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The Effect of Follicular-fluid Exposure of Endometriosis Cyst Patients toward the Levels of Bcl-2 and Cytochrome-C in Mice Oocyte

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Abstract

BACKGROUND: Endometriosis cysts adversely affect a woman's quality of life, which causes pain and reduces fertility. The World Health Organization found that the incidence of endometriosis with infertility clinical manifestations is around 10%.

AIM: The purpose of this study is to determine the presence of mice oocytes mitochondrial apoptosis exposed with endometriosis cysts follicular fluid through Bcl-2 and cytochrome C analysis.

METHODS: This study was a randomized post-test only control group design conducted at Sanglah Hospital, Bali Royal Hospital in Denpasar, and the Medical Faculty Udayana University from June 2018 to April 2019. A total of 120 mice oocytes were distributed randomly into three groups, i.e., treatment Groups 1 and 2 that added by 15% endometriosis cysts follicular fluid and control group. Bcl-2 and Cytochrome C analyzed with ELISA Technique. Comparability was tested with one-way ANOVA with a significance level of $p < 0.05$.

RESULTS: Eighteen mice oocyte replicates were normally distributed and homogeneous. The Bcl-2 levels in the treatment Groups 1, and 2 and control were 627.83 ± 146.42, 634.50 ± 140.62, and 678.83 ± 152.71, respectively; $p = 0.838$ (not significantly different). Cytochrome C levels in the treatment Groups 1, and 2, and control, respectively, were 3147.75 ± 228.50, 3104.45 ± 211.29, and 2738.28 ± 227.45; $p = 0.021$ (significantly different).

CONCLUSION: The effect of endometriosis cysts follicular fluid exposure on mitochondrial apoptosis is proven through Cytochrome C, whereas Bcl-2 is not proven.

Introduction

Endometriosis cysts are thin-walled mass sacs contain brown fluid (blood) and originate from endometrial tissue outside the uterus [1]. Endometriosis harms woman's quality of life because it causes pain and decreases the reproductive rate [2], [3], [4]. Endometriosis is closely related to infertility due to changes in folliculogenesis, reduced oocyte quality, disruption of fertilization, and decreased embryo quality. The World Health Organization found that the incidence of endometriosis with infertility clinical manifestations is around 10%. Bulletti *et al.* [5] and Augoulea *et al.* [6] showed that 30–50% of women with endometriosis have difficulty getting heredity. The success of assisted reproductive technology programs in patients with endometriosis cysts is also low due to the low level of oocyte maturity.

Endometriosis distorts the ovarian adnexa so that the transport of oocytes from the ovaries to the fallopian tube becomes disrupted. Furthermore, there is a change in the composition of the follicular fluid which causes a decrease in oocyte quality [7]. Endometriosis cyst fluid contains excessive heme due to the process of recurrent erythrocyte hemolysis which produce hemosiderin, heme, or Fe deposition [8]. Fe deposition can cause oxidative reactions through the fenton reaction which produces reactive oxygen species (ROS) in the form of superoxide anions (O_2^-) and hydroxyl radicals (OH). Excessive levels of ROS will cause unstable

mitochondrial membrane permeability and mitochondrial apoptosis characterized by low biomolecular mediator B-cell lymphoma/leukemia-2 (Bcl-2) and increased Cytochrome C. In addition, the mitochondrial damage causes decreased adenosine triphosphate (ATP) production. Low ATP levels are not only associated with low oocyte quality but also associated with decreased quality of embryonic development, implantation rates, and suboptimal placentation [9], [10].

This study aims to investigate the effect of endometriosis cysts follicular fluid exposure on mitochondrial apoptosis through the analysis of Bcl-2 and cytochrome C.

Methods

Randomized post-test only control group design experimental study conducted at Sanglah Hospital, Bali Royal Hospital in Denpasar, and the Medical Faculty Udayana University from June 2018 to April 2019. A total of 120 immature oocytes of BalbC mice were harvested from the ovaries and distributed randomly into three groups, i.e., treatment Groups 1 and 2 that added by 15% endometriosis cysts follicular fluid (TCM-199 + 15% endometriosis cysts follicular fluid) and control group (TCM-199). Follicular fluid was taken from endometriosis cyst patients who followed the assisted reproductive technology program at Graha Tunjung *in vitro* fertilization Clinic in Sanglah Hospital Denpasar.

The oocyte was waited to mature for 17–18 h in the incubator with temperature 37°C and 6% CO_2. Mature oocytes were denuded (cumulus cells were cleansed), put in 1.5 ml centrifuge tubes, and stored at −80°C. Protein Bcl-2 and cytochrome C were measured using ELISA to determine the level of mitochondrial apoptosis and expressed in pg/ml.

This study was repeated 6 times (six replicates), which used follicular fluid of different endometriosis cyst patients, so the total use of mice in this study was 162 mice with 720 oocytes. Data normality was tested by Shapiro–Wilk, and homogeneity was tested by Levene's test at a significance level $p > 0.05$. Comparability was tested with one-way ANOVA at significance level $p < 0.05$.

Results

Characteristics of Balbc strain mice

This study used 162 mice to get 720 oocytes and distributed into 18 replicate oocyte mice. The analysis of the age and weight variables of the mice used one-way ANOVA test that showed no significant differences between the treatment and control groups.

Bcl-2 levels

Bcl-2 levels on oocyte mice between treatment and control groups were tested after the treatment group was given 15% endometriosis cysts follicular fluid. The results of the significance analysis with the one-way ANOVA test are presented in Table 1.

Table 1: Mean levels of Bcl-2 in the treatment and control groups

Group	n	Bcl-2 levels	SD	F	p
Group 1	6	627,83	146,42	0.180	0.838
Group 2	6	634,50	140,62		
Control	6	678,83	152,71		

The mean concentration of Bcl-2 in treatment Group 1 was 627.83 ± 146.42, Group 2 was 634.50 ± 140.62, and the control group was 678.83 ± 152.71. Significance analysis with one-way ANOVA test showed that the value of $F = 0.18$ and $p = 0.838$, so the Bcl-2 did not differ significantly in all groups.

Cytochrome C levels

Cytochrome C levels on oocyte mice between treatment and control groups were tested after the treatment group was given 15% endometriosis cysts follicular fluid. The results of the significance analysis with the one-way ANOVA test are presented in Table 2.

Table 2: Mean levels of cytochrome c in the treatment and control groups

Group	n	Cytochrome C mean	SD	F	p
Group 1	6	3147.75	228.50	5.07	0.021
Group 2	6	3104.45	211.29		
Control	6	2738.28	227.45		

The mean of cytochrome C in treatment Group 1 was 3147.75 ± 228.50, treatment Group 2 was 3104.45 ± 211.29, and the control group was 2738.28 ± 227.45. Significance analysis with one-way ANOVA test showed that the value of $F = 5.07$ and $p = 0.021$, so the mean of cytochrome C levels in the three groups was significantly different.

Discussion

Association of Bcl-2 levels with mitochondrial apoptosis after endometriosis cysts follicular fluid exposure

Bcl-2 acts as anti-apoptosis through intrinsic pathways which play a role in protecting the mitochondrial membrane against proapoptotic proteins. Bcl-2 is

found in the outer membrane of the mitochondria, cell nucleus envelope, and endoplasmic reticulum. In this study, we did not find a significant relationship between endometriosis cysts follicular fluid administration with Bcl-2 expression, which is an indicator of mitochondrial apoptosis. Apoptosis of mitochondrial oocytes can occur through other pathways such as extrinsic pathways, p53 pathways, or independent pathways of the apoptotic mechanism without involving Bcl-2. In addition, the size and duration of endometriosis cysts are also related to the oxidant substances diffusion process into the follicular fluid, which causes the mechanism of Bcl-2 apoptosis does not work properly.

As much as, 80% of oocytes with *in vivo* maturation had mitochondria distribution spread within the cytoplasm. The distribution of mitochondria is mostly in the middle part of the cytoplasm when oocytes undergo maturation through *in vitro* processes. This condition causes unequal exposure of endometriosis cyst follicular fluid to mitochondrial oocytes when administered *in vitro*. We suspect that only the outer mitochondria are damaged by the follicular fluid [11]. Thus, these insignificant results may not be able to describe the actual condition that occurs in oocytes, which are likely to have a more significant decrease in Bcl-2 levels than the results of the study.

Association of cytochrome C levels with mitochondrial apoptosis after endometriosis cysts follicular fluid exposure

Endometriosis cysts follicular fluid was proven significantly cause apoptosis of oocyte mitochondria, which was marked by a significant difference in cytochrome C. Higher cytochrome C levels theoretically showed that oocytes exposed to endometriosis cysts follicular fluid cause mitochondrial apoptosis through intrinsic pathways. Excessive ROS post-endometriosis cysts follicular fluid administration will release calcium (Ca^{2+}) ions from the endoplasmic reticulum and cause mitochondrial apoptosis characterized by cytochrome C [9]. Eleftheriadis *et al*. showed that cytochrome C is a potential and ideal clinical marker of mitochondrial apoptosis and cell damage [12].

The presence of ROS in the cytoplasm post-endometriosis cysts follicular fluid administration can trigger the activation of the MAPK-JNK signal. The JNK kinase enzyme can phosphorylate and activate the transcription factor of Foxo3 proapoptosis. Activated Foxo3 then migrates from the cytoplasm to the cell nucleus and triggers the transcription of the Bcl-2 genes proapoptotic family called Bcl-2-like protein 11 (Bim) and other proapoptotic proteins. Bim triggers apoptosis by modulating several proteins such as B-cell lymphoma extra-large (Bcl-XL), Bcl-2, Bcl-2 associated X protein (Bax), and Bcl-2 homologous antagonist/killer (Bak) which causes the release of cytochrome C [13].

Mitochondria can be induced to release cytochrome C through various stress signals or after caspase activation, which is triggered by surface receptor ligands. The integrity of the outer membrane and the release of cytochrome C from mitochondria are regulated by the Bcl-2 protein family, which consists of antiapoptotic factors such as Bcl-2 and Bcl-XL and proapoptotic proteins such as Bax and Bak. These proteins can undergo heterodimerization with each other and interact with mitochondria. As a result, the Bcl-2 protein can prevent apoptosis by decrease the release of protein between the mitochondrial membrane, including cytochrome C and apoptosis-inducing factor [12].

Jiang *et al*. reported that the released of cytochrome C from mitochondria to the cytoplasm would activate the caspase pathway, which is the executor and mediator of cell death [14]. In addition, cytochrome C is also released by mitochondria into the cytosol when apoptosome formation process and cell death progression. When cytochrome C found in the cytosol, it will interact with the Apaf-1 molecule which activates pro-caspase-9 into caspase 9 [15]. The primary function of caspase 9 is the activation of caspase 3 which triggers apoptosis by lyses cell cytoskeleton and trigger DNA fragmentation [16], [17].

Songsasen and Collado-Fernandez *et al*. reported that energy metabolism is essential for oocyte maturation because it requires a lot of energy from various substrates, including carbohydrates, amino acids, and lipids [18], [19]. The mitochondrial apoptosis showed that mitochondria as energy producers from various substrates were not able to produce energy for the oocyte maturation [20], [21].

Conclusion

There is a significant difference in endometriosis cysts follicular fluid exposure with mitochondrial apoptosis through cytochrome C. The levels of cytochrome C are higher in the treatment group exposed to endometriosis cysts follicular fluid. There is no significant difference in the Bcl-2 oocytes levels exposed and not exposed to endometriosis cysts follicular fluid. This finding is literally indicated that the outer mitochondria are not damaged by the follicular fluid as well as not be able to describe the actual condition that occurs in oocytes.

Ethical Clearance

Ethics approval has been obtained before the study being conducted from the Ethics Committee, Faculty of Medicine, Universitas Udayana, Bali, Indonesia, with number 1954/UN14.2.2.VII.14/LP/2018

Authors' Contributions

All of authors are responsible for the study from the conceptual framework, data gathering, data analysis, until reporting the results of study.

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