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Effect of Ovariectomy on Fas Expression in Endometrial Epithelial Cells in White Rats (*Rattus norvegicus*)

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ABSTRACT
Menopause is the permanent cessation of menstruation resulting from the loss of ovarian follicle activity which marks the end of a woman's natural reproductive life. One method to make menopause in experimental animals is the ovariectomy method. During menopause, hormonal changes occur, namely a decrease in the hormone estrogen. Estrogen is a powerful antioxidant that protects against oxidative stress during reproduction. The purpose of this study is to prove that ovariectomy can increase the expression of endometrial epithelial cells in white rats (Rattus norvegicus). This research design is true experimental with a randomized post- only control group design. The experimental animals used were female white rats (Rattus norvegicus) aged 9-12 weeks with a bodyweight of 150-250 grams totaling 30 individuals which were divided into 2 groups, namely the negative control group and the positive control group. Ovariectomy was performed and waited until the 28th post ovariectomy day. The apoptotic index was examined using Tunnel Assay. The data were analyzed and the Independent Sample T - Test was conducted.

Keywords: Ovariectomy, Fas Expression, Endometrial Epithelial Cells, White Rat (*Rattus norvegicus*)

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BACKGROUND

Ovariectomy is a surgical procedure that aims to remove either one or both ovaries. When an ovariectomy is performed on one ovary, it is called a unilateral ovariectomy, while an ovariectomy performed on both ovaries is called a bilateral ovariectomy. Ovariectomy in humans aims to prevent or treat certain medical conditions such as ovarian cancer and endometriosis. However, ovariectomy in experimental animals has a different purpose. Ovariectomy in experimental animals aims to make the experimental animals into menopause for further examination of the organs of experimental animals that have undergone ovariectomy. Menopause is a physiological process in women which occurs at the age of 45-55 years which is defined as the permanent cessation of menstruation for one consecutive year. The age of menopause depends on several factors including the number of women's ova at birth, the frequency of ovum loss during their life cycle, the number of follicles in the ovaries that function to maintain the menstrual cycle. The diagnosis of menopause is made retrospectively and is different for each woman.(Mendoza-nu, Rosado-pe, and Santiago-osorio 2011) (Doshi and Agarwal 2013).

By 2025, the number of postmenopausal women is expected to reach 1.1 billion worldwide. Most women will experience disturbances during the menopausal transition, including vasomotor symptoms (VMS), sleep disturbances, mood disturbances, and vaginal dryness. (Kling et al. 2019). Disorders during menopause occur because during menopause there is a decrease in estrogen levels (Doshi and Agarwal 2013) (Safitri 2012) (Elizabeth H. Ruder, Terryl J. Hartman, Jeffrey Blumberg 2009). The decrease in the hormone estrogen triggers oxidative stress (Sa, Arronte-rosales, and Correa-mun 2012). Oxidative stress plays an integral part in the aging process. Oxidative stress occurs when there is an imbalance between free radicals and antioxidant defenses in the body. One of the powerful antioxidants is estrogen. During menopause, there is a decrease in the hormone estrogen (Doshi and Agarwal 2013)(Elizabeth H. Ruder, Terryl J. Hartman, Jeffrey Blumberg 2009). Estrogen can reduce oxidative stress by modulating the expression and function of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase as well as antioxidant enzymes (SOD, GPx, catalase), providing protection against oxidative stress during the reproductive stage. Estrogen is a powerful antioxidant that provides protection against oxidative stress during reproduction (Gamal and Elkholi 2015) (Wassmann, Wassmann, and Nickenig 2015). Estrogen is a powerful antioxidant that can reduce low-density lipoprotein oxidation in vitro and in vivo (Sa, Arronte-rosales, and Correa-mun 2012). In addition, the chemical structure of the estrogen molecule has antioxidant ability due to the presence of OH at C3 of the phenolic ring in position A which acts as a free radical scavenger preventing oxidative damage. (Sa, Arronte-rosales, and Correa-mun 2012). The antioxidant activity of estrogen is carried out in different ways, namely estrogen molecules act as free radical scavengers, neutralize excess ROS, and increase the number of antioxidant molecules such as thioredoxin and SOD. (Wang et al. 2011). In addition, estrogen can act to trap transition metals, such as Fe2+ and Cu+, reducing the formation of Fe2+ and Cu+ into oxidants again. (Sa, Arronte-rosales, and Correa-mun 2012). Estrogen increases the transcription, expression, and activity of SOD2 and SOD3 without affecting GPx and catalase. Other studies have shown that a decrease in the hormone estrogen increases oxidative stress depending on the concentration and chemical structure of this hormone. At high concentrations, estrogen tends to have a beneficial antioxidant effect by inhibiting the 8-hydroxylation of guanine DNA bases. However, at low concentrations, this hormone has a pro-oxidant effect, especially when its chemical structure contains catechol a. Oxidant effects in the form of base oxidation, formation of DNA additions, and affect genetic mater

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(Wang et al. 2010). Several studies have shown that biomarkers of oxidative stress (lipoperoxide) have higher levels in postmenopausal women than premenopausal women. However, other studies have stated otherwise that there is a decrease in lipid peroxidase and an increase in antioxidant activity in postmenopausal women compared to premenopausal women.

When estrogen production falls below a critical value, estrogen no longer inhibits the production of the gonadotropins FSH and LH . (Wang et al. 2010). As a result, the production of FSH and LH increases. Estrogen is a powerful antioxidant that provides protection against oxidative stress during reproduction (Sa, Arronte-rosales, and Correa-mun 2012) (Wang et al. 2010). However, at menopause, with a decrease in the hormone estrogen, the proliferation of endometrial epithelial cells will decrease and cell death will increase so that if this continues, there will be atrophy of the endometrial organs. Apoptosis or cell death in menopausal endometrium can occur through the intrinsic pathway (intrinsic pathway) or extrinsic pathway (extrinsic pathway). the extrinsic pathway of apoptosis in the endometrium of ovariectomized rats is characterized by an increase in TNFα and Fas. TNFα is a cytokine that acts as a death receptor linked to the Fas protein. Fas bind to its ligand (FasL). Three or more fas molecules combine to form FADD (Fas-associated death domain). This FADD attaches to the death receptor and begins to bind to the inactive form of caspase 8. This procaspase 8 molecule then splits into the active caspase 8. This enzyme then activates the executor caspase resulting in apoptosis in endometrial epithelial cells of ovariectomized rats (Sato et al. 2003). According to a study conducted by Mor (2001) withdrawal of the hormone estrogen resulted in an increase in the expression of Fas in the endometrium. Another study showed a time-dependent increase in Fas expression in the endometrium where it increased by 15% after 3 hours of estrogen withdrawal, 15% after 6 hours of withdrawal, 27% after 24 hours of withdrawal and 200% after 48 hours of withdrawal. (Song et al. 2002).

METHOD

This research is pure experimental research (true experimental) with post- test only control group design method. this study was divided into 2 groups, namely the positive control group (KP), namely the group of white rats that underwent ovariectomy ,and the negative control group (KN), namely the group of white rats that did not undergo ovariectomy. The research was conducted in several laboratories at the Faculty of Medicine, Universitas Brawijaya. The maintenance of experimental animals and the ovariectomy process were carried out in the Pharmacology laboratory of the Faculty of Medicine, Universitas Brawijaya. Examination of the apoptotic index of endometrial epithelial cells in white rats was carried out in the Biochemistry Laboratory of the Faculty of Medicine, Universitas Brawijaya. The materials used in this study include (1) experimental animal rearing materials consisting of Commfeed (ABS) brand animal food produced by PT. Japfa Comfeed Indonesia, Tbk, (2) Materials for the ovariectomy process include ketamine 40-80 mg/kg BW and xylazine 5 - 10 mg/kg BW, ethanol, povidone iodine, gut paint thread (cromik 3/0 and silk 3/0), sterile gauze (3) Materials for examination of apoptosis using Tunnel Assay: xylol, absolute ethanol, ethanol (70%, 80% and 90%), aquadest, TBS solution, proteinase K, TdT equilibration buffer, TdT labeling reaction mix, TdT enzyme, stop buffer, blocking buffer, conjugate, DAB solution, Mayer's Hematoxylin, entellan, cover glass, and tissue.

Rats were kept in the Pharmacology laboratory of the Faculty of Medicine, Brawijaya University, Malang. White rats were kept in cages made of plastic with a size of 40x30x20

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cm covered with wire and given a 1.5-2 cm thick husk mat which was replaced every 2 days. The cage is provided with a place to eat and drink. White rats were given standard feed with a portion of 40 g/day/head. Drinking needs are provided in a special place on an ad libitum basis. The room temperature is always kept constant at a temperature of 20-25 °C. The ovariectomy procedure was carried out at the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University, Malang. The ovariectomy procedure was performed after the acclimatization process, namely on the 8th day using ketamine 40 - 80 mg/kg BW (intraperitoneal) and xylazine 5-10 mg/kg BW (intraperitoneal) anesthesia. On the 28th day post ovariectomy, a vaginal swab was performed on white rats to determine the condition of the vaginal pH. The normal vaginal pH of white rats is between 5.7 - 7.3 with an average of 6.3. If it is found that the vaginal pH is more than 7.3 (alkaline condition), this condition indicates that the white rat is in a hypoestrogen state. After ovariectomy, samples of endometrial epithelial cells were taken from white mice in the positive control group and the negative control group for examination of Fas expression using IHC.

RESULTS

Before testing the effect of ovariectomy on Fas expression, the normality and homogeneity of the data were tested. The data normality test uses Shapiro Wilk because the number of samples is < 50 with the decision criteria if the significance value or *p-value* > 0.05 then the data is normally distributed. The results of the data normality test are presented in the table below:

Table 1. Normality Test of Fas Expression of Endometrial Epithelial Cells in White Rats (*Rattus norvegicus*)

Variable	Group	*p-value*	Distribution
Fas Expression	Negative Control	0,505	Normal
	Positive Control	0,404	Normal

Furthermore, the data homogeneity test was conducted to determine the data variance of all groups. The homogeneity test decision criteria if the significance value or p-value > 0.05, then the data is said to be homogeneous. The results of the data homogeneity test are presented in the following table:

Table 2. Homogeneity Test of Fas Expression of Endometrial Epithelial Cells in White Rats (*Rattus norvegicus)*

Variable	*p-value*	Description
Fas Expression	0,798	Homogen

Based on the results of the normality and homogeneity test of the data in the table above, the research variable, namely the expression of fas, has data that is normally distributed and homogeneous so that it meets the requirements for parametric testing.

The process of testing the effect of ovariectomy on fas expression by comparing the negative control group (not ovariectomized white rats) and the positive control group (ovariectomized white rats) using an independent sample t test (Independent Sample T Test) because the data for these variables are normally distributed. The results of the free sample t-test are presented in the table below:

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Table 3. Effect of Ovariectomy on Apoptotic Index of Endometrial Epithelial Cells in White Rats (*Rattus norvegicus*)

Variable	Negative Control average ± Stand.dev	Positive Control Average ± Stand.dev	*p-value*
Fas Expression	9,33 ± 3,23	30,67 ± 4,50	0,000 < α

In table 3, based on the results of the independent sample t test, shows that there is a significant difference (p = 0.000<α) in the expression of fas between the negative control group and the positive control group. Based on the mean value of fas expression between the negative control group (9.33 ± 3.23) was lower than the positive control group (30.67 ± 4.50). This proves that non-ovariectomized mice show lower fas expression when compared to ovariectomized mice.

DISCUSSION

Based on the Independent Sample T Test, it showed that between the negative control group (not ovariectomized) and the positive control group (ovariectomized without anthocyanin), the results showed an increase in the mean value of Fas expression between the positive control group (40.67 ± 6.53) compared to the negative control group. (15 ± 4.69). This means that the white mice that were not ovariectomized showed lower Fas expression compared to the Fas expression of the ovariectomized mice. This is consistent with a study conducted by Mor (2001) which stated that withdrawal of the hormone estrogen resulted in an increase in the expression of Fas and FasL in the endometrium. Another study showed that the increase in the expression of Fas in the endometrium was time dependent which increased by 15% after 3 hours of estrogen withdrawal, 15% after 6 hours of withdrawal, 27% after 24 hours of withdrawal and 200% after 48 hours of withdrawal. (Song et al. 2002). Apoptosis in the endometrium of ovariectomized rats can occur through two pathways, namely the intrinsic pathway and the extrinsic pathway (Hongmei and Vogt 2012). The extrinsic pathway of apoptosis in ovariectomized rat endometrium is characterized by an increase in TNFα and Fas (Kumar, Robbins, Leonard, S. 2010) (Locksley et al. 2001). This pathway initiates apoptosis by being mediated by transmembrane receptor interactions. This pathway involves cytokines including death receptors, namely TNFα which functions to send apoptotic signals (Locksley et al. 2001). This death receptor consists of an extracellular cysteine domain and has an acytoplasmic domain of about 80 amino acids called the death domain (Ashkenazi, Dixit, 2001). Death receptors located on the cell surface are the TNF (Tumor Necrosis Factor) receptor family, which includes TNF-R1, CD95 (Fas), and TNF-Related Apoptosis Inducing Ligands (TRAIL)-R1 and R2. There are 2 types of receptors for TNF, namely TNFR-1 and TNFR-2. TNF binds to TNFR-1 which can initiate the caspase activation pathway. Fas (Apo-1 or CD 95) is a receptor for other extrinsic apoptotic signals on cell membranes, and belongs to the TNF receptor family. FasL (Fas ligand) is a protein that binds to Fas to activate the Fas pathway. Fas is a transmembrane protein that belongs to the TNF family. The ligand that binds to the death receptor located on the transmembrane will form a trimer called FADD (Fas Associated Death Domain). The complex formed between the ligand-receptor and FADD is called DISC (Death Inducing Signaling Complex). This complex activates

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procaspase 8. Activated caspase 8 (heterotetramer) is released from the DISC into the cytoplasm. Caspase 8 is an initiator caspase which will activate the executor caspase mainly through pro-caspase 3(Gewies A 2003). The death receptor can be inhibited by a protein called FLIP which binds to FADD and caspase 8 so that it becomes inactive (Kataoka et al., 1998; Scaffidi, 1999). Apoptotic regulation also involves a protein called Toso which has been shown to block Fas induction through T cells by inhibiting the caspase 8 activation process. (Mcilwain et al. 2014).

TNFα is a cytokine that acts as a death receptor linked to the Fas protein. This Fas will bind to its ligand called FasL. When three or more Fas molecules combine, they form a domain called FADD (Fas-associated death domain). The receptor ligand with FADD binds to form a complex called DISC (Death Inducing Signaling Complex). The complex then attaches to the death receptor and binds to procaspase 8. This procaspase 8 molecule then breaks down into active caspase 8. This enzyme will activate caspases and other procaspases and activate enzymes that act as mediators in the execution phase so that apoptosis occurs in endometrial epithelial cells in ovariectomized rats. (Sato et al. 2003).

Fas expression in human endometrium is present throughout the menstrual cycle. In the late proliferative phase Fas is found in the Golgi apparatus and vesicles while in the secretory phase of the menstrual cycle, Fas is found in the plasma membrane. This pattern of changes suggests that Fas has a role in human endometrial apoptosis during menstruation and responds to hormonal changes. Under the influence of the hormone estrogen, endometrial cells in the proliferative phase of the menstrual cycle become resistant to apoptosis. Withdrawal of the hormone estrogen will activate the Fas pathway and initiate an apoptotic program in endometrial cells. The balance of cell survival and apoptotic programs is influenced by the role of estrogen and progesterone in endometrial cells (Mor, Straszewski, and Kamsteeg 2002)(Song et al. 2002).Based on the results of the research and the theory that has been stated above, the hypothesis in this study has been proven, namely that ovariectomy increases the expression of Fas which is one of the apoptotic marker proteins in endometrial organs of white rats (*Rattus norvegicus*).

CONCLUSSION

Ovariectomy is a surgical method to make rats into menopause by taking both ovaries. Based on the results of this study, it was proven that ovariectomy was able to increase the expression of endometrial epithelial cells of ovariectomized white mice (*Rattus norvegicus*) or in other words menopause was able to increase the death of endometrial epithelial cells. So from the results of this study, it can be developed to conduct further research related to efforts to reduce endometrial epithelial cell death by using certain antioxidants.

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