level and oocyte quality. Therefore, studies with a large sample size are warranted in the future to confirm whether GDF-9
expression is correlated with oocyte quality and to assess whether GDF-9 could be used as a new molecular marker to predict the oocyte developmental competence.

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