THE EFFECT OF ENDOMETRIOSIS ON MALONDIALDEHIDA (MDA) AND 8-HYDROXY-2-DEOXYGUANOSINE (8-OHdG) IN OOCYTE OF ENDOMETRIOSIS MODEL MICE AFTER OVULATION

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KEYWORDS
endometriosis, MDA, 8-OHdG, oocyte

ABSTRACT:
The mechanism of endometriosis causing infertility has not yet been fully explained. Lipid peroxidation is one of the pathways that causes a decrease in oocyte quality due to the influence of endometriosis, resulting in infertility. Malondialdehyde (MDA), which is a product of lipid peroxidation, will affect the increase in 8-hydroxy-2-deoxyguanosine (8-OHdG) so that the DNA damage occurs. The purpose of this study is to prove the effect of endometriosis on the levels of MDA and 8-OHdG in oocytes of endometriosis model mice.This study used 32 mice which are divided into endometriosis model mice (P0) and healthy mice (P1) as controls. Endometriosis model mice were made by injecting intraperitoneally the endometrial tissue from patients diagnosed with endometriosis. Oocytes obtained from each mouse were separated from the Oocyte-Cumulus Complex (OCC) then divided into two and each was examined for MDA and 8-OHdG levels by ELISA.It was found that MDA and 8-OHdG levels were significantly different in endometriosis model mice compared to healthy mice ($p<0.05$). Pearson correlation test between MDA levels and 8-OHdG levels in endometriosis model mice showed no significant relationship ($p>0.05$).MDA and 8-OHdG levels were higher in endometriosis model mice compared to healthy mice. There was no relationship between increased levels of Malondialdehyde (MDA) and increased levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) in endometriosis model mice.

Introduction

The mechanism by which endometriosis causes infertility has not yet been fully explained. From several studies, endometriosis is associated with low pregnancy rates, where endometriosis is suspected to affect the process of embryo implantation in the endometrium and pregnancy success in patients undergoing in vitro fertilization (IVF) (Xu et al., 2015). Fertilization and oocyte division rates were also reported by Carlberg et al to be lower in women with endometriosis undergoing IVF compared to women without endometriosis (Carlberg et al., 2000)

The pathophysiology of endometriosis is caused by a complex interaction between genetic, immunological, hormonal, and environmental factors (Bulun, 2019; Agostinis et al., 2021). Until now, Sampson's theory is the most widely accepted theory as the cause of endometriosis (Gupta et al, 2015). Some data also conclude that the deviaton of abnormal

immune response in the peritoneal fluid environment play a major role in the pathogenesis of endometriosis and are associated with infertility (Hendarto, 2015).

Research on endometriosis related to oxidative stress has been widely conducted. The literature shows that oxidative stress is a potential factor involved in the pathogenesis, formation, and development of endometriosis. Oxidative stress is defined as an imbalance between Reactive Oxygens Species (ROS) and antioxidants that causes an inflammatory response in the peritoneal cavity. As a result, there is continuous damage to lipids, cell membrane proteins, and cell Deoxyribonucleic Acid (DNA) (Carvalho et al., 2013). Inflammatory factors and ROS will disrupt communication between oocytes and granulosa, disrupt the folliculogenesis, resulting in decreased oocyte quality. In addition, the presence of pro-inflammatory factors and ROS also affects oocytes such as the occurrence of abnormal meiotic spindles, chromosomal misalignment and decreased production of Growth Differentiation Factor-9 (GDF-9) (Hendarto, 2015).

Increased ROS in the ovaries triggers lipid peroxidation in the Polyunsaturated Fatty Acid (PUFA) side chains of plasma membranes and intracellular organelles containing lipids (Mohammadi, 2019). Oocytes are known to have a fairly high lipid content, so they are greatly affected by the lipid peroxidation process. Lipids play various biological roles in the ovaries, including as a source of energy, a mediator of the signal delivery process, and as the basic component of plasma and organelle membranes (Dunning et al, 2014). Products from the lipid peroxidation process such as Malondialdehyde (MDA) and hydroxyl radicals will continue to accumulate as a result of the cell wall damage and intracellular organelles along with the increasing amount of ROS. Therefore, malondialdehyde is one of the secondary products of the lipid peroxidation process that is most often used as a biomarker for cell membrane damage (Enechukwu *et al*., 2019).

The impact of lipid peroxidation that characterized by high levels of MDA and hydroxyl radicals, will cause damage to oocyte DNA. Damage to nuclear DNA and mtDNA will cause errors in DNA replication and transcription, cell mitosis and mitochondrial function. Specifically, mitochondrial DNA (mtDNA) is very susceptible to oxidative damage because it is located on the inside of the mitochondrial membrane near to the electron transport chain, so that the impact will be a decrease in metabolic processes and energy production such as the synthesis of Adenosine Triphosphate (ATP) which plays a role in the oocyte maturation process (Postawski et al, 2013).

In the cell nucleus and mitochondrial DNA, the 8-hydroxy-2-deoxyguanosine (8-OHdG) is one of the results of oxidative damage induced by free radicals, especially hydroxyl radicals (HO•). The production of 8-OHdG is caused by oxidation of one of the base that make up DNA, namely guanine (G). Guanine (G) is the most potent nuclear base affected by free radicals that are oxidized in DNA so that it can cause transversion mutations or Single Nucleotide Polymorphisms (SNP) in the DNA nucleotide sequence. Currently, 8-OHdG has become a biomarker that is widely used to assess DNA damage due to oxidative damage (Valavanidis et al, 2009; Ock et al, 2012). Research conducted by Seino et al found that increased 8-OHdG affects oocyte quality and is suspected to be one of the causes of infertility ofpeople with endometriosis (Valavanidis et al, 2009). High levels of 8-OHdG in oocytes indirectly decrease the ATP production by mitochondria. Adenosine Triphosphate (ATP) is the source of cell energy and all complex processes occur in oocytes before ovulation and fertilization (Hoshino, 2018).

This study aims to prove the effect of endometriosis on MDA and 8-OHdG. Malondialdehyde (MDA) was chosen as the main indicator or biological marker to show the degree of lipid peroxidation due to free radicals on the cell membrane, while 8-OHdG is the most frequently used marker to assess DNA damage due to free radicals. Considering that human research to determine the effect of endometriosis on oocytes is constrained by ethics, this study uses mouse oocytes as a model of endometriosis.

Material and Methods

This study used mice (Mus musculus) as experimental animals. 32 mice were used which were divided into 2 groups, namely the endometriosis model mouse group (P0) and the healthy mouse group as a control (P1). We made endometriosis model mice by intraperitoneally injecting the human endometrium from endometriosis sufferer. Tostimulate the endometrial growth, estrogen injections were given. Fourteen days after intraperitoneal endometrial injection, it is expected to become endometriosis model mice. In the control group, placebo injections were given. All groups of mice were then superovulated by administering the injections of Pregnant Mare Serum Gonadotropin (PMSG), Human Chorionic Gonadotropin (hCG), and mated with male mice that had undergone vasectomy.

The induction of estrusby mating with male mice is needed to trigger ovulation in experimental mice. Seventeen hours after hCG injection, all mice were terminated to collect oocyte-cumulus complexes (OCCs) from the fertilization sac. Cumulus cells and oocytes were then separated using the hyaluronidase enzyme. Because the mice experience multiple ovulations, oocytes obtained from each mouse were divided into 2 for MDA and 8-OHdG examination using ELISA technique. The data obtained were then analyzed using Statistical Package for the Social Sciences (SPSS) software.

Results

The samples used in this study were 32 mice that had met the inclusion and exclusion criteria. This study involved 2 groups of mice, namely the endometriosis model group and the control group (healthy female mice). Each group consisted of 16 mice.

Before conducting the MDA and 8-OHdG level difference test, a normality test was conducted to determine the mean difference test to be used. The mean difference test of MDA levels used is the unpaired t-test and it is found that MDA levels were significantly higher in the endometriosis model mice group compared to the control group with a mean value in the endometriosis group is 1.238 nmol/mL and the control group is 1.214 nmol/mL **(Table 1).**

Table 1. The Result ofMDA Level Analysis Test

Variable	Mean±SD MDA Level (nmol/mL)	P value
Endometriosis model mice group	1,238±0,009	0,001*
Control mice group	1,214±0,011	

*unpaired t-test,SD = standard deviation

The 8-OHdG levels using the unpaired t-test also obtained significantly higher results in the endometriosis model mice group compared to the control group with a mean value in the endometriosis group is 11.775 pg/mL and the control group is 6.143 pg/mL **(Table 2).**

Table 2. The Result of 8-OHdGLevel Analysis Test

Variable	Mean±SD 8-OHdG (pg/mL)	P value
Endometriosis model mice group	11,775±0,571	0,001*
Control group	6,143±1,069	

*unpaired t-test SD = Standard Deviation

The results of the correlation test between MDA levels and 8-OHdG levels in the endometriosis model mice group showed no significant relationship **(Table 3).**

Table 3. The Result ofCorrelation Test between MDA Levels and 8-OHdG Levels in Endometriosis Model Mice

Variable	P value	Correlation Coefficient (r)
MDA and 8-OHdG	0,772*	-

*Pearson Correlation Test

Discussion

Oxidative stress is highly correlated with the presence of endometrial tissue outside the uterine cavity. There are a lot of evidences discussing the oxidative stress in patients with endometriosis. Oxidative stress can produce inflammation in the peritoneal cavity through various pathways. Genetic factors that modulate oxidative stress play an important role in initiating inflammatory reactions and the formation of new nerve fibers and blood vessels. The balance between ROS and antioxidants is necessary to ensure that most metabolic processes run smoothly. When this balance shifts towards excessive ROS, oxidative stress occurs. Oxidative stress is a relevant factor in the occurrence of infertility due to reduced oocyte quality or damage to the ovarian somatic cells (Gupta et al., 2015; Immediata et al., 2022).

One of the consequences of uncontrolled oxidative stress (imbalance between pro-oxidant and antioxidant levels) is cell, tissue, and organ injury caused by oxidative damage. It has long been known that high levels of free radicals or reactive oxygen species (ROS) can cause direct damage to lipids. The main sources of endogenous ROS production are mitochondria, plasma membranes, endoplasmic reticulum, and peroxisomes through various mechanisms including enzymatic reactions and/or auto-oxidation of some compounds, such as catecholamine and hydroquinone (Ayala et al, 2014).

The two most common ROS, which can profoundly affect lipids, are hydroxyl radicals (HO·) and hydroper-oxyl (HO·2). Hydroxyl radicals (HO·) are small, highly mobile, water-soluble, and chemically most reactive form of active oxygen species. These molecules have a short half-life and are produced from O2 metabolism in cells and under various stress conditions. A single cell can produce about 50 hydroxyl radicals per second. In a full day, each cell will produce 4 million hydroxyl radicals that can attack biomolecules (Ayala et al, 2014).

In this study, Malondialdehyde (MDA) was shown to be increased and differ significantly between endometriosis model mice and control mice. Malondialdehyde has a relatively stable chemical structure and will continue to accumulate along with the increasing number of ROS, so it can be used as a cumulative biological marker of the lipid peroxidation process in the ovaries. MDA is the most mutagenic product of the lipid peroxidation reaction (Yalçınkaya et al., 2013). In the oogenesis process, the effect of oxidative stress is not yet clearly understood. Oxidative stress is expected to affect the process of chromosome spindle formation leading to aneuploidy (Paine et al., 2013).

The oxidative stress that occurs after ovulation has the potential to directly affect mitochondrial function. It is because DNA, proteins, and lipids in mitochondria are very susceptible to oxidative stress and can cause mtDNA damage due to the absence of protective histones and the absence of DNA repair mechanisms. Previous study by Sohal and Dubey 1994, Liu et al 2009 and Melov et al 1999 found a relationship between oxidative stress and mtDNA damage, loss of mitochondrial potential membrane, increased ROS by the electron transport chain (ETC) process and decreased ATP production (Lord and Aitken, 2013).

Our study found significant differences between the oocytes of endometriosis model mice and control mice. Study conducted by Broi et al. 2016 suggested that oxidative stress in the follicle compartment of infertile women with endometriosis, as represented by high 8-OHdG concentrations, is associated with oxidative damage, oocyte anomalies, and contributes to the etiopathogenesis of endometriosis-related infertility (Broi et al, 2016).

Chromosomal damage due to increased ROS and DNA damage in oocytes can be in the form of abnormal meiotic spindles and chromosome misalignment (Hendarto, 2012). Repair of DNA damage due to oxidative stress can actually be done, but it does not happen. A study by Sasaki et al 2019, found a decrease in the expression of DNA double-strand break (DSB) repair such as Brca1, Mre11, Atm, and Rad51. The decrease in Brca1 mediated by RNA in oocytes resulted in abnormal spindle formation and chromosome misalignment (Sasaki et al., 2019).

Women with endometriosis have higher oxidative stress and higher levels of free radical-induced DNA damage (Gupta et al, 2015). This opinion is supported by the study of Broi et al, where there was a significant increase in 8-OHdG in peritoneal fluid examination of patients with endometriosis. These data indicate the oxidative stress damage to DNA is in the microenvironment, which can endanger gamete quality. According to Seino et al 2002, high concentrations of 8-OHdG in granulosa cells of infertile women undergoing in vitro fertilization (IVF) were negatively correlated with fertilization rates and embryo quality. Supporting these findings, Tamura et al. 2008 showed that higher concentrations of 8-OHdG in the peritoneal fluid of women undergoing IVF were correlated with higher levels of oocyte degeneration, so it is considered that the presence of 8-OHdG in the follicle compartment has a toxic effect on oocytes (Tamura et al. 2008 ; Broi et al, 2016)

Based on the correlation test that we conducted between MDA and 8-OHdG levels, there was no significant relationship between them. Although increased levels of 8-OHdG and MDA were found in endometriosis patients and are considered as an indication of the activation of oxidative stress pathway, in this study it was not proven to be related with increased of 8-OHdG as a marker of DNA damage in oocytes. This is different when associated with the theory that lipid peroxidation in endometriosis sufferer's oocytes produces higher MDA through enzymatic and non-enzymatic processes and causes DNA damage. These results indicate that oxidative stress that occurs in lipid peroxidation may not directly affect DNA damage or that high levels of inflammatory factors in the peritoneal fluid can directly affect DNA damage without going through lipid peroxidation. Therefore, further comprehensive research is needed to see if there are other pathways that link the effect of oxidative stress to DNA damage.

Conclusion

It was found that the levels of Malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (8-OHdG) were higher in the oocytes of endometriosis model mice compared to control mice. There was no significant relationship between the levels of Malondialdehyde (MDA) and the levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) in endometriosis model mice.

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