RESEARCH ARTICLE

A high dose of total recombinant FSH suppresses granulosa cell apoptosis and maintains oocyte quality in endometriosis: A cross-sectional study [version 1; peer review: 1 not approved]

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Abstract

Background: Endometriosis is one of the most common conditions causing infertility and an indication to undergo *in vitro* fertilization (IVF). High apoptosis rate and oxidative stress in patients with endometriosis are believed to negatively affect the IVF success rate. However, there have been conflicting results on the effect of endometriosis on IVF success, and there have been limited studies that directly assess endometriosis and its effect on oocyte quality. This study was performed to explore the correlation between mRNA BAX/BCL-2 expression and oocyte quality in endometriosis compared to non-endometriosis subjects.

Methods: This was a cross-sectional study. 15 endometriosis and 15 non-endometriosis subjects were recruited through convenience sampling at Cipto Mangunkusumo Hospital, Jakarta. All subjects underwent follicle stimulation with recombinant follicle-stimulating hormone (FSH). Granulosa cells were collected and tested for BAX and BCL-2 expression and the results were compared to the oocyte quality and fertilization rate of the patients.

Results: The total dose of recombinant FSH received by the endometriosis group was significantly higher compared with that of the non-endometriosis group ($p = 0.005$). There was a difference in

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BAX level ($p = 0.029$) and BCL-2 level ($p < 0.001$) between groups. However, the BAX/BCL-2 ratio did not differ significantly ($p = 0.787$) between groups. No significant correlation was found between the BAX/BCL-2 ratio and any of the oocyte quality parameters measured.

Conclusion: We found that there is a significantly higher dose in total dose recombinant FSH received by the endometriosis group compared with the non-endometriosis group. We also found that there was no significant difference in BAX/BCL-2 ratio between the endometriosis and non-endometriosis groups.

Keywords

Apoptosis, BAX/BCL-2 ratio, Endometriosis, Oocyte Quality, r-FSH

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Introduction

Endometriosis is a condition in which endometrial tissues and glands are found outside the uterine cavity. This condition might cause pelvic pain and infertility. Ectopic endometrial tissue causes chronic inflammation, widespread fibrotic change, and adhesion^{1,2}. Endometriosis occurs in 25–40% of women with infertility, and 30–50% of women with endometriosis also have infertility^{3,4}. Endometriosis has been found to cause folliculogenesis and oocyte maturation disturbances, an increase in oxidative stress, and an imbalance in inflammatory cytokines that result in infertility^{5–8}. *In vitro* fertilization (IVF) is one of the treatment options for patients with endometriosis. In 2016, in Indonesia, there were 8152 IVF cycles were performed, 7.03% of them were in women with endometriosis⁹.

Studies on endometriosis affecting IVF success rate have shown conflicting results¹⁰. Some meta-analyses report that endometriosis affects the rate of pregnancy, miscarriage, and live birth during IVF. However, there has not been any adequate study that evaluates oocyte quality as an outcome of endometriosis. Oocyte quality is defined as the oocyte's ability to undergo maturation and fertilization¹¹. Some studies that evaluated oocytes quality from endometriosis patients found that oocyte quality were poorer regardless of sperm quality and uterine cavity condition^{12,13}. Increased apoptosis rates have been found in endometriosis ovaries, and this has been linked to decreased oocyte quality¹⁴. Studies on apoptosis show a difference between endometriosis ovaries and non-endometriosis ovaries due to increased oxidative stress¹⁵. This condition triggers apoptosis through intrinsic and extrinsic pathways as well as meiotic spindle dysfunction and direct destruction through lipid peroxidase^{16,17}. Apoptosis is regulated by specific genes that code protein and initiate the whole process. B-cell lymphoma/leukemia 2 (BCL-2) plays a crucial role in regulating apoptosis. This protein family consists of two categories: apoptosis inhibitors, BCL-2 and BCL-2-like 1 (BCL-2-L1 or BCL-XL); as well as an apoptosis trigger, BCL-2-associated X protein (BAX)¹⁸. This study aimed to measure the gene expression of these apoptosis regulators, BCL-2 and BAX, and evaluate their correlation with oocyte quality in endometriosis and non-endometriosis IVF patients. The proposed conclusion was that there was significant difference in BAX/BCL-2 mRNA expression in patients with endometriosis and non-endometriosis.

Methods

Sample selection

Subjects were selected through convenience sampling. In total, 30 women were recruited for the study, who were undergoing IVF treatment at Yasmin Kencana Clinic, Cipto Mangunkusumo Hospital, Jakarta. Sample recruitment was done in June 2016 to August 2017.

All subjects found to positively have endometriosis, determined from a clinical examination and imaging procedures done by an obstetrician/gynecologist, were included in this study as a case group. However, subjects found to have other ovarian disturbances, history of ovarian removal, smoking, alcohol consumption, and sperm factor were excluded. For control group, we included all

subjects with ovarian disturbance other than endometriosis and excluded subjects with male infertility factor.

Granulosa cell sampling was conducted in the IVF Laboratory, while RNA isolation and quantitative real-time polymerase chain reaction (PCR) were done in the Integrated Laboratory Faculty of Medicine, Universitas Indonesia, between August and October 2017.

This study was conducted according to ethical standards in the Declaration of Helsinki and approved by the Faculty of Medicine Universitas Indonesia-Dr. Cipto Mangunkusumo General Hospital Research Ethical Committee with approval number LB.02.01/X.2/871/2017. All subjects in this study had the research clearly explained to them and have signed informed consent.

Data collection

RNA and DNA extraction from granulosa cells. Follicular fluid and granulosa cells were taken during ovum pickup, as part of routine IVF treatment. Granulosa cells were obtained during follicular aspiration and separated from the oocyte. These cells were then kept in a tube containing 500 uL RNA Later solution and stored at a temperature of -80°C.

RNA was isolated from granulosa cells using the QIAamp RNA Blood Mini Kit (QIAGEN) according to the modified QIAamp RNA Blood Mini Handbook¹⁹. Buffer RLT and beta-mercaptoethanol were set with a 600 uL:6 uL ratio for each sample. Briefly, the granulosa cell samples in RNA Later solution were thawed and centrifuged at 20°C for 5 minutes (8000 × g). The supernatant was discarded, and RLT buffer mixed with beta-mercaptoethanol was added and homogenized. The sample was moved to a QIAshredder spin column and centrifuged for 8 minutes at 20°C (8000 × g). 600 uL Ethanol 70% was added to flow-through liquid, homogenized, and moved into QIAamp spin column. The QIAamp spin column was then centrifuged for 15 seconds (16.000 × g). The flow-through was then discarded and the column was washed using 600 uL RW1 buffer, centrifuged for 15 seconds (16.000 × g), and the flow-through was discarded. 500 uL RPE buffer was then added into the column for final washing, centrifuged for 15 seconds (16.000 × g), and the flow-through was discarded. This step was repeated twice. 25 uL RNase-free water was then added into the column as an elution solution to bind the RNA and the column was incubated for 10 minutes. The column then underwent centrifuge for 1 minute (16.000 × g) and the RNA collected at the collection tube under QIAamp spin column. The RNA concentration was measured with Nano Drop and stored at -80°C.

cDNA was synthesized from RNA using the Qiagen Quantitect Reverse Transcription Kit according to the modified Quantitect Reverse Transcription Mini Handbook protocol²⁰. The RNA template was mixed with RNase-free water with certain ratio according to RNA concentration obtained to gain equal cDNA concentration for all samples. This RNA and RNase-free water mix will obtain total volume of 14 uL. gDNA Wipeout buffer was

then added into the mix and then incubated at 42°C for 10 minutes. Into each sample was then added Reverse-transcription master mix 1 uL, Quantiscript RT buffer 4 uL, and RT Primer mix 1 uL. the sample was then incubate at 42°C for 15 minutes and 95°C for 3 minutes. cDNA samples were then stored at -20°C until ready to be used.

Real-time PCR for BAX and BCL-2 expression

Quantitative real-time PCR was conducted using Qiagen Quantitect SYBR Green PCR Kit according to the modified Quantitect SYBR Green PCR Mini Handbook protocol²¹. qPCR was done by using absolute quantification, which measures sample by referring to standard curve. Standard curve was constructed using gBlocks oligonucleotide which made from amplicon product from each BAX and BCL-2 primer. The quantitative real-time PCR was performed using primers in Table 1.

PCR master mix was firstly prepared and contain of 12.5 uL SYBR Green PCR mix, 0.25 uL forward primer, 0.25 uL reverse primer, and 10 uL nuclease-free water for each sample. After homogenized, the cDNA samples were then added into tube containing PCR mix and placed in real-time PCR instrument (Qiagen Rotor Gene-Q real-time PCR). Thermal profile of the instrument was set as shown in Table 2, while annealing temperature was set according do melting temperature in Table 1 for each gene, 58°C for BAX and 60°C for BCL-2.

Construction of standard curve was performed using the same PCR master mix composition, except for the cDNA samples. The cDNA was replaced by gBlocks oligonucleotide which has known concentration and diluted 5 times. The concentration of each

gBlocks used were 1 until 1×10^{-8} ng/uL. Each gBlocks concentration was run duplicate to obtain standard curve with coefficient of determination value close to 1.

The standard curve was then used to measure each sample.

Oocyte quality assessment. Oocyte quality was measured by assessing morphology, maturation index, and fertilization rate. This assessment was performed under an Inverted Microscope (Olympus). Oocyte morphology was classified with Xia criteria, and mean score was calculated from the total number of oocytes obtained per subject²². The maturation index was defined as percentage of oocytes in metaphase II, whereas the fertilization rate was defined as percentage of metaphase II oocytes which successfully fertilized.

Data analysis

During the stimulation phase, subjects were exposed to recombinant FSH, and the total dose of FSH might influence the result of the study; therefore, the total dose of FSH provided to the participants was noted.

BAX/BCL-2 ratio was the independent variable, whereas the oocyte quality was the dependent variable. Statistical analysis was conducted with IBM SPSS (Statistical Package for Social Sciences) version 22, to test distribution and comparison between the endometriosis group and the control group for each variable. A correlation test was done to test any association between BAX/BCL-2 ratio and oocyte quality indicators in both groups. Variables with normally distributed data were tested with an independent t test, whereas other variables were tested with the Mann-Whitney test.

Table 1. BAX and BCL-2 Primer List.

Gene	Primer	Sequence	Amplicon size	Tm (°C)
BAX	Forward	CGGCCTCCTCTCTACTT	106	58
BAX	Reverse	GCCTCAGCCCATCTTCTTC	106	58
BCL-2	Forward	ACCGGGAGATAGTGATGAAGTA	128	60
BCL-2	Reverse	GGGCTGGGAGGAGAAGA	128	58

Table 2. Thermal Profile for Quantitative Real-time PCR.

Step	Time	Temperature (°C)
PCR activation	15 minutes	95
Denaturation	15 seconds	95
Annealing	30 seconds	variable
Extension	30 seconds	72
Number of Cycles	40	

Results

A total of 30 subjects took part in this study: endometriosis group (n = 15) and control group (n = 15). Characteristics of these subjects are in Table 3. The patients' age in both groups showed a normal distribution, with a mean age of 33.27 ± 4.448 for endometriosis group and 32.67 ± 3.559 for non-endometriosis group. Total dose of recombinant FSH received was calculated and there was a significant difference between the groups; mean dose of FSH received by the endometriosis group was 3760.0 ± 1054.15 while the non-endometriosis group was 2763.3 ± 700.82 ($p=0.005$). The total number of oocytes obtained during ovum pickup was normally distributed in both groups (Table 3). The mean number of oocytes was lower in the endometriosis group, although the difference was not significant.

Gene expression of BAX and BCL-2 were significantly different between the groups, with the concentrations of both genes being lower in the endometriosis group (Table 4). However, there was no statistically significant difference between the BAX/BCL-2 ratio between groups ($p=0.787$).

Oocyte quality was measured with three indicators: morphology, maturation index, and fertilization rate. We found no oocyte quality difference in the endometriosis group compared with the non-endometriosis group (Table 4).

Discussion

BAX and BCL-2 are two proteins that play a crucial role in cell apoptosis through the intrinsic pathway. BAX is an apoptosis initiator, whereas BCL-2 is anti-apoptotic. A previous study by Tommi *et al.* found that the ratio between BAX and BCL-2 is one of the important indicators of apoptosis activity in cells²³.

The increases in BAX/BCL-2 ratio correspond to increased apoptosis activity and vice versa.

In this study, we found significant differences in BAX and BCL-2 mRNA concentrations in both groups. The expression of both genes was lower in the endometriosis group compared with the non-endometriosis group ($p = 0.029$ in BAX and $p<0.001$ in BCL-2). However, there was no difference in the BAX/BCL-2 mRNA ratio between groups ($p = 0.787$). This result showed that there is no difference in apoptosis activity in endometriosis granulosa cells compared with non-endometriosis cells. This finding is inconsistent with a previous study by Wiweko *et al.*, which stated that BAX mRNA concentration in endometriosis granulosa cells is higher than in the control group²⁴. However, the lower level of BAX mRNA does not imply less apoptotic activity. One interaction model of BAX and BCL-2 protein showed that while BAX induces apoptosis, BCL-2 inhibits the process²⁵. In this case, the low level of BCL-2 in endometriosis might result in decreased anti-apoptotic activity. There was also a study that showed fewer follicles in rats with BCL-2 deficiency, and that higher BCL-2 expression would suppress apoptosis and atresia²⁶.

Filali *et al.* reported the essential role of BCL-2 mRNA in granulosa cells in determining oocyte quality. BCL-2 mRNA expression was found to be higher in the granulosa cells of mature oocytes, whereas there was no difference in BAX mRNA in the same cells. Moreover, BCL-2 mRNA correlates with the fertilization rate due to the lower apoptosis activity²⁷. BAX/BCL-2 ratio has a more important role as an apoptosis marker, as mentioned earlier. However, in this study, we found no significant difference in apoptosis activity. This finding is inconsistent with a previous

Table 3. Endometriosis and non-endometriosis patient characteristics undergoing IVF treatment.

Characteristics (mean $\pm$ SD)	Endometriosis (n=15)	Non-en (n=15)	p-value
Age (years)	33.27 $\pm$ 4.45	32.67 $\pm$ 3.56	0.686
FSH dose (IU)	3760 $\pm$ 1054.15	2763.3 $\pm$ 700.82	0.005
Oocyte number	9.93 $\pm$ 5.216	12.27 $\pm$ 7.620	0.336
Oocyte score	2.61 $\pm$ 0.60	2.50 $\pm$ 0.57	0.611

Table 4. Gene expression of BAX and BCL-2, maturation index and fertilization rate for endometriosis and non-endometriosis patients undergoing IVF treatment.

Gene expression (mean (95%CI))	Endometriosis (n=15)	Non-en (n=15)	p-value
BAX ng/uL)	7.47 (0.270 – 38.2)	73.00 (0.119 – 66200)	0.029
BCL-2 (ng/uL)	0.10 (0.011 – 15.5)	9.87 (0.278 – 38.1)	0.001
BAX/BCL-2 ratio	74.69 (0.03 – 591.41)	94.80 (0.01 – 56101.69)	0.787
Maturation index	0.8 (0.50 – 1.00)	1.0 (0.25 – 1.00)	0.225
Fertilization rate	0.67 (0.17 – 1.25)	0.67 (0.00 – 0.93)	0.693

study by Wiweko *et al.* in 2017, which showed a higher BAX/BCL-2 ratio in the endometriosis group compared with the control group²⁸.

In the present study, the oocyte number in the endometriosis group was lower than in the non-endometriosis group, but was not significant ($p = 0.336$). This finding is in line with studies by Rossi *et al.* and Yang *et al.* that showed a smaller number of oocytes in endometriosis subjects^{29,30}. Regarding oocyte quality, we found no differences in the mean oocyte score, maturation index, and fertilization rate between the groups. The mean oocyte score showed no significant difference ($p = 0.611$). The oocyte score was assessed by Xia criteria, which analyzes oocyte morphology based on perivitelline space, polar body II, and cytoplasm. This result is inconsistent with Shebl *et al.*, who reported that fewer normal oocytes were observed in patients with endometriosis compared with control subjects³¹. Similarly, the maturation index and fertilization rate in both groups did not show a marked difference ($p=0.225$ and $p=0.693$, respectively). This is inconsistent with other related studies that showed maturation index and fertilization rate in endometriosis were poor; Rossi *et al.* and Yang *et al.* reported that less MII oocytes were found in endometriosis patients^{29,30}. On the other hand, Luca *et al.* stated that there was no difference in the number of MII oocytes in an endometriosis group compared with a control group³².

Inconsistent with our finding, Barnhart *et al.* showed a lower fertilization rate in endometriosis patients³³. The fertilization rate in patients with severe endometriosis was higher compared with subjects with mild/moderate endometriosis and control subjects. However, Yang reported that a decrease in fertilization rate only occurred in subjects with endometriosis grade III/IV, not those with grade I/II, compared with control subjects³⁰.

In this study, there was no correlation between BAX/BCL-2 mRNA ratio and mean oocyte score, maturation index, and fertilization rate in both groups. This result suggests that similar apoptosis activity will result in similar oocyte quality. Although we found no difference in BAX/BCL-2 mRNA ratio and oocyte quality, there was a significant discrepancy in recombinant FSH dose given to each group ($p=0.005$). The mean FSH dose in the endometriosis group was 3760 IU, whereas the control group 2763.3 received IU. Shen *et al.* reported that FSH might play a role in protecting granulosa cells from oxidative stress, although

the mechanism is currently unknown. FSH also protects mitochondria in granulosa cells undergoing oxidative stress³⁴. Our study shows that subjects with endometriosis may benefit from higher dose of recombinant FSH to produce oocytes with similar quality to those in the control group. Therefore, further studies on FSH and its correlation with apoptosis activity and oocyte quality during ovum pickup are needed.

Conclusions

Although we found lower levels of BAX mRNA and BCL-2 mRNA in patients with endometriosis, the ratio of these mRNAs was not statistically significant between groups. There was also no correlation between BAX/BCL-2 mRNA ratio and oocyte number, FSH dose, and oocyte quality in both groups. However, we noted a significantly higher dose of recombinant FSH given to the subjects with endometriosis compared with those in the non-endometriosis group. We suggest for future studies a similar protocol in collecting granulosa cells from size-matched follicles to minimize the discrepancy in BAX and BCL-2 mRNA concentrations. Moreover, examination and records on basal antral follicle and anti-müllerian hormone concentration should become a part of the standard IVF protocol.

Data availability

Figshare: DATA_A High Dose of Total Recombinant FSH Suppress Granulosa Cells Apoptosis, <https://doi.org/10.6084/m9.figshare.7483574.v1>³⁵.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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The manuscript entitled "A high dose of total recombinant FSH suppresses granulosa cell apoptosis and maintains oocyte quality in endometriosis: A cross-sectional study" - this study was conducted in 15 patients with endometriosis and 15 control patients without endometriosis. The draft is oversimplified with very preliminary results, and most data were published elsewhere. This study has a lack of novelty.

1. The sample size was too small to interpret the results with a proper power. The sample size estimation to get a proper power should be performed before conduction of the study.
2. Since the sample size is small, the baseline characteristics of patients with endometriosis should be described more clearly, such as BMI, duration of infertility, primary or secondary infertility, baseline FSH/LH/E2/P4, antral follicle count, AMH, or the revised American Fertility Society (rAFS) score, etc. Such information is basic and important. The stage of endometriosis could also be a confounding factor to affect the results.
3. The size of endometriosis of ovary, unilateral or bilateral should also be mentioned.
4. The title is interesting. However, their data didn't support their title. The gene expression of BAX and BCL-2 is only an association in patients with endometriosis. There was no ex-vivo functional assay, such as comparing the apoptosis activity in granulosa cells from patients with endometriosis under different doses of recombinant FSH, to support the title of manuscript. Thereafter, the title should be considered to be rewritten.
5. The conclusion section in the abstract should be rewritten. It was loosely organized and did not incorporate the title of this manuscript.
6. Whether this was a retrospective or prospective study according to the statement in the methods section could not be determined clearly. Please describe it clearly.
7. What are the diagnosis criteria of endometriosis in this manuscript, especially for clinical

examination mentioned in the methods section? Besides, the grade of endometriosis should be illustrated.

8. The authors collected follicular fluid during ovum pickup, but we did not see any data from the analysis of follicular fluid.
9. Please describe the protocol of controlled ovarian hyperstimulation more clearly, including the medications used for COH, the method(s) of insemination, etc.
10. It was not proper to interpret the data from two small groups as normal distribution.
11. The goal of this study is not clear. One can not be sure if the authors tried to compare the results from endometriosis and non-endometriosis, or if they wanted to compare the dose effect of recombinant FSH in patients with endometriosis. Please make it more obvious.
12. The basic characteristics of patients should be more detailed, including AMH and/or AFC, COH protocol, COH duration, medication for triggering, maximal E2 level, etc.
13. In Table 4, the mean value of maturation index in the non-endo group is 1, but the range of maturation index is 0.25-1.00. Is the mean value correct?
14. The results of this manuscript were different from previous studies, but the authors did not explain the possible reason for the different findings in the discussion section. They published similar studies in 2017. This manuscript is very similar to their previous studies. Thereafter, there were no new insights from this draft.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Endometriosis in ART

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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