fshr and lhr mRNA expression in granulosa cells of poor responder

N Hanifah¹, B Wiweko² and A Bowolaksono¹
¹Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok 16424, Indonesia
²Department of Obstetric and Gynecology, Faculty of Medicine, Universitas Indonesia, Depok 16424, Indonesia

Corresponding author’s email: alaksono@sci.ui.ac.id

Abstract. In Vitro Fertilization (IVF) is one of the most commonly used procedures to help pregnancies in couples who have infertility problems. One of the problems of infertility is poor ovarian response and the woman who experiences it is known as poor responder. Poor responders do not have an adequate ovarian response to gonadotropin in ovarian stimulation. The success of fertilization in poor responders tends to be low due to low quantity and is generally followed by low oocyte quality. Gonadotropins consisting of FSH and LH, play a role in follicle development and ovulation. The follicle response in capturing gonadotropins depends on the exact bond between the hormone and its receptor (FSHR and LHR) in the granulosa cells surrounding the oocyte. The purpose of this research is to know the expression level of fshr and lhr mRNA in granulosa cells of poor responders through real-time PCR method which then tested statistically using t-test. Fourteen samples of each poor responders and normal women were used in this research. The results showed insignificant differences between expression level of fshr and lhr mRNA in granulosa cells of poor responders and normal women (p > 0.05).

Keywords: fshr, lhr, poor ovarian response, real-time PCR

1. Introduction
Ovarian stimulation is the first step in in vitro fertilization procedure (IVF). Ovarian stimulation aims to obtain a number of fertilized oocytes [1]. The occurrence of an ovarian response that has minimal response to ovarian stimulation is referred to as the incidence of poor ovarian response and the woman who experiences it is known as a poor responder. Poor responders consistently have low oocyte yield and low pregnancy success [2, 3]. Based on the consensus of the European Society of Human Reproduction and Embryology working group on poor ovarian response definition (ESHRE) in Bologna, a woman is said to be a poor responder if the patient meets two of the three criteria: maternal age (> 40 years) a low, and a history of ovarian stimulation with abnormal oocyte retrieval results (< 4). The ovarian response to gonadotropin administration is one of the factors that influence the success of cycle [4]. Gonadotropin has an important role in the development of follicles and ovulation [5]. Gonadotropin consists of a group of hormones that stimulate follicles, namely Follicle Stimulating Hormone (FSH) and Leutenizing Hormone (LH) [6].

Oocytes depend on the aid of granulosa cells that are able to respond to FSH and LH for maturation and ovulation [5]. Patients with poor response do not respond adequately to gonadotropin administration
so as to have low oocyte gain [3, 7]. Until now, no ovarian stimulation has been found that results in successful outcomes of IVF procedures for patients with poor response. The study by Rosen et al. [8] indicates fertilization rate can be used an oocyte quality parameter to assess the success of the cycle as it is considered to be a predictor of the success rate of pregnancy in IVF procedure. Expression of the gonadotropin receptor and its ability to respond to hormones is an important factor in ovarian stimulation. An understanding of the expression of FSHR and LHR is needed as a preliminary study in an effort to improve the success of IVF procedures in people with poor response. Therefore, it is necessary to research the expression of fshr and lhr mRNA in granulosa cells of poor responders.

2. Materials and method

2.1. Granulosa cell samples materials
Granulosa cell samples were taken from patients undergoing the In Vitro Fertilization (IVF) program at RSCM Yasmin Kencana during OPU (Ovum Pick Up). Isolation of granulosa cells and their preservation in RNAlater® QIAGEN were performed by embryologists.

2.2. RNA isolation
Isolation of granulosa cell RNA using QIAamp RNA Blood Mini Kit [QIAGEN]. RNA totals were obtained using cell lysis solution, wash buffer, and specially designed spin columns of the kit. First, the samples were centrifuged for 5 minutes at 3,000 g. The supernatant was removed then RLT buffer and β-mercaptoethanol were added. Samples were filtered out by centrifuging for 2 minutes at 17,000 g. Then, 600 μL of 70 % Ethanol was added to the supernatant obtained and the samples were centrifuged for 15 seconds at 8,000 g. Next, the samples were centrifuged using wash buffer for 15 seconds at 8,000 g and the supernatant obtained was removed. This step was repeated twice with the last one was done for 3 minutes at 17,000 g. After that, 50 μL of RNA-free-water was added for RNA elution. The samples were centrifuged for 1 minute at 17,000 g and stored in micro tubes. Then, the samples concentration and purity of isolated RNA were measured by spectrophotometer and then stored at -80 °C.

2.3. cDNA synthesis (reverse transcription)
Synthesis of cDNA was done using Quantitect Reverse Transcription Kit [QIAGEN]. Initially the RNA and Rnase-free water volumes in each sample were computed to obtain a mixture with a final RNA concentration of 15 ng/20 μL in genomic elimination DNA reaction. The volume of each sample varies and depends on the concentration of RNA obtained. After genomic DNA elimination, each sample was mixed with 6 μL of reverse transcription mix for cDNA synthesis. The results obtained are then stored at -20 °C.

2.4. Quantitative real-time PCR (Q-PCR) with absolute quantitative method
Real-time PCR was performed using Qiagen Quantitect SYBR Green PCR Kit [QIAGEN]. The standard curve was formed using a concentrated G-Block oligonucleotide which was then diluted. The resulting data of the sample amplification was interpolated by the standard curve formed.

2.5. Statistical analysis
The mRNA expression data based on the quantitative method with the standard curve was obtained in nominal form with the unit ng/μL. Data processing was done using SPSS software 16.0 version. The results obtained were tested for normality using the Saphiro-Wilk normality test and Levene homogeneity test. T-tests were performed to determine the mean differences between the expression of fshr and lhr mRNAs in poor responder and normal women.
3. Results and discussion

3.1. Expression of fshr mRNA on poor responder’s granulosa cells

The t-test performed on the quantification of fshr mRNA between poor responder and normal women showed the p value of 0.196 ($p = 0.196$). The p value obtained is greater than 0.05 ($p > 0.05$) therefore, there was no significant difference between the expression of the fshr mRNA in the female granulosa cell in response to the normal woman. The number of developed follicles and the number of matured oocytes were the most commonly used criterion in determining the incidence of adverse ovarian response. However, the sample of poor responders used in this study was based not only on the number of matured oocytes obtained. The results of this study were different with the results obtained by Cai et al. [9].

Humaidan et al. [10] divided the group of poor responder women into four major groups, ie poor responder women with adequate ovarian reserves (FAB $\geq 5$) but have a minimum response (mature oocyte $< 4$) and poor responder women with low ovarian reserves (FAB $< 5$). Each group was subdivided into two groups by age, ie poor responders young (< 35 years) and elderly ($\geq 35$ years). Based on these groupings, the samples used by Cai et al. [9] have adequate ovarian reserve but have low mature oocyte gain ($< 4$) due to minimal response to ovarian stimulation.

Differences in the results of the study may be caused by differences in the sample population of poor responder women used. This is supported by the study of Thiruppathi et al. [11] which compared the expression of FSH receptors in female granulosa cells of poor responder and normal women that showed no significant difference between FSHR expressions in both granulosa cell groups. The study also does not differentiate poor responders groups.

This research did not differentiate the sample population of poor responder women so it has different results from previous studies. The difference in the results of the study, ie, the average value of fshr mRNA in poor responder woman is higher than normal women, although not significant, is thought to be due to the use of poor responders sample populations dominated by women with low ovarian reserves group, not those with low ovarian response group. Ovarian reserves are known to decrease as age increases [12], and 11 of 14 samples of poor responder women are aged $\geq 35$ years.

3.2. Expression of lhr mRNA on poor responder’s granulosa cells

The t-test performed on the quantification of lhr mRNA between poor responder and normal woman showed the p value of 0.111 ($p = 0.111$). The p value obtained was greater than 0.05 ($p > 0.05$) therefore it could be concluded that there was no significant difference between the expression of lhr mRNA between poor responder and normal woman. The average difference shows that lhr mRNA expression of poor responder women was lower than that of normal women although not significant. These results were consistent with research conducted by Papamentzeloupolu et al. [13] who found that women with lower LHR expression had a lower ovarian response as well. These results were based on the correlation of lower LHR expression in granulosa cells and the longer the time required for ovarian stimulation.

The results of studies conducted by Regan et al. [14] also showed reduced density of LH receptors in older women's granulosa cells compared to younger women. The expression of lhr mRNA on poor responder’s granulosa cells has a lower mean value than normal women although not significant. These results are thought to show lower levels of follicular development than in normal women. The expression of LH receptors on follicles is regulated by FSH and estrogen. Expression of LH receptors increases during follicle growth along with the follicle's ability to secrete estrogen [15]. In other words, increased expression of LH receptors may indicate a more advanced development in a group of follicles [16]. Estrogen especially estradiol, primarily function in oocyte maturation and correlate with follicular development, but it is also known that poor responder women have low estradiol levels, which may indicate the development of impaired follicles [17].

The result of the research is not statistically significant, which could be caused by the number of samples that did not meet the requirement for estimated number of sample count. There were only 14
out of 18 samples (based on the estimated number of samples), while there were 14 samples of normal women. In addition, many influential factors such as indifferenzation of poor responder samples based on poor responders grouping population. The age grouping of women is considered necessary because the quantity and quality of oocytes clearly decreases in the elderly women group (> 35 years) [18]. Limitations of number and sample data become obstacles in the uniformity of background selection in both groups of samples so as to produce biased conclusions. Further research is required with more sample quantities and rigorous sample selection to produce accurate analysis and valid conclusions.

4. Conclusion

$fsr$ and $lhr$ mRNA expression in granulosa cells of poor responder did not have significant differences compared to expression of $fsr$ and $lhr$ in normal women.

References

[1] Bansal K 2011 Manual of Intrauterine Insemination (IUI), In Vitro Fertilization (IVF), and Intracytoplasmic Sperm Injection (ICSI) 2nd edition (New Delhi: Jaypee Brothers Medical Publishers Pvt Ltd.)

[2] Oehninger S 2011 F, V & V Obgyn 3 101-8

[3] Ubaldi F, Vaiarelli A, D’Anna R and Rienzi L 2014 BioMed Res. Int. 2014 352098

[4] Ferraretti A P et al. 2011 Hum. Reprod. 26 1616-24

[5] McKenzie L J 2011 Granulosa cell and their impact on human ART in Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice ed. Gardner D K et al. (UK: Cambridge University Press)

[6] Bowen R 2004 Gonadotrops: Luteinizing and Follicle Stimulating Hormones available at http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/hypopit/lhfsh.html

[7] Badawy A, Wageah A, Gharib M E and Osman E E 2011 J. Reprod. Infertil. 12 241-8

[8] Rosen M P et al 2010 Fertil. Steril. 94 1328-33

[9] Cai J et al 2007 Fertil. Steril. 87 1350-6

[10] Humaidan P, Alviggi C, Fischer R and Esteves S C 2016 F1000Res. 5 2911

[11] Thiruppathi P, Shafati S, Dias J A, Radwanska E and Luborsky J L 2001 Mol. Hum. Reprod. 7 697-704

[12] Visser J A, Jong F H d, Laven J S E and Themmen A P N 2006 Reproduction 131 1-9

[13] Papamentzelopoulou M et al. 2012 J. Assist. Reprod. Genet. 29 409-16

[14] Regan S L P et al. 2017 Mol. Cell. Endocrinol. 446 40-51

[15] Zeleznik A J 2004 Reprod. Biol. Endocrinol. 2 1-7

[16] Mihm M and Evans ACO 2008 Reprod. Domest. Anim. 43 48-56

[17] Santos M J d 1 et al. 2013 Hum. Reprod. 28 224-9

[18] George K and Kamath M S 2010 J. Reprod. Sci. 3 121-3