Gene expression of follicle-stimulating hormone receptors in granulosa cells in poor ovarian responders

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Abstract. Women with poor ovarian response do not produce adequate follicles and express low estradiol levels after standard ovarian stimulation protocols; therefore, their chances of becoming pregnant are reduced. The follicle-stimulating hormone (FSH) binds to specific follicle-stimulating hormone receptors (FSHRs) in the ovary, thereby activating adenylyl cyclase/cyclic adenosine monophosphate, a gene expression regulator. The present study aimed to evaluate whether the FSHR levels in poor ovarian responders differed from those in normal ovarian responders. This cross-sectional study was conducted in the Cipto Mangunkusumo’s Hospital. The study cohort included women with poor ovarian responses (n = 17) and control women with normal ovarian responses (n = 17) who underwent IVF procedures. The FSHR levels in ovarian tissues were measured using qPCR and then compared between the two groups. No statistically significant differences were found in the FSHR levels between the groups. A polymorphism mutation may be present in the FSHR gene of poor ovarian responders.

1. Introduction

The rate of poor ovarian responders during assisted reproductive technology varies between 9% and 24\%. In Indonesia, the number of IVF cycles for patients aged above 40 years increased from 12.6\% to 13.15\% during 2013–2016 according to the data in the Perftri. Poor ovarian responders are defined based on three criteria laid out by the ESHRE working group: advanced maternal age or any other risk factor for poor ovarian response (POR), a previous POR event, and an abnormal ovarian reserve test.

In poor responders, the FSH level increases due to the lack of negative feedback from inhibin-B. FSH acts on the follicle-stimulating hormone receptor (FSHR) in ovarian cells, activating the adenylyl cyclase/cyclic adenosine monophosphate (cAMP), a gene expression regulator. Herein, we assessed...
whether the expression of FSHR in ovarian tissues is different between normal ovarian responders and poor ovarian responders.

2. Materials and Methods

2.1 Patient eligibility
Patients undergoing IVF cycles who met the inclusion criteria provided signed informed consents to participate in the study. Patients were divided into two groups: poor responders and normal responders. Poor ovarian responders were defined as women presenting at least two of the Bologna criteria: (i) advanced maternal age (≥ 40 years) or any other risk factor for POR, (ii) a previous POR, and (iii) an abnormal ovarian reserve test (ORT) (AFC < 5–7 follicles or AMH < 0.5–1.1 ng/mL).

2.2 Place and time of study
This study was conducted at the Yasmin Clinic, Cipto Mangunkusmo’s Hospital, in Jakarta, Indonesia.

2.3 Standard patient assessments
Patients underwent routine anamnesis, physical examination (general status and obstetrics status), ultrasonography, and laboratory examination. Serum and granulosa cells were collected during ovum pick-up. Granulosa cell samples from aspirated follicles were obtained from all participants. Granulosa cells automatically separate from oocytes, whereas the cumulus cells remain adhered to the oocyte. Granulosa cell samples were placed in a tube containing 500 µL RNA and frozen at −80°C until used in mRNA measurements.

2.4 ELISA examination
Anti-Müllerian hormone (AMH) levels were measured from serum samples using the ELISA method. ELISA was performed using the AMH Gen II ELISA kit (Beckman Coulter, USA) following the manufacturer’s instructions. Briefly, the calibrator, control, and test samples were incubated in microtiteration wells coated with anti-AMH antibody. After washing, the anti-AMH antibody labeled with biotin was added to each well. Incubation and washing were conducted for a second time. Then, streptavidin–horseradish peroxidase (HRP) was added to each well. After the third incubation and washing steps, the tetramethylbenzidine (TMB) substrate was added to each well. Then, an acid solution was added, and the absorption of the colored product formed after conversion of the enzymatic substrate was measured at a wavelength of 450 nm to determine the AMH concentration based on the calibrator’s absorption curve.

2.5 RNA isolation from granulosa cells and real time PCR
The RNA isolation kit from Qiagen RNeasy Mini Kit based on the protocol of the RNeasy Mini Handbook was used with minor modifications. First, our samples were thawed at room temperature. The samples were then vortexed for 3 s and centrifuged for 3 min at 8000 ×g at room temperature. The supernatants were discarded. Then, after addition of 600 µL of RLT buffer with β-Mercaptoethanol, the cell membranes were disrupted in a sonicator. The next steps are the same as those described in the manufacturer’s protocol. Table 1 shows the composition of the buffer used for obtaining pure RNA.

| Ingredients                        | Volume   |
|------------------------------------|----------|
| gDNA Wipeout Bufer, 7×             | 2 µL     |
| Template RNA                       | Variable |
| RNase free water                   | Variable |
| Total Volume                       | 14 µL    |

Table 1. Genomic composition of the DNA elimination buffer
2.6 Real time PCR
The Quantitect SYBR PCR Master Mix, template cDNA, forward and reverse primers, and RNase free water were used for RT PCR as described in Table 2. The results of the RT PCR expression data for FSHR expression in granulosa cells was used for data analysis.

| Table 2. Component of master mix RT PCR reaction |
|-------------------------------------------------|
| **Component** | **Volume/Reaction** | **Final Concentration** |
| Quantitect SYBR Green PCR Master Mix | 25 µL | 1× |
| Primer F | Variable | 0.3 µM |
| Primer R | Variable | 0.3 µM |
| RNase Free Water | Variable | |
| Template cDNA | Variable | ≤500 ng/reaction |
| Total Volume | 50 µL | |

3 Results
A total of 34 samples were analyzed in this study: 17 from the poor responders group and 17 samples from the responders group. The average age was significantly higher in the poor responder group than in the normal responders group (p = 0.035). The AMH level was statistically lower in the poor responder group than in the normal responder group (p = 0.004). Patient characteristics are presented in Table 3. The FSHR expression in granulosa cells of poor responders was higher than that in normal responders (1.259 × 10−8 vs. 0.887 × 10−8), but the difference was not statistically significant (p = 0.658) (Table 4).

| Table 3. Patient characteristics. |
|----------------------------------|
| **Characteristic** | **Poor Responders n = 21** | **Normal Responders n = 21** | **P** |
| Age, mean (DS) | 36.62 (3.17) | 33.9 (4.72) | 0.035a |
| AMH level follicular fluid (ng/mL), median | 1.24 (0.34–14) | 2.55 (1.55–8.32) | 0.004b |
| FSH Recombinant dose (IU), median | 2925 (900–3750) | 2325 (1125–3450) | 0.105b |
| Oocyte count, median | 4 (1–9) | 10 (3–20) | 0.001b |

| Table 4. FSHR Expression in granulosa cells of poor responders and normal responders |
|----------------------------------|
| **FSHR Expression** | **Poor Responders n = 17** | **Normal Responders n = 17** | **P** |
| FSHR Expression in granulosa cells (ng/µL), mean (standard deviation) | 1.259 × 10−8 (2.729 × 10−8) | 0.887 × 10−8 (2.137 × 10−8) | 0.658 |
Table 5. Correlation between FSHR Expression in Granulosa Cells with Age, AMH Levels, and FSHR Dose

| Characteristics                  | Correlation          | FSHR Expression in Granulosa Cells n=17 | Poor Responders | Normal Responders |
|----------------------------------|----------------------|----------------------------------------|-----------------|-------------------|
| Age                              | Correlation coefficient (r) | P*                                   | −0.156 | −0.246 |
| AMH in Follicular Fluid (ng/mL)  | Correlation coefficient (r) | P*                                   | −0.009 | 0.108 |
| FSHR Dose (IU)                   | Correlation coefficient (r) | P*                                   | 0.31   | 0.056 |

4. Discussion
Badawy et al. found that the granulosa cells of poor responders express lower FSHR levels, although they express normal androgen levels [1]. However, in our study, FSHR expression was slightly higher in poor responders than in normal responders, but the difference was not significant.

The administration of testosterone improves granulosa cell proliferation and increases the number of growing follicles. Nielsen showed a strong correlation between androgen levels in the follicular fluid and androgen receptors in granulosa cells with FSH expression in granulosa cells. Moreover, FSHR expression in granulosa cells will also improve after supplementation with testosterone; thus, it is possible that androgens sensitize granulosa cells to the FSH trigger. This study tried to find a positive correlation between DHEA levels and FSHR expression in poor responders, but the correlation was not statistically significant. Based on these facts, follicle sensitivity to FSH stimulation may be enhanced by supplementation with androgens. Therefore, poor responders may benefit from such an approach to enhance FSHR expression in granulosa cells; however, more studies are needed. Genetic polymorphisms in the FSH receptor gene may lead to poor quality of mRNA production, reduced expression of FSH and LH receptors in granulosa cells; this possibility should be addressed in future studies.

As a limitation, this study did not obtain androgen levels under basal conditions in the follicle because our samples were derived from women who were given recombinant FSH stimulation. Future studies need to investigate interactions between androgen levels and androgen and FSH receptors. In addition, this study did not examine FSH polymorphisms in any of our patients, and this may have influenced our findings.

5. Conclusion
There were no significant differences found in the FSHR levels in granulosa cells between poor ovarian responders and normal groups.

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