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The Mechanism of Endoplasmic Reticulum Stress and the Cyclin D2 Expression on Ovarian Granulosa Cell Cycle in Infertile Women with Endometriosis

Jimmy Yanuar Annas[1*], Hendy Hendarto[2], Erry Gumilar Dachlan[2], Samsulhadi[2], Soegiharto Soebijanto[3], Eu Leong Yong[4]

[1]Medical Doctor Program, Faculty of Medicine, Universitas Airlangga, Surabaya 60131, Indonesia
[2]Department of Obstetrics and Gynecology, Faculty of Medicine-Dr. Soetomo Teaching Hospital, Universitas Airlangga, Surabaya 60131, Indonesia
[3]Department of Obstetrics and Gynecology, Faculty of Medicine-Dr. Cipto Mangunkusumo General Hospital, Universitas Indonesia, Jakarta 10430, Indonesia
[4]Department of Obstetrics and Gynecology, National University of Singapore, Singapore 119074, Singapore

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ABSTRACT

The endometriosis related to secretory cell namely granulosa cell provides its important organelle of endoplasmic reticulum. This study aimed to analyze the influence of TNF-α against the characteristic of granulosa cell, especially the function in its organelle of endoplasmic reticulum and the cell cycle. This was an observed cross-sectional study. Samples were found by laparoscopy at the time of 17 mm sized follicle was detected. The assessment of peritoneal fluid's TNF-α level was done by ELISA, the marker of mRNA-GRP78, eIF2α, and CHOP taken from isolated granulosa cell were assessed by qPCR, and flow cytometry was used to observe cell cycle while Western blotting spent to evaluate Cyclin D2. We observed 13 women with endometriosis and 4 women with non-endometriosis. The study showed that peritoneal fluid TNF-α was higher in endometriosis group compared to non-endometriosis (48.66±3.9 vs 37.6±3.8 ng/mL, p<0.05). The study apparently indicated sharply increased of all marker's mRNA-GRP78, eIFα and CHOP with respectively 3.32, 8.54 and 1.78- fold, but Cyclin D2 also increased (97.056% vs 86.217%; p>0.05) in endometriosis women group. The granulosa cell's cycle showed the higher result on G0/G1 phase but lowering S/M phase on endometriosis group compared to control. It seemed the influence of peritoneal fluid TNF-α occurred against GRP78. Endometriosis women having higher TNF-α level suggested to lead ER stress were playing the important role in the granulosa cell cycle.

Keywords: endometriosis, granulosa cell, TNF-α

Correspondance:
Jimmy Yanuar Annas, MD
Department of Obstetrics and Gynecology, Faculty of Medicine-Dr. Soetomo Teaching Hospital, Universitas Airlangga, Surabaya 60131, Indonesia
E-mail: jimmyyanuar@fk.unair.ac.id

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INTRODUCTION

Endometriosis is a benign disease, but it has negative impact on women's reproductive performance and quality of life. Clinical symptoms that are often displayed by endometriosis are not typical, such as dysmenorrhea, dyspareunia and or infertility (1). As many as 30% of women in infertility couples suffer from endometriosis (2,3). Fecundity and even IVF-ET outcome in women with endometriosis are lower than those without (4). Infertility pathogenesis in severe endometriosis is mostly caused by distortion of pelvic anatomy due to adhesion and disruption of fallopian tubes function that prevent them from facilitating fertilization. In mild to moderate endometriosis, despite normal internal genitalia, the patients also suffer from infertility. Pathogenesis of infertility in endometriosis is unknown (4,5).

Disturbance in folliculogenesis or oogenesis is suspected to play some roles in infertility endometriosis. The Growth Differentiation Factor 9 (GDF9) in follicular fluid is lower in endometriosis patients than in non-endometriosis (6). Previous study found lower intrafollicular Anti Mullerian Hormone (AMH) in patient with mild endometriosis compared to non-endometriosis, but the Antral Follicle Count (AFC) was not significantly different (7). Another study isolated granulosa cell from follicular fluid obtained during Ovum Pickup after long protocol of ovarian stimulation in Advanced Reproductive Technology (ART), and found that granulosa cell cycle was in S phase (8). Whereas, in non-endometriosis group, it was in M phase. Those are the evidence that folliculogenesis is impaired in endometriosis, and it seems there is a problem in the proliferation of granulosa cell which causes lower AMH level and impaired granulosa cell cycle. All the above evidence explains endometriosis with impaired folliculogenesis and apparently is related loosely to the problem with proliferation in granulosa cell causing lower AMH level and disturbed granulosa cell. However, the cause of this phenomenon is not yet understood.

Endometriosis is a chronic inflammation disease with high pro-inflammatory cytokines and oxidative stress milieu in peritoneal fluid (2,3,9). Interaction between endometriosis implants and macrophage could result continuous in activation of Nuclear Factor kappa B (NF-kB) that leads to the increase in proinflammation cytokines, especially TNF-α(10). The presence of steroidogenesis activity in ectopic endometriosis cell also can give positive feedback to TNF-α production in peritoneal fluid. On the other hand, the ovary is located in fossa ovary in Douglas pouch, which is the lowest part of pelvic cavity and always be drowned in the pro-inflammatory cytokine-rich peritoneal fluid of women with endometriosis. Besides, other studies also showed endoglin concentration in the peritoneal fluid increased proportionally with the severity of endometriosis (11).

We hypothesized that pro-inflammation cytokine (TNF-α level) in peritoneal fluid could cause unfolded protein response (UPR) (increase of GRP78) in granulosa cell isolated in the follicle fluid aspirated from dominant follicle which caused translation attenuation of Cyclin D2 and in turn caused granulosa cell cycle arrest at G0/G1 phase. This study aimed to analyze the influence of TNF-α against the characteristic of granulosa cell, especially the function in its organelle of endoplasmic reticulum and the cell cycle.

METHODS

This was an observational study with cross-sectional design. This study took place in Graha Amerta Infertility Clinic, Tertiary-Care of Dr. Soetomo Teaching and General Hospital, Surabaya from January 2016 to May 2018. Tissue samples were taken from women aged 30-37 years who underwent laparoscopy for evaluation of tubal and peritoneal factors as part of diagnostic work-up for subfertility. Inclusion criterion was regular menstrual cycles which received infertility management which indicated to do laparoscopy surgery at the time where 17 mm or larger sized follicle was detected. Informed written consent was obtained from all participants. The study was approved by the IRB. Exclusion criteria were a history of hormonal therapy in the previous three last months, previous pelvic inflammatory disease sign, such as filmy adhesions around the peri-tubal area, tubal phimosis, hydrosalpinx and hugh Curtis sign, and non-endometriosis ovarian tumors.

During laparoscopy, follicular fluid of dominant follicle and 10 cc of peritoneal fluid were aspirated using 21-gauge laparoscopy aspiration needle. Severity of endometriosis was scored using Revised American Fertility Society (R-AFS) scoring system for endometriosis. The subjects in this study were divided into two groups, endometriosis group and non-endometriosis group. Endometriosis lesions were resected for histopathologic examination. Hematoxylin Eosin staining was used to identify endometrial cells in tissue samples.

The level of TNF-α in the peritoneal fluid was examined using Enzyme Linked Immunosorbent Assay (ELISA). Granulosa cell in aspirated follicular fluid was isolated for evaluation. mRNA level of GRP78, eIF2α, CHOP, Cyclin D2 and of cell granulosa were evaluated by Real Time PCR (qPCR), and Cyclin D2 protein of granulosa cell was evaluated using Western Blot. Granulosa cell cycle and apoptosis were evaluated using flow cytometry.

RESULTS

For this study, seventeen women were enrolled. Control subjects (n=4) did not have endometriosis on laparoscopy. Thirteen women were in the endometriosis group. Three of the 13 (23.07%) patients had stage one or minimal, five (38.5%) had stage two or mild, four (30.8%) had stage three or moderate, and one (0.08%) had stage four or severe endometriosis. There were three patients at stage three and four with endometrioma cysts measuring 2 to 4 cm on one side of the ovary.

Fig. 1 shows comparison of TNF-α levels in the peritoneal fluid in two groups. The TNF-α levels in the peritoneal fluid of women with endometriosis (48.66±3.9 ng/mL) were higher than that in non-endometriosis women (37.6±3.8 ng/mL). Statistical test showed the significance value of $p<0.05$, which indicated that the difference was statistically significant.

Fig. 2 shows the results of qPCR examination of granulosa cells isolated from follicular fluid. This examination aims to quantitatively observe the GRP78, eIF2α, CHOP and Cyclin D2 mRNA (*CCND2*) in granulosa cells isolated from dominant follicular fluid.

Table 1 shows an increase in GRP78 mRNA expression of 3.32-fold, eIF2α of 8.54-fold, CHOP of 1.78 fold, followed by CCND2 mRNA expression of 3.9 fold in the endometriosis compared to non-endometriosis group. These data indicated that there was an increase in the transcription of UPR, eIF2α, CHOP and CCND2 chaperones in granulosa cells of women with endometriosis.

Table 1: mRNA fold change value in endometriosis group

		No endometriosis	Endometriosis
.avgCT	CCND2	36.25067	37.20018
	CHOP	34.9827	37.06679
	eIF2α	34.45613	34.2823
	Grp78	31.26739	32.45294
	Gapdh	29.86736	32.78806
avgΔCT	CCND2	6.383311	4.412112
	CHOP	5.115344	4.278728
	eIF2α	4.588775	1.49424
	Grp78	1.400029	-0.33513
	Gapdh	0	0
ΔΔCT	CCND2	0	-1.9712
	CHOP	0	-0.83662
	eIF2α	0	-3.09453
	Grp78	0	-1.73516
	Gapdh	0	0
2^-ΔΔCT	CCND2	1	3.920937
	CHOP	1	1.785856
	eIF2α	1	8.54177
	Grp78	1	3.329156
	Gapdh	1	1

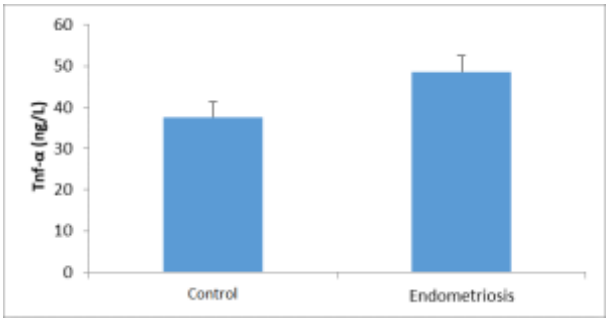

Figure 1: Comparison of TNF-α in peritoneal fluid between groups

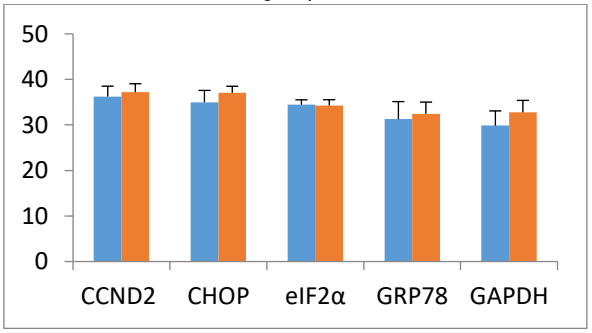

Figure 2: Comparison of mRNA from qPCR examination between groups

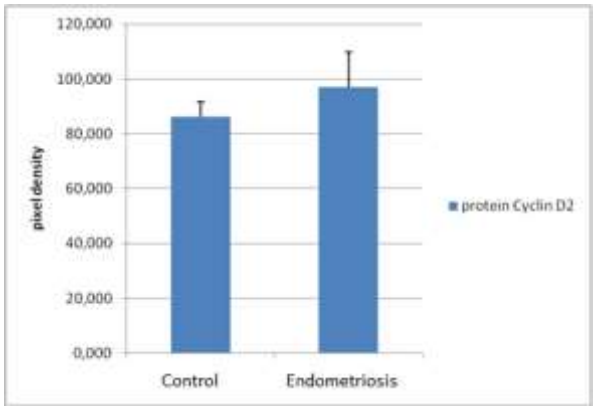

Figure 3: Western Blot of Cyclin D2 (34 kDa)

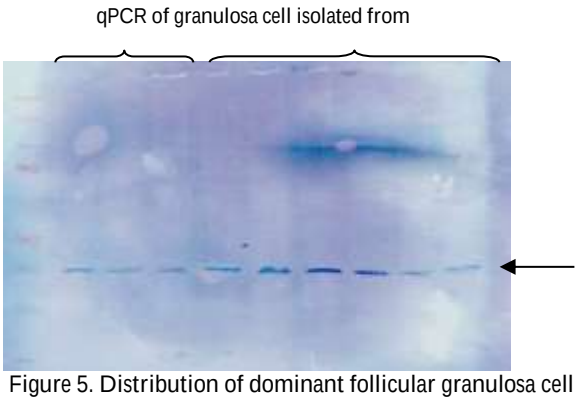

Figure 5. Distribution of dominant follicular granulosa cell cycle

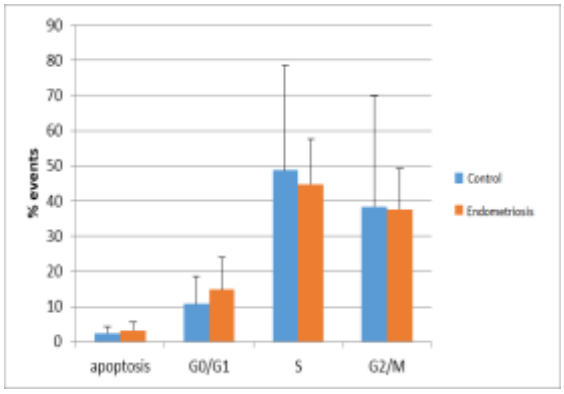

Figure 4: Cyclin D2 pixel density

After it was found that CCND2 mRNA increased in qPCR examination, we performed a Western Blot (WB) examination to prove whether CCND2 mRNA increase was also followed by protein synthesis. Cyclin D2 is a protein with a weight of 34 kDa (Fig.3). Fig. 4 shows the comparison of pixel density protein between the two groups on densitometry examination.

Fig. 5 shows that endometriosis and non-endometriosis groups have the same cell distribution pattern. Both had the highest distribution in phase S followed by phases M, G0/G1 and apoptosis. In the endometriosis group, granulosa cells were higher in the G0/G1 phase, the S phase and M phase were lower than in non-endometriosis. Apoptosis showed the lowest distribution. The endometriosis group had higher number of apoptotic cells than non-endometriosis. Statistical test showed P value <0.05, indicating that the difference was significant.

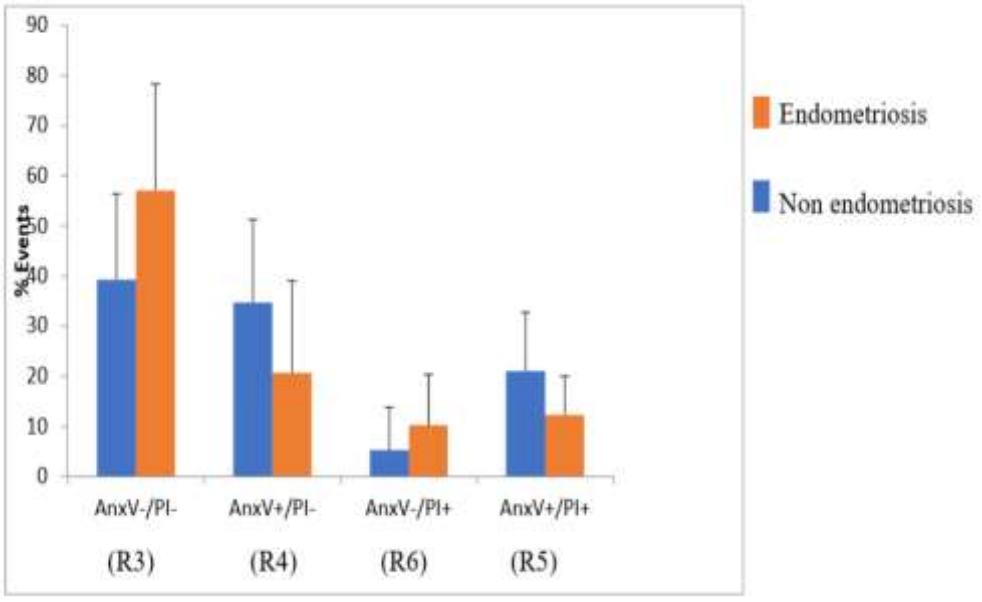

Figure 6: Distribution of the number of viable, apoptotic, and necrotic granulosa cells

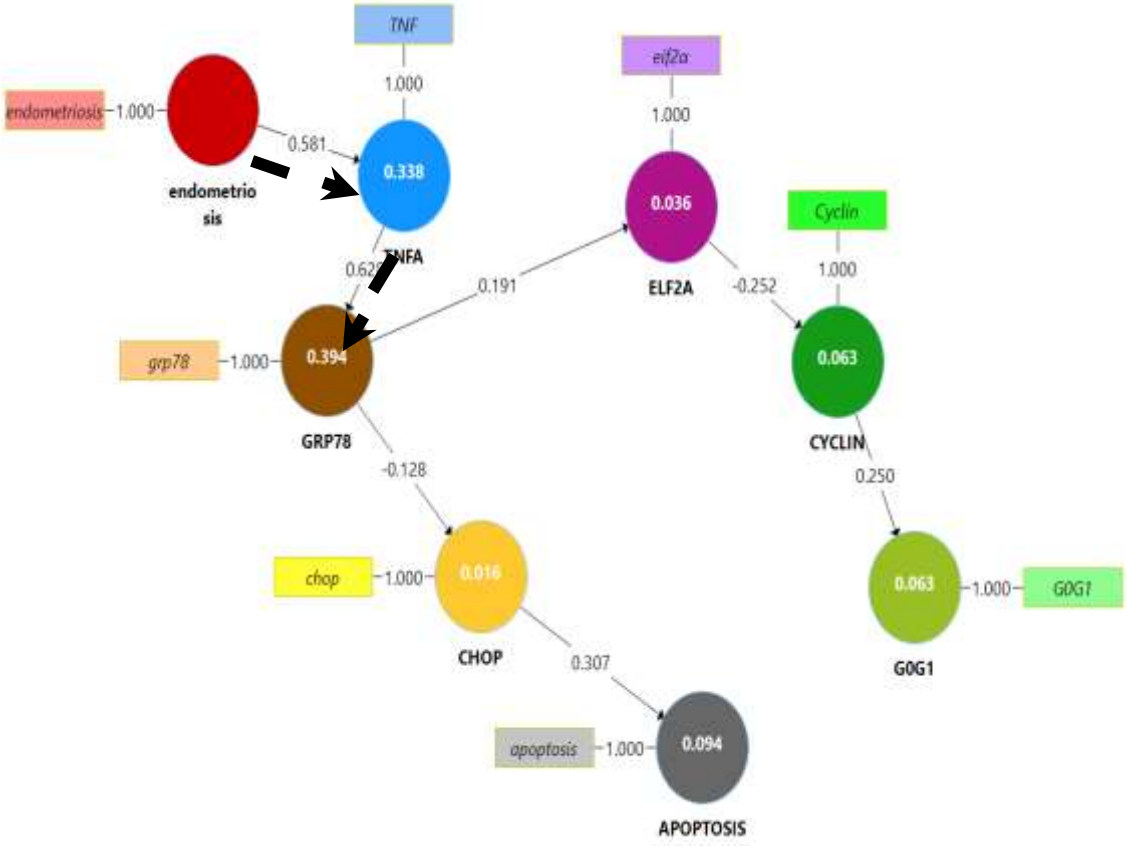

Figure 7: Path analysis between variables

Fig. 6 shows the comparison of the number of viable, apoptotic and necrotic granulosa cell between groups examined using annexin V and propidium iodide (PI) staining. Annexin V was bound to phosphatidylserine, and PI was bound to Deoxyribonucleic Acid (DNA), and then evaluated using flow cytometry. The fluorescence intensities of flow cytometry were grouped into 4: annexin V negative & PI/R3 negative (viable cells), annexin V positive & PI/R4 negative (early apoptosis), annexin V positive & PI/R5 positive (late apoptosis), and annexin V negative & PI/R6 positive (necrosis/ necroptosis).

Fig. 6 shows that the endometriosis group has a higher number of viable cells (R3) than non-endometriosis group. In apoptosis, R4 and R5 in non-endometriosis group were higher, whereas in the endometriosis group, it was higher in R6 or annexin V negative, PI positive. Statistical test showed p value <0.05, indicating that the difference was significant.

This study used T test on LISREL 9.3 program to assess correlations between variables. Variables were estimated for the T test were R-AFS score, TNF-α level of peritoneal fluid, GRP78, eIF2α, CCND2, CHOP, apoptosis, and granulosa cells cycle of isolated from dominant follicular fluid (Fig. 7). If the T test >1.96, the correlation was regarded as significant. Fig. 7 shows that R-AFS - TNF-α of peritoneal fluid - GRP78 scores of granulosa cells have significant correlation with coefficients of 0.338 and 0.394 (Bold arrow). The GRP78-eIFα-Cyclin D2 coefficient was higher than the coefficient of GRP78-CHOP-apoptosis.

DISCUSSION

Endometriosis women having significant higher TNF-α level suggested to lead ER stress were playing role in the granulosa cell cycle. It has been already known where their environment are rich in proinflammatory cytokines and ROS (2,9). The though and continuously interaction between implanted endometriosis lesions and macrophages lead to intensely NF-kB activation, in turn pushing macrophage producing increased TNF-α, Interleukin 1 (IL-1), and inducible Nitric Oxide Synthase (iNOS) (2,10,12).

In endometriosis group, most of the patients had mild and moderate stage. Three of them showed endometrioma, although in small size (2 to 4 cm). Chromotubation test in endometriosis patients also did not show bilateral abnormalities or patents. It was possible that in these patients the contribution to fertility disorders was obtained from endometriosis. Besides, women with endometriosis have risk to endometrial cancer whereas the Natural Kill (NK) cell is lower. Also, women who are diagnosed with epithelial ovarian cancer (EOC) have risk to endometrial premalignancies (13,14). Moreover, there are angiogenic factors which play important roles in growth of endometrial cancers (15).

The cause of infertility in severe stage endometriosis is frequent anatomical distortion in the pelvic cavity, which can then interfere with the fertilization process. Adhesions that occur in the fallopian tubes and ovaries can inhibit the fallopian tube's mobility, thereby interfering with the ovum pick-up process. In mild-moderate endometriosis, the

structure of the internal genital anatomy is generally still normal. In fact, infertility also occurs as we obtained in this study's subject. The cellular immune factors may play a role in the pathogenesis of endometriosis (14,16). Several studies have shown that mild and moderate endometriosis also showed chronic inflammation in the pelvic cavity produced by the body's immune interaction with endometriosis implants (2,3,17).

High Cyclooxygenase-2 (COX2) level of ectopic endometrial cells appear and activate PGE2 production in the peritoneal fluid. This PGE2 can reduce macrophage scavenger activity, thus failing to eliminate endometriosis cells (18). On the other hand, these endometriosis lesions have aromatase enzymes, so they can produce estrogen locally. This estrogen gives positive feedback to PGE2 so that more proinflammatory cytokines are produced (19).

The results of the qPCR and GRP78 mRNA expression were higher than those in non-endometriosis women. The accumulation of unfolded protein in the lumen of the endoplasmic reticulum (ER) causes UPR reaction by activating the UPR sensor, IRE1α, activating transcription factor 6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK). This UPR has two functions, to improve misfolded protein by increasing GRP78 and GRP94 to increase folding capacity, which will phosphorylate eIF2α so antioxidant activation occurs, and attenuation of global translation to reduce the burden of traffic on the ER lumen (20,21).

Prolonged ER stress will cause UPR decompensation, then eIF2α will activate CHOP and initiate apoptosis via caspase 12 or 4 (20,21). IRE1α can also bind TNF Receptor-associated Factor 2 (TRAF2) so that TNF-α exposure during RE stress is easier, resulting in cell death through both apoptosis and necroptosis (22,23). Cell death through apoptosis and necroptosis has common upstream, which is through the Tumor necrosis Factor Receptor 1 (TNFR1) (22,24). Cellular Fas-associated death domain (FADD) such as IL-1β Converting Enzyme Inhibitory Protein (c-FLIP) will regulate cell death through apoptosis or necroptosis (24). Further study is needed to elucidate pathway of necrosis or necroptosis.

CCND2 mRNA and the expression of Cyclin D2 protein were higher in the endometriosis group than those in non-endometriosis. In UPR, PERK will phosphorylate eIF2α, causing global attenuation to reduce traffic in ER lumen, including the translation of Cyclin D2 protein. In vitro studies showed that UPR, through PERK and eIF2α, can suppress Cyclin D1 and cell cycle arrest occurs in G0/G1 (25). The study sample was taken at the end of the follicular phase when estrogen level was high (3). Follicle Stimulating Hormone (FSH), activing, and estrogen can have a positive effect on cyclin D2 (26). Even though in this study there was an increase in eIF2α, which should have suppressed Cyclin D2, the possibility of positive effects of estrogen and FSH was still higher, so that Cyclin D2 increased, although it was not statistically significant.

The distribution of the granulosa cell cycle in the endometriosis and non-endometriosis groups has the same distribution pattern. The distribution of G0/G1 and apoptotic phases in the endometriosis group was higher than those in non-endometriosis, whereas the distribution of S and M phase was higher than that in endometriosis group. The distribution in G0/G1 phase in endometriosis group was higher than that in non-endometriosis, although Cyclin D2 expression was higher. Cyclin D2 will form a complex with Cyclin Dependent Kinase (CDK) 4/6, which then phosphorylates the protein Retinoblastoma (pRB) (27). The G1-S phase rate depends on the Cyclin-Cdk complex with protein 21 (P21), which is the Cdk inhibitor (CKI). ATF4 in ER stress can increase CKI p21, so that, although cyclin D increases, cell cycle arrest occurs (28). Phosphorylated eIF2α can activate ATF 4 in ER stress (20,21).

This study found viable and dead cell distribution from the examination of flow cytometry and annexin V. These results showed that the percentage of viable granulosa cells and necrosis/necroptosis in women with endometriosis was higher than that in non-endometriosis. UPR and cell cycle arrest are mechanisms in which cells survive in an oxidative stress environment. Oxidative stress conditions determine when cells will enter and stop at phase G0/G1 to stay alive. If the level of oxidative stress has exceeded the cell adaptation limit, then eIF2α will activate CHOP so that apoptosis occurs, or IRE1α will bind TRAF2thus TNF-α exposure can more easily cause death through necroptosis by activating Receptor Interacting serine/threonine kinase 3 (RIPK3) and Mix Lineage Kinase Domain Like Pseudo kinase (MLKL). The ER stress triggers cell death through necroptosis through TNFR1, RIPK3 and MLKL activation (22).

Path analysis showed that GRP78 changes due to TNF-α affect the granulosa cell cycle. However, other factors may also have an effect so that the expression of cyclin D2 was high. The positive effect of estrogen combined with the effect of suppression of ER stress on CKI or p21 caused higher expression of cyclin D2 in endometriosis. The ability of GRP78 to improve the unfolded protein accumulation in the ER lumen was still high, so changes in the granulosa cell cycle were still had the similar distribution despite high G0/G1 phase and low S phase in endometriosis. Patients in this study also still had regular menstrual cycle.

This study showed that endometriosis had a negative impact on dominant follicular granulosa cells. Granulosa cells have the role of steroidogenesis in folliculogenesis and communication with oocytes in oogenesis. ER stress in granulosa cells can be one of the basis for infertility in women with minimum to moderate grade of endometriosis. ER stress and changes in granulosa cell cycles in endometriosis women could have been worsened in increased granulosa cell work load, such as ovarian stimulation in ART. In in-vitro observation, Gonadotropin stimulation on ER stressed granulosa cell could lead in increase apoptosis (29). Further study is needed to investigate ER stress in cumulus oophorous complex and oocyte quality as we could not determine whether the isolated granulosa cell in this study was mural or cumulus oophorous complex.

This study has limitations. Seventeen women agreed to participate over the study after fulfilling the inclusion criterion. Thirteen subjects were allocated in endometriosis group and four in non-endometriosis group. At first, sample size in the study determined ten subjects in each group. However, reaching appointed control number in proper time

was very hard and seemed beyond limitation of the study. Statistical calculations of this study used multivariate test of General Linear Model to adapt the number of research subjects of endometriosis and non-endometriosis groups that were not equal in number.

CONCLUSION

Endometriosis women having significant higher TNF-α level suggested to lead ER stress were playing role in the granulosa cell cycle. Further study is needed.

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