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Effect of Exposure to Follicular Fluid in Endometrioma Patients on the Presence of Polar Body I, Distribution Pattern and Intensity of Mitochondria Oocyte Fluorescence

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ABSTRACT

***Introduction*: Mitochondria are small organelles that are directly involved in many essential cellular functions. Mitochondria are very sensitive to the surrounding environmental conditions and are easily affected by various free radicals or ROS. Endometriosis is a disease associated with increased ROS. The effect of endometriosis on oocyte mitochondrial abnormalities or dysfunction has received limited attention. This study aims to determine the effect of exposure to follicular fluid in endometrioma patients on the presence of polar body I, distribution pattern, and intensity of mitochondrial fluorescence in mice oocytes.**

***Methods*: The study design was a randomized post-test only control group design using oocytes of immature Swiss mice exposed to follicular fluid from endometrioma patients. Follicular fluid was taken at the time of picking oocytes from infertility patients who participated in the FIV-ISIS program. Immature oocytes were matured in vitro (IVM) in culture media with follicular fluid added from endometrioma and non-endometriotic patients as a control. The presence of polar body I (oocyte maturation), fluorescence intensity (amount/metabolic activity) and mitochondrial distribution pattern were compared in the two groups. Data analysis with SPSS 16.0 program. Variable analysis was done by chi square test and independent t test.**

***Results*: Polar body I was significantly lower (30% vs 75%) in the treatment group compared to the control group (p=0,01). The pattern of diffuse distribution (30% vs 70%) was significantly lower in the treatment group compared to the control group (p=0,027). The mean fluorescence intensity (556,54±268.96 vs 818,07±228.17) was significantly lower in the treatment group compared to the control group (p<0,001).**

***Conclusion*: The effect of exposure to follicular fluid in endometrioma patients significantly reduced the presence of polar body I, caused a change in distribution pattern and decreased the intensity of mitochondrial fluorescence in mice oocytes.**

Keywords: Endometriosis, mitochondria, polar body I, ROS.

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I. Introduction

Mitochondria are small organelles in the cytoplasm that are directly involved in many essential cellular functions, including ATP production, reactive oxygen species (ROS) and control of apoptosis. Mitochondrial dysfunction associated with infertility has been shown to occur in women with metabolic diseases or disorders such as diabetes, obesity, as well as in the aging process of oocytes (aging) [1].

Endometriosis has a strong correlation with infertility, however, despite decades of research, the etiology and pathogenesis of this disease are not well understood. The data show low fertilization, implantation and pregnancy rates, and high abortion rates in endometriosis patients undergoing in vitro fertilization (FIV) programs. Patients with endometriosis also show a lower number, quality of oocytes and embryos, and a lower rate of blastocyst formation [2], [3].

Until now, there is no comprehensive evaluation in the literature of the effect of endometriosis on oocyte quality, from both a clinical and biological perspective. Mitochondrial function can be observed experimentally so that research on the number and metabolic activity of mitochondria (fluorescence intensity) and mitochondrial distribution patterns can improve understanding of the mechanism of infertility in patients with endometriosis [3], [4].

Based on this background, the researchers wanted to know the effect of exposure to follicular fluid in patients with endometriosis cysts (endometriomas) on mouse oocytes. It is hoped that this will clarify understanding, so that in the future it will be possible to develop new strategies in therapeutic treatment to restore mitochondrial function in the event of defects and a decrease in oocyte quality. This will greatly help to increase fertility and the success rate of FIV in patients with endometriosis.

II. Methods

This research was a laboratory experimental analytical study with a Randomized Post-test Only Control Group Design approach. The study was carried out for 6 months starting from January 2019 to June 2019. The target population in this study was Swiss mouse oocytes. Inclusion criteria were Swiss mice oocytes aged 12-14 weeks, body weight 30-40 grams, and matured after IVM. The exclusion criteria were immature mouse oocytes after IVM. Mice oocytes that died or degenerated at the time of IVM were excluded from the study.

Observation/maintenance, physical examination, randomization of samples, ovarian stimulation, and ovary collection of mice were carried out at the Animal Laboratory Unit, Pharmacology Section, Faculty of Medicine, Udayana University Denpasar. The selection process, in vitro maturation of immature oocytes, incubation on culture media with follicular fluid added, staining of mitochondria with MitoTracker red dye and examination of polar body I were carried out at the Biomedical Laboratory, Faculty of Medicine, Udayana University Denpasar. Examination of fluorescence intensity and mitochondrial distribution pattern using a fluorescence microscope was carried out at the Center for Veterinary Medicine (BBVET) Denpasar.

The study was divided into treatment and control groups. conducted on immature mouse oocytes which were randomly divided into 2 groups, then matured in vitro (IVM) in TCM-199 medium + follicular fluid from endometrioma patients (treatment group) and TCM-199 medium + non-endometriotic follicular fluid (control group). Data analysis was performed using SPSS 16.0 for Windows. The comparative test was carried out using an independent t-test and chi-square test.

III. Results

The total number of mice used was 20, from which 115 immature oocytes were obtained which were distributed randomly in both study groups and then maturation in vitro was carried out on the media according to the treatment and control groups. After the in vitro maturation process, the presence of polar body I (oocyte maturation) was assessed in both groups. In the treatment group, there were 20 mature oocytes (41,7%) while in the control group there were 37 oocytes (67,3%).

In this study, an independent t-test was conducted on the age and weight characteristics of mice in both study groups. The results of the independent t-test showed that there was no difference in the mean age and weight of mice between the two research groups with the p-value for each variable >0,05.

TABLE I: Characteristics of Age and Weight in the Treatment and Control Groups

Variable	Treatment Group n=10	Control Group n=10	p
	Mean±SD	Mean±SD	
Age (day)	88,9±5,76	91,0±4,67	0,38
Weight (gram)	34,2±1,93	34,7±1,82	0,56

The treatment effect analysis was tested based on the presence of polar body I in mice oocytes between the two groups after being treated with follicular fluid exposure in endometrioma patients. The chi-square test showed that the presence of polar body I in the treatment group was significantly different from the control group, where the presence of polar body I was lower (41,7% vs 67,3%) in the treatment group compared to the control group (p=0,01).

TABLE II: The Difference in the Presence of Polar Body I between the Treatment and Control Groups

Variable		Treatment Group	Control Group	p
		n (%)	n (%)	
Polar body I	Yes	20 (41,7)	37 (67,3)	0,01
	No	28 (58,3)	18 (32,7)	

The treatment group appeared to have a pattern of peripheral, semiperipheral, and diffuse distribution of 10%, 60%, and 30%, respectively. Meanwhile, the control group did not have a peripheral distribution pattern, but 30% and 70% semiperipheral and diffuse distribution patterns appeared, respectively (Fig. 1). Based on the chi-square test, there were significant differences in the distribution of mitochondria in the treatment group and the control group. The pattern of diffuse distribution was significantly lower (30% vs 70%) in the treatment group compared to the control group (p=0,027).

TABLE III: DIFFERENCES IN MITOCHONDRIAL DISTRIBUTION PATTERNS BETWEEN TREATMENT AND CONTROL GROUPS

Groups	Peripheral	Semiperipheral	Diffuse	p
	n (%)	n (%)	n (%)	
Treatment (n=20)	2 (10)	12 (60)	6 (30)	0,027
Control (n=20)	0 (0)	6 (30)	14(70)	

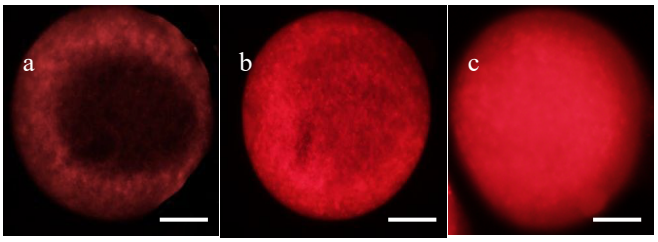

Fig 1. Photo representation of the distribution pattern of mouse oocytes observed with a fluorescence microscope. (a-c) represent three different distribution patterns of mouse oocyte mitochondria: (a) peripheral-mitochondrion distributed only in the periphery of the oocyte, (b) semiperipheral-mitochondrion heterogeneously distributed, (c) diffuse-mitochondrion homogeneously distributed throughout the cytoplasm. MitoTracker red coloring. Magnification 200x, white line = 50µm.

The mean fluorescence intensity in mouse oocytes between the two groups was compared and the mean fluorescence intensity in the treatment group was 556,54±142,97 and the control group average was 818,07±235,75. The significance analysis using the independent t-test showed that the p value <0,001. This means that the mean fluorescence intensity in the treatment group was significantly lower than the control group (p<0,05).

TABLE IV: DIFFERENCES IN FLUORESCENCE INTENSITY BETWEEN THE TREATMENT GROUP AND THE CONTROL GROUP

Variable	Treatment Group n (20)	Control Group n (20)	p
	Mean±SD	Mean±SD	
Fluorescence intensity	556,54±142.97	818,07±235.75	<0,001

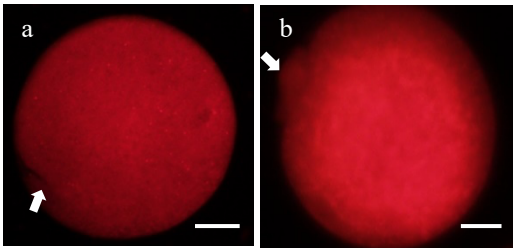

Fig 2. Photo representation of the intensity of the mitochondrial fluorescence of mice oocytes in the treatment group (a) and control group (b), the photo was taken with an immunofluorescence microscope after staining with MitoTracker red. Magnification 200X, white line = 50µm, white arrow = polar body I.

IV. DISCUSSION

In this study, the mean presence of polar body I in the two study groups showed a significant difference, where the presence of polar body I was lower (41,7% vs 67,3%) in the treatment group than the control group (p=0,01). This shows that exposure to follicular fluid in endometrioma patients can cause a significant decrease in the presence of polar body I.

The polar body I is clinically a marker of nuclear maturation, which means that the oocyte is in a haploid state and ready to be fertilized. Nuclear maturation stages consist of GVBD, arrest in meiosis (prophase) I, extrusion of polar body I, and arrest in meiosis (metaphase) II. All of these stages are essential for the oocyte to be fertilized. Analysis of the morphological parameters of oocytes with endometriosis showed that the occurrence of polar body dysmorphism (fragmentation) was significantly higher in oocytes in the endometritis group. In addition to the increased abnormality of polar body I, incomplete extrusion or delayed extrusion and division of polar body I also appeared in the endometriosis group. Polar body I abnormalities are associated with decreased oocyte potential for fertilization [5]-[7].

This study showed that there was a significant difference in the distribution of mitochondria in the two groups. The pattern of diffuse distribution was significantly lower (30% vs 70%) in the treatment group compared to the control group (p=0,027). In general, good oocyte quality was associated with a diffuse or even distribution of mitochondria throughout the cytoplasm of mature oocytes. Mitochondrial distribution is also believed to be important just before embryonic division, to ensure that each blastomere receives sufficient mitochondria to survive the early stages of embryogenesis [8].

The semiperipheral and peripheral distribution patterns were significantly higher in the treatment group indicating that mitochondria were heterogeneously distributed or mostly in the periphery of the cytoplasm. This pattern is often seen in oocytes of poor quality and immature oocytes. Mitochondria in immature oocytes are distributed mainly in the periphery, this may be related to the higher energy requirements in the cortex, because oocytes require the role of cumulus cells at this stage and the close relationship between oocytes and cumulus cells is maintained through gap junctions [8], [9].

Mitochondria are the most abundant organelles in the cytoplasm, functioning primarily in providing ATP for the oocyte. In addition, mitochondria play an important role in several functions including maintaining cellular homeostasis and regulation of cell survival. Recent evidence suggests the role of mitochondria in oocyte development and reproduction. Various methods have been developed to measure mitochondrial quantity, including morphometric analysis of the number of organelles, quantification of the number of mtDNA copies, and measurement of mitochondrial activity [8], [10].

In this study, mitochondrial-specific dye (MitoTracker red), fluorescence microscopy, and the ImageJ program were used to assess differences in the number and metabolic activity of mouse mitochondrial oocytes in the treatment and control groups by assessing the fluorescence intensity. MitoTracker red is excellent for analyzing the morphological characteristics of mitochondria. The advantage of this dye is that it can bind specifically to mitochondrial proteins in the intermembrane space and remains stable after the fixation process [11].

This study showed a significant decrease in the mean intensity of mitochondrial fluorescence (556,54±268,96 vs 818,07±228,17) in oocytes of mice exposed to follicular fluid in patients with endometriomas compared to controls (p<0,001). This shows that the quantity or activity of

mitochondria has decreased in the oocytes of mice exposed to the follicular fluid of endometrioma patients.

The decrease in fluorescence intensity in the treatment group of this study represents an increase in the number of depolarized mitochondria or a decrease in membrane potential (ΔΨm) because MitoTracker red is a "membrane-potential-dependent dye". Mitochondrial membrane potential (Δψm) is the “driving force” of ATP synthesis by mitochondria so it is the main indicator of the level of mitochondrial activity, reflects the process of electron transport and oxidative phosphorylation, and is closely related to the capacity of mitochondria to produce ATP. Depolarization below the Δψm threshold indicates mitochondrial dysfunction and is a prerequisite for mitophagy mechanisms that play a role in maintaining mitochondrial homeostasis [12]-[14].

In general, a normal human MII oocyte contains approximately 105 mitochondria. The number of mitochondria correlates with their capacity to produce ATP. Consequently, an adequate number of mitochondria with an even distribution in mature oocytes is believed to be essential to meet the energy requirements of each blastomere during early embryonic development [10].

Endometriosis is a disease associated with increased ROS, both in the follicular and systemic environment. Follicular fluid from patients with endometriosis contains ROS, which can cause DNA damage and impaired oocyte maturation as a potential cause of infertility. Mice oocytes respond to DNA damage by stopping or delaying meiosis I (metaphase I arrest) through the activity of the Spindle Assembly Checkpoint (SAC) and DNA Damage Response (DDR) pathways [15].

Mitochondrial function is a marker of oocyte quality and developmental potential. Disrupted mitochondrial activity reduces ATP production, is involved in meiotic chromosomal nondisjunction and can inhibit cell division and embryonic development. Since mammalian mitochondria are inherited from the mother, healthy, good-quality oocytes indicate a higher capacity for fertilization and potential for embryonic development [10], [16], [17].

Overall, studies examining mitochondrial dynamics in relation to oocyte developmental competence provide evidence that the minimal number and distribution of mitochondria play a role in the successful development of oocytes and embryos. The follicular environment is very important for the oocyte maturation process, changes in the composition of the follicular fluid can affect oocyte maturation and quality. The toxic effects of oxidative stress and inflammatory mediators on the follicular fluid of endometrioma patients may be the mechanism underlying the findings in this study. This toxic effect can cause changes in the microenvironment for oocyte development, resulting in mitochondrial dysfunction, impaired maturation, and decreased oocyte quality and quantity.

V. Conclusion

Exposure of follicular fluid of endometrioma patients to mouse oocytes significantly reduced the intensity of mitochondrial fluorescence, caused changes in mitochondrial distribution patterns, and decreased the presence of polar body I.

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