Correlation between anti-Müllerian hormone serum level and Bax/Bcl-2 mRNA expression ratio from granulosa cells in patients with PCOS

B Wiweko1,2,3*, A Beelonie2, Asmarinah4, N Purwito4, A Bowolaksono3,5, N Hanifah5, A M Sholihah5, K Mutia3, N Muna3, P A Iffanolid3, O Riayati2 and R R Febri3

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 Reproduction, Infertility, and Family Planning Research Center, Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia, Jakarta, 10430 Indonesia
4Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
5Department Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, 16424, Indonesia

*E-mail: budiwiweko@gmail.com

Abstract. Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age. Affected women usually have lower apoptotic rates of granulosa cells than unaffected women, resulting in lower oocyte quality. The Bcl-2 associated X protein (Bax), responsible for cell apoptosis, is low in women with PCOS, whereas the anti-apoptotic Bcl-2 is increased. In addition, the anti-Müllerian hormone (AMH) is also thought to suppress follicle apoptosis in the ovaries. This study evaluate whether the Bax/Bcl-2 ratio is correlated with the AMH serum level in women with PCOS.

A cross-sectional study was conducted based on medical records from the Cipto Mangunkusumo’s Hospital. Data from 20 women with PCOS and 20 control women who have undergone in vitro fertilization procedures were evaluated. The serum expression levels of AMH, Bax, and Bcl were also measured. This study found no differences between Bax or Bcl-2 levels or in the Bax/Bcl-2 ratio between the PCOS and the control groups (p = 0.38, p = 0.233, and p = 0.31, respectively). The mean AMH serum level in the PCOS group was significantly higher than that in the control group (p < 0.05). However, when AMH levels and the Bax/Bcl-2 ratios were tried to be matched, there was no significant correlation (p = 0.71, r = −0.109). Serum levels of AMH are not significantly correlated to the mRNA expression levels of Bax or Bcl-2 in granulosa cells.

1. Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine dysfunction in women of reproductive age with a prevalence of 5%–10%. Patients usually present with symptoms of hyperandrogenism, oligomenorrhea, chronic anovulation, and hyperinsulinemia (Rotterdam criteria). Hyperandrogenism in women can alter folliculogenesis causing early immature oocyte releases. Immature oocytes are of low...
quality and lead to low fertilization rates, especially in women who undergo in vitro fertilization (IVF) procedures [1-3]. In those cases, although ovulation rates after induction can reach 80%, the rate of pregnancies that end in live births reach only 40%–50%. These outcomes mean that the actual infertility rate in women affected by PCOS is higher than that in non-affected women. Of the patients with PCOS who undergo IVF, 16.9% suffer from ovarian hyperstimulation syndromes (OHSS), probable due to the low apoptotic rates of their granulosa cells. A significant and consistent correlation between PCOS and OHSS has been reported [4-6].

The anti-Müllerian hormone (AMH) is thought to suppress follicle apoptosis or atresia in the ovaries. AMH is produced in granulosa cells from small preantral (PA), larger PA, and small antral follicles. The AMH level influences first folliculogenesis. Folliculogenesis is affected by gonadotropin hormone. The level of AMH is thought to decrease with age, and thus aging increases atresia [7,8].

In addition, women with PCOS are also reported to have lower follicle apoptotic rate and 75 times higher AMH level in granulose cells. AMH has also been shown to inhibit the recruitment of primordial follicles, prevent selection of follicles by follicle-stimulating hormone and aromatase inhibition. AMH is also could not suppressed by gonadotropin-releasing hormone (GnRH) agonist agent or steroid contraception which suppress gonadotropin serum. {1.2 [EN] Meaning unclear. Please clarify} The evidence suggests that AMH inhibits follicular atresia, while the gonadotropin hormone helps follicles to grow [8,9].

Vitamin D has been shown to downregulate AMH signaling and causes apoptosis. These findings support AMH role as an anti-apoptosis agent and support the idea that vitamin D and leptin promote apoptosis. In swine’s ovary, granulosa cell, leptin medication in vitro increases p53 accumulation and apoptosis related to BAX and proliferation substrate (PCNA, cycline B1). Bcl-2 associated x (Bax) gene, which is thought to be responsible for cell apoptosis, is low in women with PCOS, whereas Bcl-2 gene which responsive for anti-apoptosis increases [9,10]. {1.2 [EN] Meaning unclear. Please clarify}

In addition, women with PCOS are thought to experience lower apoptosis rates due to low Bax/Bcl-2 levels. If these mechanisms are confirmed, new strategies for treatments for ovarian insufficiency, to delay the ovarian aging and menopause could be sought. No studies have evaluated AMH’s control of ovarian follicle atresia; therefore, we designed our study to evaluate the bax/bcl-2 ratio apoptosis marker levels in women with PCOS and look for correlations with oocyte quality.

2. Materials and Methods
2.1 Patient Eligibility
This study enrolled women diagnosed with PCOS based on their AMH levels (and Rotterdam criteria), and who underwent IVF procedures in the PCOS group. For the control group, we enrolled women with infertility dysfunction caused by factors other than ovarian problems. All participants signed informed consent forms. The criteria of exclusion were women with endometriosis disease or ovarian dysfunction other than PCOS, active smokers, women using hormonal contraception during the stimulation cycle, and women who did not agree to participate.

2.2 Place and time of study
The study was conducted in the Yasmin Clinic, of the Cipto Mangunkusmo’s hospital, in Jakarta during July 2016.

2.3 Participant assessment
Potential study patients were selected based on anamnèsis, physical examination (general status and obstetrics status), availability of ultrasound and laboratory examination results. Patients were examined based on standard operations in the Yasmin Clinic. Serum samples and granulosa cells from aspirated follicles were obtained from all participants. Granulosa cells separate automatically from oocytes, while the cumulus cells still stick to the oocyte. Granulosa cell samples were placed in a tube containing 500 ul RNA and frozen at −80 °C until used in mRNA measurements.

2.4 Enzyme-linked immunosorbent assay
AMH levels were measured from serum samples using the enzyme-linked immunosorbent assay (ELISA). The AMH Gen II ELISA kit (Beckman Coulter, USA) was used following the manufacturer’s instructions. Briefly, the calibrator, control, and test samples were incubated in microtitration wells coated with anti-AMH antibody. After washing, we added anti-AMH antibody labeled with biotin to each well. Incubation and washing were conducted for a second time. Then, streptavidin-horseradish peroxidase was added to each well. After the third, incubation and washing steps, we added the tetramethylbenzidine substrate to each well. Then, an acid solution was added, and we detected the enzymatic substrate changing degree under wave length absorption at 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 was used based on protocol RNeasy Mini Handbook with minor modifications. We first thawed our samples at room temperature. The samples were then vortexed for 3 second and centrifuged for 3-minute at 8000 g at room temperature. The supernatants were discarded. Then, after addition of 600 ul of RLT buffer with β-mercaptoethanol, the cell membranes was disrupted in a sonicator. The next steps were the same as those described in the manufacturer’s protocol.

2.6 Real time PCR
The Quantitect SYBR PCR master mix, template cDNA, primer forward and reverse, RNase free water for the RT-PCR were used, as described on Table 2. The result of the RT-PCR expression data for Bax and Bcl-2 granulosa cells, was paired with the oocyte maturity based on morphology for the study’s data.

Table 1. Genomic Composition of DNA elimination mix.

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

Table 2. Component of master mix RT-PCR reaction.

| Component                                | Volume/Reaction | Final Concentration |
|------------------------------------------|-----------------|---------------------|
| Quantitect SYBR Green PCR master mix     | 25 μL           | 1x                  |
| 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           |                     |

2.7 Statistical and Analysis data
The primary outputs for our study included the serum AMH levels and the mRNA expression levels for Bax and Bcl-2 from granulosa cells obtained during oocyte extraction after ovum pick-up. We analyzed all the data statistically using the SPSS 20 software. We considered a P value as significant when it was <0.05.

The demographic data distributions for AMH serum level, and mRNA Bax and Bcl-2 expressions were calculated. Data were presented as mean and standard distribution if the data were normally distributed, or as median and range if the data were not normally distributed. This study analyzed the correlation between Bax/Bcl-2 ratio and AMH serum using Pearson’s correlation for normally distributed data, or using Spearman correlation if the data were not normally distributed.

3. Results
3.1 Samples Characteristics
The mean age of women in the PCOS group was 33.8 ± 3.18 years, and that in the control group was 35.15 ± 4.22 years. This difference was not statistically significant (p = 0.295). The body mass index for women in the PCOS groups were 25.02 ± 3.38 kg/m2, and that in the control group was 23.95 ± 3.7 kg/m2 (p = 0.345).

3.2 rFSH level Characteristics
Women in the PCOS group had a median 2250 (ranging from 1200–20225), and those in the control group had a median level of 2662.5 (ranging from 1125–3675).

|                      | PCOS (n = 20) | Control (n = 20) |
|----------------------|--------------|-----------------|
| Age                  |              |                 |
| Mean                 | 33.82        | 35.15           |
| Standard             | 3.18         | 4.22            |
| Deviation BMI        |              |                 |
| Mean                 | 25.02        | 23.95           |
| Standard             | 3.38         | 3.7             |
| Deviation rFSH       |              |                 |
| Median               | 2250         | 2662.5          |
| Range                | 1200–20225   | 1125–3675       |
| AMH                  |              |                 |
| Median               | 11.59        | 2.05            |
| Range                | 2.9–24.09    | 0.3–2.42        |
| BCL-2                |              |                 |
| Median               | $3.505 \times 10^{-8}$ | $2.035 \times 10^{-8}$ |
| Range                | $4 \times 10^{-9}$–$5.57 \times 10^{-7}$ | $1.08 \times 10^{-9}$–$2 \times 10^{-6}$ |
| BAX                  |              |                 |
| Median               | $3.3 \times 10^{-5}$ | $1.6 \times 10^{-5}$ |
| Range                | $2.61 \times 10^{-8}$–$2.19 \times 10^{-4}$ | $2.09 \times 10^{-9}$–$1.33 \times 10^{-4}$ |
| BAX/BCL-2            |              |                 |
| Median               | 520.12       | 1141.68         |
| Range                | 0.11–2875    | 1.87–48826.29   |

3.3 AMH level characteristics
Women in the PCOS group had a median of 11.59 (range, 2.9-24.09), whereas those in the control group had a median of 3.04 (range 0.3-2.42). We found the difference to be statistically significant (p < 0.005).

3.4 Bax and Bcl-2 level characteristics
There was no differences in Bcl-2 or Bax levels between PCOS and control group women (p = 0.233, p = 0.380, respectively).

3.5 Correlation between AMH level and Bax/bcl-2 ratio
There was no significant correlations between the AMH levels and the bax/bcl-2 ratios in either group (p = 0.354 for the control group, and p = 0.697 for the PCOS group).

4. Discussion
The Bcl2-associated x protein (Bax) and the B-cell leukemia/lymphoma gene-2 (Bcl2) proteins are responsible for apoptosis and anti-apoptosis functions, respectively. We found no differences in their expression levels in granulosa cells between those in PCOS women and those in control women (p > 0.05). Moreover, we found no significant differences between the Bax/bcl-2 ratios associated to the control and PCOS groups (p > 0.05). Our study contradicts the results of a previous study showing significant differences between PCOS and control groups in terms of the Bax/Bcl-2 ratio. This, could be due to the fact that our granulosa cell samples were derived from ovaries that had been stimulated, or maybe because of the processes they underwent during the IVF preparations. The cultured environment may have influenced our results, but this was unavoidable, because simulating the natural environment is difficult. Studies have addressed this issue. Cumulus cells taken from in vivo matured oocytes were shown to increase the expression of genes associated with expansion (tumor necrosis factor-alpha-induced protein 6, TNFAIP6) and oocyte maturation regulation (inhibin beta A, INHBA, and follistatin, FST). And, cumulus cells obtained from oocytes matured in vitro showed expression of genes that respond to stress (heat shock protein, HSPA5, and HSP90AB1). Studies have also reported that FF cultured in bovine tissues was of better quality than those matured in vitro. From our study, FF might have been induced due to our protocol.

Our IVF protocol included the use of a GnRH agonist to trigger oocytes. Tili et al., observed that administration of gonadotropin inhibits apoptosis and atresia in granulosa cells. Its mechanism is thought to be through reduction of Bax expression while maintaining Bcl-2 levels. At the same time, Das et al in their study showed that GnRH agonist administration in vivo could increase follicular atresia and apoptosis in follicles from rats that had been given chorionic gonadotropin and the levels correlated with the anti-apoptosis/proapoptosis ratio. This results are similar to those by Choi et al., where GnRH administration suppressed Bcl-2 expression. These results confirm that GnRH administrations may have become cofounding variables in our study influencing our results.

In our study, women in the PCOS group had higher AMH levels than those in the control group. And, although the negative correlation between the AMH level and the Bax/Bcl-2 ratio in the PCOS group, was not significant, a higher AMH level should lower the Bax/bcl-2 ratio. The number of samples in our study was low and that could also have affected the power of the statistical analysis estimates. Studies have shown that the AMH level can predict oocyte numbers in women with PCOS and other infertility disorders. The alteration of the Bax/Bcl2 ratio in the ovaries probably also contributes to this effect.

5. Conclusion
There was no correlation between AMH serum level and BAX/BCL2 expression in granulosa cells of women suffering Polycystic Ovary Syndrome.
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