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The Effect of α-Tocopherol on Oxidative Stress and Ovarian Function in Rats Exposed to Tobacco Smokes

Sigara Dumanına Maruz Kalan Sıçanlarda Tokoferalinin Oksidatif Stres ve Over Fonksiyonlarına Etkisi

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ABSTRACT

Purpose: The purpose of the current study was to evaluate the role of α- tocopherol in inhibiting ovarian oxidative stress, changes in ovarian follicles, estradiol levels, and the thickness of the endometrium in rats exposed to tobacco smoke.

Material and Methods: Forty female rats were divided into five groups consisting of a control group; group exposed to tobacco smoke; groups exposed to tobacco smokes receiving α - tocopherol supplementation at doses of 100; 200, and 400 mg/kg. Exposure to tobacco smoke was induced using smoke pumping equipment which was designed and made available in the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University. Analysis of MDA level was done colorimetrically. Analysis of the number of ovarian follicles and endometrial thickness was done histopathologically with hematoxylin eosin staining. Analysis of estradiol level was done by ELISA technique.

Results: Exposure to tobacco smoke in rats can increase ovarian MDA level (0.388 ± 0.085) significantly compared to the control (0.121 ± 0.026) (P> 0.05). α-tocopherol in various doses can reduce MDA level significantly compared to the group exposed to tobacco smoke (P <0.05), but has not reached the levels of the control group. Exposure to tobacco smoke can reduce the number of primary and secondary follicles significantly compared to the control (P <0.05), and tended to decrease the number of De Graff follicles in the ovaries despite not significant (P> 0.05). Administration of α-tocopherol in different doses increased the number of secondary follicles which reached the number in the control group (P> 0.05). The number of primary follicles in the α-tocopherol group of the first dose (3.8 ± 1.095) was comparable to the control group (3.8 ± 1.095) (P> 0.05). The numbers of secondary follicles in the α-tocopherol group of the first dose (5.2 ± 1.304) and of the second dose (5.4 ± 0.548) were significantly higher than the control group (3.6 ± 0.548) (P < 0.05). Tobacco smoke lowered estradiol level (10.815 ± 2.374) significantly compared with no exposure (21.354 ± 4.215) (P < 0.05). Administration of α-tocopherol in different doses increased estradiol level, reaching the level in the control group (P > 0.05). Tobacco smoke lowered thickness of endometrium (209.491 ± 38.635) significantly compared with no exposure (600.265 ± 76.563) (P < 0.05). The α-tocopherol of the first dose (604. 569 ± 33.621) and of the second dose (609.459 ± 37.105) increase estradiol levels, reaching the level in the control group (600.265 ± 76.563) (P > 0.05).

Conclusion: Regarding the results describe above, it was concluded that α-tocopherol could prevent oxidative damage and loss of ovarian function due to exposure to tobacco smoke characterized by decreased levels of MDA, increases in

primary and secondary follicles, as well as the hormone estradiol. Furthermore, it also increased the thickness of endometrium.
Key Words: oxidative stress; ovaries; endometrium; estrogen; antioxidant

ÖZET

Amaç: Bu çalışmanın amacı, sigara dumanına maruz kalan sıçanlarda α-tokoferolün overlerin oksidatif stresi, over foliküllerinin değişiklikleri ile östradiol düzeyleri ve endometriyum kalınlığı üzerine etkilerini değerlendirmektir.

Materyal ve Metod: Kırk dişi sıçan beş gruba ayrıldı. Bu beş grup içerisine kontrol grubu ve sigara dumanına maruz bırakılan gruplar; 100, 200 ve 400 mg/kg dozlarında α-tokoferol takviyesi yapılan, dahil edildi. Sigara dumanı için duman pompalama ekipmanı Brawijaya Üniversitesi Tıp Fakültesi Farmakoloji Laboratuvarında tasarlandı ve üniversitemiz kullanımına sunuldu. Kolometrik olarak MDA seviyesi analizi yapıldı. Over folikülü sayısının analizi ve endometriyum kalınlığı hematoksilen eozin boyama ile histopatolojik olarak yapıldı. ELİSA tekniğiyle östradiol seviyesinin analizi yapıldı.

Bulgular: Kontroller ile (0.121 ± 0.026) (p> 0.05), sigara dumanına maruz kalan sıçanların over MDA düzeyleri (0.388 ± 0.085) karşılaştırıldı. Sigara dumanına maruz kalan sıçanların over MDA düzeylerinin önemli ölçüde arttığı gözlendi. Çeşitli dozlarda α-tokoferol verilen gruplar sigara dumanına (P <0.05) maruz kalan grup ile karşılaştırıldığında MDA düzeyinin önemli ölçüde azaldığı ancak kontrol grubunun seviyesine ulaşılmadığı gözlendi. Sigara dumanına maruz kalan grupla kontrol grubunun (P<0.05) birincil ve ikincil folikülleri kıyaslandı. Ovarlerde De Graff folikül sayısının azalma eğiliminde olmasına rağmen bu sonuç anlamlı olarak değerlendirilemedi (P>0.05). Farklı dozlarda α-tokoferol uygulaması ikincil folikül sayısı artırarak kontrol grubunda (P> 0.05) gözlemlenen rakamlara ulaşılmasını sağladı. İlk doz α-tokoferol uygulanan gruptaki (3.8 ± 1.095) birincil folikül sayısı, kontrol grubuyla karşılaştırıldı (3.8 ± 1.095) (P> 0.05). Tokoferol grubunda İlk doz (5,2 ± 1,304) ve ikinci doz (5.4 ± 0.548) sekonder folikül sayıları kontrol grubuna (3.6 ± 0.548) (p <0.05) göre anlamlı ölçüde yüksek bulundu. Sigara dumanıyla östradiol düzeyinin (10.815 ± 2.374) azalması kıyaslandığında aralarında anlamlı bir fark bulunmadı (21.354 ± 4.215) (P < 0.05). Farklı dozlarda uygulanan α-tokoferol, östradiol seviyelerini artırarak kontrol grubu (P> 0.05) seviyesine ulaştığı gözlendi. Sigara dumanına maruz bırakılan ve bırakılmayan gruplar (600,265 ± 76,563) (p <0.05) karşılaştırıldığında endometriyum kalınlığının azaldığı (209.491 ± 38.635) gözlendi. Tokoferolün birinci doz (604. 569 ± 33,621) ve ikinci dozunda (609,459 ± 37,105) östradiol seviyeleri artarak kontrol grubu (600,265 ± 76,563) (P> 0.05) seviyelerine ulaştı.

Sonuç: Yukarıda belirtilen bu sonuçlara göre, α-tokoferol nedeniyle azalmış MDA düzeyleri, primer ve sekonder folikül artışlarının yanı sıra sigara dumanına maruz kalmayla östradiol hormon, oksidatif hasarı ve overlerin fonksiyon kaybını önleyebilir sonucuna varıldı. Aynı zamanda endometriyum kalınlığıda artış gösterdi.

Anahtar Kelimeler: Oksidatif stres, overler, endometriyum, östrojen, antioksidan

INTRODUCTION

Tobacco contains more than 200 components and when burned will form more than 7000 compounds[1]. Tobacco smoke is a dynamic and complex aerosol consisting of particulate matter phase and gas phase which can evaporate[2]. Tobacco smoke is a pollutant in the room as well as underlying various human diseases such as cardiovascular disease, chronic obstructive pulmonary disease, emphysema, and cancer[3-5]. Smoking also affects women's reproductive health and fertility[6]. Women in Indonesia are not only active smokers but also passive smokers.

Reactive oxygen compounds contained in the gas phase and tar phase will result in oxidative damage to various organs[7,8]. Many studies in animals and humans revealed that reactive oxygen compounds in the female reproductive organs are involved in the modulation of the physiological spectra, including oocyte maturation, steroidogenesis in the ovary, corpus luteal function and luteolysis[9,10]. Increased oxidative stress in the ovary can bring about pregnancy complications or spontaneous abortion and infertility[8]. Tobacco smoke also results in oxidative stress on follicles in the ovaries and influences folliculogenesis by inhibiting the growth of follicles[6]. In addition, tobacco smoke causes endometrial hyperplasia,

subendometrial vascularization, and changes in the intensity of blood flow in the endometrium. Benzo(a)pyrene can affect ovaries thereby estrogen production is inhibited. These compounds interfere with blood flow in the endometrium during the menstrual cycle, thereby reducing the thickness of the endometrium[11,12]. Therefore, it is necessary to find any substances capable of counteracting the negative effects of tobacco smoke on the female reproductive organs.

Fat soluble vitamin E has antioxidant activity[13]. Vitamin E consists of tocopherols and tocotrienols, in which both have isoforms α-, β-, γ-, and δ. Of these various subtypes, α-tocopherol is the best antioxidant in biological systems and most likely acting as a peroxyl radical trapping[14]. The α-tocopherol is an antioxidant in the lipid compartment to protect against lipid peroxidation[15]. Other biological functions of the tocopherol are changing gene expression, modulation of cell signaling and proliferation[16]. α-tocopherol is found in significant amounts in ovaries and follicular fluid[17]. α-tocopherol can also enhance the capacity of the immune system at each stage of the menstrual cycle in female athletes[18]. Therefore, this study aimed at evaluating the role of α-tocopherol in inhibiting ovarian oxidative stress, changes in ovarian follicles, estradiol levels, and the thickness of the uterus in female rats exposed to tobacco smoke.

MATERIAL and METHODS

Animal

Adult female Sprague Dawley rats weighing 150-200 g were obtained from the Laboratory Pharmacology Brawijaya University. All experimental procedures were compliant with the Medical Faculty Brawijaya University Committee Guidelines on the Use of Live Animals in Research, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 2010. The rats were kept in a room with a 12-h light/dark cycle at 22 ◦C and were provided access to food and water ad libitum. Female rats were randomly divided into the following four groups: the control group (n = 8), the tobacco smoke exposure group (TS, n = 8), the TS + 100 mg/kgBW α-tocopherol group (TS + toc100; n = 8), the CS + 200 mg/kgBW α-tocopherol group (TS + toc200; n = 8; n = 8), and the CS + 400 mg/kgBW α-tocopherol group (TS + toc400; n = 8).

Tobacco Smoke Exposure Procedure

Tobacco smoke exposure exposure was done by smoking pump equipment that was designed and available in Pharmacology Laboratorium, Medical Faculty, Brawijaya University of Malang. The rats in the control was exposed to fresh air under similar conditions. Female rats placed into whole-body exposure chambers (26 x 12 x 12 cm^3) made from fiberglass and were exposed to cigarette smoke for 3-4 min per cigarettes, twice a day in the morning and afternoon, for eight weeks. During exposure, the temperature was maintained at 22–25°C, and relative humidity was approximately 40%. The present study used clove cigarettes of “Gudang Garam” brand. These cigarettes contained 45.77 mg/cigarette of tar and 2.76 mg/cigarette of nicotine. CO level of tobacco smoke was 16.66 mg/cigarette.

Supplementation

Alpha-tocopherol used in this study is the Vitamin E of Brand Nature - E (1 capsule = 100 IU). Doses of α-tocopherol in the study refers to previous studies, i.e. 100 mg / kg body weight / day, 200 mg / kg body weight / day, 400 mg / kg body weight / day [19]. The vitamin E was administered orally with the probe with sesame oil solvent.

Malondialdehyde Level

Ovaries of 100 mg were cut into small pieces and then ground in a cold mortar placed on a block of ice with a temperature of 40°C. The grinding was done by one person until homogeneous by using a timer of + 4 minutes. Then add 1 mL of phosphate buffer solution. Furthermore

homogenate was transferred into a test tube and the supernatant. 1 mL of ovarian supernatant was added with 500 ml of distilled water, then added 250 µL TCA12.5 %, 250 µL HCl 1N, and 100 µL of Na-Thio. At each addition of reagent, the solution was homogenized by vortex. Furthermore, the solution was incubated in a bath with a temperature of $100^{O}C$ for 30 minutes and will be colored pink, then cool at room temperature. The solution was then centrifuged at 2500 rpm for 10 minutes. Supernatant above was removed, transferred to a new test tube. Sample absorbance was measured by spectrophotometry at a wavelength of 532 nm for TBA test, then the absorbance and the concentration of MDA solution can be read.

The number of Follicles

The numbers of primary, secondary, tertiary and de Graaf follicles were calculated from the right ovary cut transversely and then preparation was made and stained histologically with HE and the follicles were calculated using Dotslide Olympus Camera XC 10. The entire cross-section was analyzed and further identified with magnification of 400 X.

Determination Phase

The estrous phase was determined to know the execution time of experimental animals, which was done on week 8 of treatment period where interval time between swap was 12 hours. Cotton buds, cover glass, glass objects, Giemsa and the microscope were prepared for vaginal swap. Put cotton buds soaked with 0.9% physiological saline into the vaginal opening and rotate 360 to obtain vaginal discharge, and then put the vaginal discharge on glass objects, dried and then soaked in methanol 9% for 10 minutes. It was then stained with Giemsa for 30 minutes. After stained with Giemsa, it was then washed in running water and dried, then observe using a microscope with a magnification 40 times. Results of a vaginal swap for phase determination of white rats were based on the presence of and quantity of vaginal epithelial cells[20].

Dissection

Dissection was performed on week 8 of the treatment. Before the rats were killed, the vaginal swap was done to determine the estrous cycle in rats; rats that were on proestrus phase would be killed and if rats were not the proestrus phase, wait until the proestrus phase.

Measurement of Estradiol

Estradiol in serum was measured immunoenzymatically using an ELISA method (Cusabio, Catalog Series CSB-E05110r, China).

The Thickness of the Endometrium

The thickness of the endometrium was determined by calculating the average thickness of the endometrium with the highest and lowest sizes at each incision for sample using Dot Slide camera.

Ethics

This research has been approved by research ethics committee Faculty of Medicine University of Brawijaya, Malang, Indonesia

Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using 1-way ANOVA with SPSS 15.0 statistical package. Post Hoc test was used if the ANOVA was significant. $p < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the levels of MDA in the control group and groups exposed to tobacco smoke with or without the administration of α-tocopherol. Exposure to tobacco smoke in rats can increase ovarian MDA level (0.388 ± 0.085) significantly compared to the control (0.121 ± 0.026) (P> 0.05). The α-tocopherol in various doses can reduce MDA level significantly compared to the group exposed to tobacco smoke (P <0.05), but has not reached the levels of the control group.

Exposure to tobacco smoke could reduce the number of primary and secondary follicles significantly compared to the control (P <0.05), and

tended to decrease the number of De Graff follicles in the ovaries despite not significant (P> 0.05). Administration of α-tocopherol of the second dose (2.2 ± 0.447) in De Graff follicles significantly increased the number of follicles compared to control (1.2 ± 0.447) (P> 0.05). Administration of α-tocopherol in different doses increased the number of secondary follicles, reaching the number in the control group (P> 0.05). The number of primary follicles in the α-tocopherol group of the first dose (3.8 ± 1.095) was comparable to the control group (3.8 ± 1.095) (P> 0.05). For the groups administered with α-tocopherol of the second dose (5.8 ± 0.837) and of the third dose (5.2 ± 0.447), the number of primary follicles was significantly higher than the control group (3.8 ± 1.095) (P < 0.05). The numbers of secondary follicles in the α-tocopherol group of the first dose (5.2 ± 1.304) and of the second dose (5.4 ± 0.548) were significantly higher than the control group (3.6 ± 0.548) (P < 0.05) (Table 2).

Tobacco smoke lowered estradiol level (10.815 ± 2.374) significantly compared with no exposure (21.354 ± 4.215) (P < 0.05). Administration of α-tocopherol in different doses increased estradiol level which reached the level in the control group (P > 0.05) as showed in Table 3.

Tobacco smoke lowered the thickness of endometrium (209.491 ± 38.635) significantly compared with no exposure (600.265 ± 76.563) (P < 0.05). The α-tocopherol of the first dose (604. 569 ± 33.621) and of the second dose (609.459 ± 37.105) increased estradiol levels, reaching the level in the control group (600.265 ± 76.563) (P > 0.05) (Table 4).

Table 1. Level of malondialdehyde in exposed groups and control female rats

		Exposure groups			
Level	Non exposure	TS	TS + toc100	TS + toc200	TS + toc400
MDA (ng/100 mg)	0.121 ± 0.026	0.388 ± 0.085[a]	0.252 ± 0.040[ab]	0.246 ± 0.033[ab]	0.309 ± 0.053[ab]

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure)l group; [b]p<0.05; in comparison with tobacco smoke exposed groups.

Table 2. Number of follicle in exposed groups and control female rats

		Exposure groups			
Follicle (number)	Non exposure	TS	TS + toc100	TS + toc200	TS + toc400
De Graaf	1.200 ± 0.447	0.600 ± 0.548	1.800 ± 0.447b	2.200 ± 0.447ab	0.800 ± 0.447cd
Primary	3.800 ± 1.095	2.600 ± 0.894a	3.800 ± 1.095b	5.800 ± 0.837abc	5.200 ± 0.447abc
Secondary	3.600 ± 0.548	1.400 ± 0.548a	5.200 ± 1.304ab	5.400 ± 0.548ab	3.000 ± 0.707bcd

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure)l group; [b]p<0.05; in comparison with tobacco smoke exposed groups; [c]p<0.05; in comparison with tobacco smoke exposed groups + α-tocopherol 100 mg / kg body weight / day; [d]p<0.05; in comparison with tobacco smoke exposed groups + α-tocopherol 200 mg / kg body weight / day.

Table 3. Estradiol in exposed groups and control female rats

		Exposure groups			
Level	Non exposure	TS	TS + toc100	TS + toc200	TS + toc400
Estradiol (pg/ml)	21.354 ± 0.026	0.388 ± 0.085a	0.252 ± 0.040b	0.246 ± 0.033b	0.309 ± 0.053b

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure)l group; [b]p<0.05; in comparison with tobacco smoke exposed groups.

Table 4. Level of endometrial thickness in exposed groups and control female rats

		Exposure groups			
Level	Non exposure	TS	TS + toc100	TS + toc200	TS + toc400
Thickness (μm)	0.121 ± 0.026	0.388 ± 0.085a	0.252 ± 0.040b	0.246 ± 0.033b	0.309 ± 0.053abcd

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure)l group; [b]p<0.05; in comparison with tobacco smoke exposed groups; [c]p<0.05; in comparison with tobacco smoke exposed groups + α-tocopherol 100 mg / kg body weight / day; [d]p<0.05; in comparison with tobacco smoke exposed groups + α-tocopherol 200 mg / kg body weight / day.

DISCUSSION

Components of tobacco smoke will accumulate on the female reproductive organs[10,21]. As a result, the various components of tobacco smoke will produce negative effects on sterosidogenesis and gametogenesis in ovaries, oocyte maturation, ovulation, oocyte cumulus complex, gamete and embryo transport in the oviduct, and fertilization and implantation[12,22,23].

In this study, tobacco smoke in rats can increase ovarian MDA levels significantly compared to control (P > 0.05). This indicates that exposure to tobacco smoke triggers oxidative damage through lipid peroxidation of the ovarian structure[11,24,25]. Oxidative damage reflects an imbalance between reactive oxygen compounds and antioxidant defenses[26]. Nicotine will increase lipid peroxidation by blocking the enzymatic antioxidants and cause damage to cell membranes[27]. Furthermore, increased oxidative stress will support the senescence of cells, such as inhibition of follicle development in pre-antral and antral stages and decreased viability of follicles[6]. In fact, function of follicles as hormone-producing could also be affected.

Follicular development and ovulation depend on proliferative changes and differentiation of granulosa cells and theca cells that undergo steroidogenesis when stimulated by intraovarian gonadotropin and cytokines. Exposure to tobacco smoke can reduce the number of primary and secondary follicles significantly compared to the control (P <0.05), and tended to decrease the number of De Graff follicles in the ovaries despite not significant (P> 0.05). This indicates that the primary and secondary follicles are more sensitive to exposure to tobacco smoke compared De Graff follicles. This difference in sensitivity requires further research. One of the active components playing a role in the inhibition of follicular development is nicotine, lowering the number of primary, secondary and De Graaf follicles compared to control[28]. The mechanism of reduction in the follicle is through follicular development delay due to apoptosis[6,11,29].

In this study, exposure to tobacco smoke also lowers estradiol levels (10.815 ± 2.374) compared with no exposure (21,354 ± 4,215) (P > 0.05). Such a decrease in hormone estradiol is caused by the benzo(a)pyrene as a component of tobacco smoke that inhibits the growth of follicles in the ovaries thereby resulting in decreased production of estradiol[11]. Serum estradiol concentration is regarded as one of the parameters that reflects hormonal influence on the endometrium. Estradiol level in the tissues is not only controlled by serum estradiol level but also dependent on hormone metabolism in the target tissue[30]. The concentration of estradiol in the endometrium is significantly higher than in the peripheral circulation[31]. Tobacco smoke is steroidogenic pathway inhibitor that leads to a decrease in the synthesis of estradiol[32]. Several mechanisms of tobacco smoke-induced steroidogenesis reduction may include increased hepatic estrogen metabolism[33], the high concentration of sex hormone binding globulins (SHBG), leading to low concentration of free active estrogen[34], an increase in catechol-estrogen complex formation[35] as well as the effects of the enzyme aromatase suppression by tobacco alkaloid derivatives[36].

During the reproductive period, endometrium is dynamic and will experience cycles of proliferation, differentiation, and decay. In premenopausal women, endometrium is proliferative or secretory in nature, depending on the phase of the menstrual cycle[36]. Capability of the endometrium to provide proper environment for conception, implantation, early gestation and placentation is an important point for pregnancy and fertility[37]. Exposure to tobacco smoke also reduces the thickness of endometrium (209.491 ± 38.635) significantly compared with no exposure (600.265 ± 76.563) (P < 0.05). This is caused by carbon monoxide and nicotine contents of the tobacco smoke that decreases blood flow in the uterus[38], a decrease in endometrial stem cell recruitment[39], as well as decreased production of estrogen, triggering atrophic endometrium as in postmenopausal women[36].

The α-tocopherol in various doses can reduce MDA level significantly compared to the group exposed to tobacco smoke (P <0.05), but has not reached the levels of the control group. Administration of alpha-tocopherol of the second dose improves De Graff follicles significantly compared to the control group (P> 0.05). Administration of α-tocopherol in different doses increases the number of secondary follicles, reaching the number in the control group (P> 0.05). The number of secondary follicles in the α-tocopherol group of the first dose and of the second dose is significantly higher than the control group (3.6 ± 0.548) (P < 0.05). The second and third doses can increase the number of primary follicles significantly compared to the control group (P> 0.05). The α-tocopherol in different doses is able to increase the hormone estradiol level significantly in white rats compared to a group exposed to tobacco smoke, reaching the level of the group with no exposure. In addition, the α-tocopherol of the first and second doses can increase the thickness of the endometrium, reaching the thickness of the group without exposure. Vitamin E (60 mg/kg/day) orally can reduce apoptosis of oocytes in rats exposed to nicotine[27]. Administration of a range of doses of alpha-tocopherol can increase levels of the hormone estradiol significantly in rats. Administration of 20 µm alpha-tocopherol can increase the cultured pig oocyte maturation[17].

It is concluded that the α-tocopherol can prevent oxidative damage and loos of ovarian function due to exposure to tobacco smoke. Inhibition of oxidative damage characterized by decreased levels of MDA will trigger improvement of ovarian function in the form of increased primary and secondary follicles, as well as the hormone estradiol. Furthermore, it also increases the thickness of endometrium.

Declaration of interest

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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