Correlation between androgen levels in follicular fluid and granulose cell FSHR expression in poor responder women

B Wiweko1,2,3*, N M D Suratih4, N Hanifah5, A M Sholilah5, N Muna3, K Mutia3, P A Ifanolidia3, A Bowolaksono5, R Muharam1,2,3 and A Hestiantoro1,2,3

1Yasmin IVF Clinic, Dr. Cipto Mangunkusumo General Hospital, Jakarta, 10430, Indonesia
2Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
3Human Reproductive, Infertility and Family Planning Research Center, Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
4Department of Obstetrics and Gynecology, Persahabatan Public Hospital, Jakarta, 13230, Indonesia
5Department of Biology, Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok, 16424, Indonesia

*E-mail: budiwiweko@gmail.com

Abstract. Androgen levels are involved in folliculogenesis and follicular maturity. The increase of follicular androgen levels in poor responders can increase FSH receptor expression. However, a previous study found that there was no difference in androgenic follicular fluid levels in poor responder. This discovery led to a theory of differences in FSH receptor density. The objective of this research was to investigate the correlation between androgen levels in follicular fluid with granulose cells FSH receptor expression in poor responder women. This cross-sectional study was completed at the Yasmin IVF Clinic, dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia. Forty-two subjects who underwent IVF were asked to participate and their follicular fluid testosterone and DHEA levels were measured. The patients were classified into two groups consisting of 21 poor responder patients and 21 normo-responder patients. Granulose cells were collected from 17 patients in each group to measure the FSH receptor expression. Correlation between androgens in follicular fluid with granulose cells FSH receptor expression was analyzed using the Spearman test. There were significant between-group differences in mean age with poor responders presenting as older and with lower AMH levels. Testosterone levels from follicular fluid were significantly higher in poor responders, but similar findings were not observed with DHEA levels, which did not rise to the level of statistical significance. There were a significantly lower number of oocytes and cleavage embryos in the poor responder group. Granulose cell FSH receptor expression was higher in the poor responder group, although this difference also did not rise to the level of statistical significance. There was no correlation between testosterone follicular fluid level and granulose cell FSH receptor expression. We found the DHEA follicular fluid level was positively correlated with granulose cell FSH expression, even though the difference was not statistically different. There was no significant correlation between intra-follicular androgen levels and fertilization rates in both poor responders and non-poor responders. Additional studies are needed to find correlations between the other factors affecting poor responders.
1. Introduction
Oral health is essential and has an important role in everyday life. In Indonesia, oral health remains one of the main problems suffered by the community. The Indonesian Household Health Survey in 2001 showed that oral health problems are the complaints of 60% of the Indonesian population. Dental and oral diseases, which are usually found among the people of Indonesia, include dental caries, periodontitis, gingivitis, stomatitis aphthosa, and halitosis [1]. Halitosis or bad breath is defined as an unpleasant breath arising from physiological and pathological factors derived from oral or systemic sources and is one of the most frequent oral health problems. Several studies conducted in industrialized countries showed a prevalence of halitosis of as high as 50% with various severities [2]. Besides health problems, halitosis can also greatly affect the social life of patients.

About 80%–90% of halitosis comes from the oral cavity, and the accumulation of bacteria on the posterior part of the tongue is one of its main causes [3]. Halitosis can be caused by various factors, such as foods and drinks, poor oral hygiene, periodontal diseases, tongue coating, xerostomia (dry mouth), and systemic diseases [2]. Moreover, halitosis can be caused by upper and lower respiratory tract disorders, digestive disorders, and use of certain drugs [4]. Volatile sulfur compound (VSC) is the major cause of halitosis. The VSC components, are hydrogen sulfide gas (H2S), methyl mercaptan (CH3SH), and dimethyl sulfide ((CH3)2S). VSC is formed through the reactions of non-volatile materials in the mouth, especially protein, with anaerobic bacteria in the oral cavity [2].

Prevention efforts and treatment of halitosis are brushing the teeth and the tongue, using mouthwash, and improving one’s diet. Treatments such as brushing the teeth and tongue or using an antiseptic mouthwash have been proved to reduce hydrogen sulfide and methyl mercaptan, which are the components of VSC [5].

The use of mouthwash is a simple effort to overcome halitosis. Particularly in Indonesia, the market offers a wide variety of brands with different active ingredients of mouthwashes. Chlorhexidine is the most commonly used antibacterial agent. However, although chlorhexidine is one of the most effective oral antiseptic agents, research shows that the long-term use of chlorhexidine has some side effects, such as staining on the teeth and tongue and reduced sensitivity of taste buds [6,7].

Developments in dentistry have produced several discoveries of new products that can be used as supporting periodontal treatment, one of which is chlorine dioxide (ClO2). ClO2 is a strong oxidizing agent that can kill bacteria through a protein synthesis mechanism [8]. It contains oxygen, which can be used as an antiseptic on wounds and accelerates healing, and is effective for halitosis, gingivitis, periodontitis, and bleeding gums [10-12]. ClO2 and chlorite anion (ClO2-) together can oxidize VSC to become a non-malodor product and destroy amino acids, such as cysteine and methionine, which are VSC precursors, in the process of oxidation [12]. Mouthwashes containing ClO2 have been widely used in developed countries, such as Japan and the United States, and ClO2 mouthwash has been reported to be effective in reducing bad breath in the morning (morning breath malodor) up to 4 h after application in healthy subjects [13]. In Indonesia, mouthwashes containing ClO2 are not too popular among the public. Moreover, studies on the efficacy of the use of mouthwash containing ClO2 against halitosis in Indonesia have not yet been conducted. Therefore, this research is conducted to analyze the efficacy of using mouthwash containing ClO2 as the active ingredient to address halitosis. The results are expected to improve the knowledge of dentists in dealing with halitosis and to help people to choose the right mouthwash to overcome halitosis.

2. Materials and Methods
This study used a blind randomized clinical trial by taking samples randomly before and after the study. Data retrieval was conducted before and after a subject gargled with mouthwash provided by the researcher. The entire protocol of this study was reviewed and approved by the Research Ethics Committee of the Faculty of Dentistry, University of Indonesia. The sample comprised 40 people who were chosen randomly, met all the criteria for inclusion, and were exempted from the exclusion criteria. The 40 subjects were divided evenly into two groups: the test group, which was required to rinse with
mouthwash containing ClO$_2$ (i.e., Oxyfresh® “Oxygene® Mouthrinse”), and the control group, which was required to rinse with aquadest.

One day prior to the measurement, the subjects were instructed not to consume pungent foods to prevent overload levels of VSC. All subjects were also instructed not to eat, drink, gargle, brush their teeth, and consume chewing gum for at least 2 h prior to the initial measurements to obtain an initial score that was not too diverse among subjects. To eliminate the psychological factors that could become confounding factors in the study, the subjects were not told to which group they would be included prior to the measurement. The Oxyfresh® “Oxygene® Mouthrinse” used in this study was non-colored and clear, similar to aquadest, so that the subjects would be unaware of the type of mouthwash they would use.

The VSC scores were measured by OralChroma™, and organoleptic measurement was performed at 0 min before the subjects gargled (baseline), 30 min after the subjects gargled, 2 h after the subject gargled, 4 h after the subject gargled, and 6 h after the subject gargled. The VSC scores were analyzed by the Wilcoxon statistical test. A significance level of 0.05 ($p = 0.05$) and confidence level of 95% ($\alpha = 0.05$) were obtained.

3. Results

There were 42 samples eligible for this study, obtained from 21 poor responders and 21 non-poor responders. During the study, we found four samples from each group without granulose cells. Therefore, for measurement of FSH receptors expression, we were limited to 17 samples in each group.

This study found significant differences in average age, which was higher in the poor responder group compared with the non-poor responder groups ($P = 0.035$). AMH levels were statistically lower in the poor responder group compared with the non-poor responder group ($P = 0.004$). Subject characteristics are presented in Table 1.

| Table 1. Subjects’ characteristics. |
|------------------------------------|
| Poor Responder                   | Non-Poor Responder                  | $P$   |
| Age, mean (DS)                   | 36.62 (3.17)                        | 33.9 (4.72) | 0.035* |
| AMH level follicular fluid (ng/mL) median | 1.24 (0.34–14)                       | 2.55 (1.55–8.32) | 0.004b |
| Follicular fluid DHEA (ug/dL), median | 123.5 (7.4–377.9)                   | 112.4 (43.9–293.8) | 0.734b |
| Follicular Fluid Level Testosterone (ng/mL), mean (DS) | 11.94 (2.56)                          | 9.5 (2.01) | 0.001a |
| FSH Recombinant dose (IU), median | 2925 (900–3750)                     | 2325 (1125–3450) | 0.105b |
| Oocyte count, median             | 4 (1–9)                             | 10 (3–20) | 0.001b |
| Cleavage Rate (DS)              | 3.46 ±3.18                          | 6.21 ±3.24 | 0.009a |

| Table 2. Granulose cell’s FSHr expression on poor responder and non-poor responder. |
|------------------------------------------|
| Poor Responder                         | Non-Poor Responder                  | $P*$ |
| Granulose Cell FSHr (ng/μL), mean (DS) | 1.259 × 10$^{-8}$                     | 0.887 × 10$^{-8}$ | 0.658 |
|                                          | (2.729 × 10$^{-8}$)                  | (2.137 × 10$^{-8}$) |

* Mann–Whitney test
Table 3. Correlation between granulose cell FSH expression with follicular fluid androgen levels

| Follicular Fluid Androgen | Poor Responder (n = 17) | Non-Poor Responder (n = 17) |
|---------------------------|-------------------------|-----------------------------|
| Testosterone (ng/mL)      | Coefficient Correlation (r) | 0.056                        | -0.051                        |
| DHEA (ng/mL)              | Coefficient Correlation (r) | 0.316                        | 0.218                        |
|                           | P*                      | 0.830                        | 0.844                        |
|                           | P*                      | 0.216                        | 0.400                        |

* Spearman test

Table 4. Correlation between granulose cell's FSH expression with age, AMH level and FSHr dose.

| Granulose Cell’s FSHr Expression | Poor Responder (n = 17) | Non-Poor Responder (n = 17) |
|----------------------------------|-------------------------|-----------------------------|
| Age                              | Coefficient Correlation (r) | -0.156                      | -0.246                      |
|                                  | P*                      | 0.55                        | 0.34                        |
| Follicular Fluid AMH (ng/mL)     | Coefficient Correlation (r) | -0.009                      | 0.108                       |
|                                  | P*                      | 0.97                        | 0.68                        |
| FSHr Dose (IU)                   | Coefficient Correlation (r) | 0.31                        | 0.056                       |
|                                  | P*                      | 0.281                       | 0.844                       |

* Spearman test

This study found higher intra-follicular levels of both DHEA and testosterone in poor responders. Intra-follicular testosterone levels in the poor responder group were significantly higher in the non-poor responder group (p = 0.001). We found no statistically significant differences in DHEA intra-follicular levels, although the levels in the poor responder group were higher than those in the non-poor responder, although the difference did not rise to the level of statistical significance (p = 0.734). We also found statistically different results on oocyte counts and cleavage rates, which were higher in non-poor responders (p = 0.001 and p = 0.009).

For FSHr expression analysis, we obtained 17 out of 21 sample expressions for each group. We found FSHr expression in granulose cells was higher in poor responders compared with non-poor responders (1.259 × 10⁻⁸ compared with 0.887 × 10⁻⁸), although the difference did not rise to the level of statistical significance (p = 0.658).

There was no correlation between follicular fluid testosterone levels and FSHr expression in either group. Meanwhile, intra-follicular DHEA levels in poor responders were positively correlated with
FSHr expression ($r = 0.316$), although the relationship did not rise to the level of statistical significance ($p = 0.218$).

Total dose of rFSH was correlated with FSHr expression within the poor responder group even though there was no statistically significant difference ($r = 0.31; p = 0.281$).

4. Discussion

Oocyte count and cleavage rate on the poor responder group was significantly lower because of lower ovarian reserve, resulting from older age and lower intra-follicular AMH levels. This result corresponded with previous studies by Bancsi [12] and Wiweko [13].

Follicular fluid is important during pregnancy because it forms the surrounding environment in which the oocyte grows and could therefore be used as biochemical predictor of oocyte quality. As stated before by Gleicher [7], androgens will influence granulose cells through genomic signaling (by androgen receptors) and non-genomic signaling. A major synergetic relationship between androgens and FSH can be seen during the follicle maturation phase, especially in the pre-antral and early antral phase.

This study corresponded by Drummond found many androgen receptors within the ovarian in the pre-antral phase [14]. Although androgens also play important roles in the pre-antral phase, in this study, we found it was hard to gain follicular fluid during the pre-antral phase. We found only one study performed by Nielsen et al., which collected pre-antral follicular fluid from the ovarian cortex for ovarian preservation.

As previously disclosed by Hugues, androgens enhance FSH receptor counts on granulose cells, therefore escalating folliculogenesis [15]. We found higher testosterone levels among the poor responder group compared with the non-poor responder group. Meanwhile, DHEA levels were not significantly different, even though there were higher levels in the poor responder group.

This result corresponded with a study performed by de Los Santos et al. and Wiweko, which found higher levels of testosterone and DHEA, but not to the level of statistical significance [13,16]. A previous study performed by Barad and Gleicher offered DHEA to enhance androgen levels and achieved a mature oocyte count as a result [7,17].

Recently, a study that emphasized FSH receptor expression has attracted special interest. Badawy et al. found that poor responders had lower FSHr on granulose cells although both groups shared the same levels of androgen [18]. Meanwhile in our study, we found the FSHr expression was higher in the poor responder group, although the difference was not statistically significant ($1.259 \times 10^{-8}$ vs. $0.887 \times 10^{-8}$).

Granulose cell proliferation improves, and growing follicles will increase after testosterone supplementation. Expression of FSHr on granulose cells will also improve after testosterone supplementation, so it is possible that androgen enhances follicle growth by sensitizing granulose cells by FSH trigger. Our study found positive correlations between DHEA levels and FSHr expression in poor responders, even though the difference was not statistically significant.

Our results are in accordance with a study by Nielsen, which showed a strong correlation between androgen levels in follicular fluids and androgen receptors in granulose cells with FSH expression in granulose cells. This result supports the idea that androgens will enhance follicle response and FSHr expression in granulose cells. On the basis of these facts, follicle sensitivity to FSH stimulation could be enhanced by providing more androgen supplementation. Therefore, in poor responders, we will need more androgen to enhance FSHr expression in granulose cells. Androgen (DHEA/testosterone) supplementation on poor responders, in order to achieve higher FSHr expression and produce more mature oocytes, should improve success IVF success rates among poor responder patients.

In existence of genetic backgrounds could affect the polymorphisms of androgen receptors and FSH receptors and, in return, will affect the boundary between the androgen and androgen receptors, poor quality of mRNA production, poor density of FSH receptors, and LH receptors in granulose cell.

This study sought to correlate androgen levels in follicular fluid with expression of FSH receptors in granulose cells in poor responders. Given non-invasively, we expect this process will emerge as a
cornerstone for giving androgen supplementation to poor responder patients and for optimizing outcomes of controlled ovarian stimulation. This is the only known study of FSH receptor expression in Indonesia. Because of this, all samples were analyzed in a single entrusted laboratory, so we do not believe our laboratory results are biased.

A key limitation to this study was that the androgen levels examined did not represent basal intrafollicle conditions because of the already given stimulation by using FSH recombinant. Further studies are needed to explore interactions between androgen levels and androgen receptors and FSH receptors. Instead of it, no genetically control over subject, so may possibly lower probability of having polymorphism of FSH receptor and androgen as well which may affect the final result.

5. Conclusion
There was no significant correlation between intra-follicular androgen levels and fertilization rates in both poor responders and non-poor responders. Additional studies are needed to find correlations between the other factors affecting poor responders.

Acknowledgement
This study was supported by a research grant from PITTA University of Indonesia (international publication indexed grants for students' final assignment).

References
[1] WHO 2004 Infecundity, Infertility, and Childlessness in Developing Countries 2004; DHS Comparative reports No.9
[2] Oudendijk J F, Yarde F, Eijkmans M J, Broekmans F J and Broer S L 2012 The poor responder in IVF: is the prognosis always poor?; a systematic review Hum. Reprod. Update 18 1-11.
[3] Allahbadia A P, La Marca A, Fauser B C, Tarlatzis B, Nargund G and Gianaroli L 2011 ESHRE consensus on the definition of ‘poor response’ to ovarian stimulation for in vitro fertilization: the Bologna criteria Hum. Reprod. 26 1616-24.
[4] PERFITRI, Laporan IVF di Indonesia 2013, Jakarta: Perhimpunan Fertilisasi In Vitro Indonesia
[5] PERFITRI, Laporan IVF di Indonesia 2015, Jakarta: Perhimpunan Fertilisasi In Vitro Indonesia
[6] Galey-Fontaine J, Cédrin-Durnerin I, Chaïbi R, Massin N, Hugues J N 2005 Age and ovarian reserve are distinct predictive factors of cycle outcome in low responders Reprod. Biomed. Online 10 94-9.
[7] Gleicher N, Weghofer A and Barad D H 2011 Defining ovarian reserve to better understand ovarian aging Reprod. Biol. Endocrinol. 9 23
[8] Nelson S M, Yates R W and Fleming R 2007 Serum anti-Mullerian hormone and FSH: Prediction of live birth and extremes of response in stimulated cycles implications for individualization of therapy Hum. Reprod. 22 2414-21
[9] Erickson G F and Shimasaki S 2001 The physiology of folliculogenesis: the role of novel growth factor Fertile Steril. 76 943-9
[10] Meldrum D R, Chang R J, Giudice L C, Balasch J and Barbieri R L 2013 Role of decreased androgens in the ovarian response to stimulation in older women Fertile Steril. 99 5-11.
[11] Mader S S 2010 Human Biology. (New York: McGraw Hill Companies Inc.) p.xviii;588:A14,G18.
[12] Bancsi L F, Broekmans F J, Eijkmans M J, de Jong F H, Habbema J D, and te Velde E R 2002 Predictors of poor ovarian response in in vitro fertilization: A prospective study comparing basal markers of ovarian reserve Fertile Steril. 77 328-36.
[13] Wiweko B and Shafira N 2016 Analisis kadar androgen cairan folikel terhadap laju fertilisasi pada perespon buruk dan perespon normal. Tesis program pendidikan dokter spesialis obstetric dan ginekologi, November 2016 (Jakarta: Faculty of Medicine, Universitas Indonesia).
[14] Drummond A E 2006 The role of steroids in follicular growth Reprod. Biol. Endocrinol. 4 16.
[15] Hugues J N, Massart P and Cedrin-Durnerin I 2013 Assessment of theca cell function: A prerequisite to androgen or luteinizing hormone supplementation in poor responders Fertile Steril. 99 333-6.

[16] De los Santos M J, García-Laez V, Beltrán D, Labarta E, Zuzuarregui J L, Alamá P, Gamiz P, Crespo J, Bosch E and Pellicer A 2013 The follicular hormonal profile in low-responder patients undergoing unstimulated cycles: is it hypoandrogenic? Hum. Reprod. 28 224-9.

[17] Barad D H, Weghofer A and Gleicher N 2011 Utility of age-specific serum anti-Mullerian hormone concentrations. RBM online 161 59-66.

[18] Badawy A, Wageah A, El Gharib M and Osman E E 2011 Prediction and diagnosis of poor ovarian response: The dilemma J. Reprod. Infertil. 12 241-8.