CukurovaMedicalJournal

Araştırma Makalesi / Research Article

The Effects of α–Tocopherol on Endometrial Uterine Vascularization, Uterine Cervix Oxidative Stress and Proliferation in Female Rats Exposed to Tobacco Smoke

Sigara Dumanına Maruz Kalan Dişi Sıçanlarda α-Tocopherol'ün Endometrial Uterin Vaskülarizasyonu, Rahim Serviks Oksidatif Stres ve Proliferasyon Üzerine Etkisi

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CukurovaMedicalJournal 2014;39(4):689-696.

ABSTRACT

Purpose: This study aimed toknow whether administration of α-tocopherolable to increase uterine endometrial vascularization, inhibit uterine cervix oxidative stress, and augment cervical proliferation in female rats exposed to tobacco smoke.

Material and Methods: Twenty five, 8 weeks old female Wistar rats (*Rattusnorvegicus*) were divided into 5 groups (n=5 each), including control group (non exposure group); the rats were exposed to tobacco smoke (CS); the rats were exposed to tobacco smoke and treated with α–tocopherol at dose of 100 mg/kg/day (CS + AT_1); rats were exposed to tobacco smoke and treated with α–tocopherol at dose of 200mg/kg/day (CS + AT_2); rats were exposed to tobacco smoke and treated with α–tocopherol at dose of 400 mg/kg/day (CS + AT_3). The expression of endometrial VEGF was observed by immunohistochemical staining using anti-VEGF primary antibody and *biotinylated universal secondary antibody.* MDA levels were measured colorimetrically. The number of spiralis arterioles and proliferation of uterine cervix epithelial cells were counted histologically withhematoxilin eosin staining. This research has been approved by research ethics committee Faculty of Medicine University of Brawijaya, Malang, Indonesia (Number 477/EC/KEPK-S2-KB/10/2013).

Results: Tobacco smoke significantly lowered VEGF level, the number of spiralis arterioles, anduterine cervix epithelial cells compared with control group ($P < 0.05$). Administration of α-tocopherol in different doses significantly increasethe VEGF level to reachs the level in the control group ($P > 0.05$). Second and third doses of α-tocopherolsignificantly increased the number of spiralis arterioles compared with CS group ($P < 0.05$), to reached the similar level in the control group ($P > 0.05$).Exposure to tobacco smoke in rats can increase uterine cervix MDA level significantly compared to the control ($P< 0.05$). The α-tocopherol in various doses cansignificantly reduce MDA level compared to the CS ($P <0.05$), but has not reached the levels of the control group. The number of epithelial cells in α-tocopherol-administered groupwas not significantly different compared with CS group ($P > 0.05$).

Conclusion: Administration of α–tocopherolcan increase the vascularization in endometrial uterine of rats exposed to tobacco smoke. Besides, α–tocopherolinhibit uterine cervix oxidative stress, but can't induces the proliferation of uterine cervix epithelial cells.

Key Words: Lipid soluble antioxidant;oxidative stress, angiogenesis; reproduction.

ÖZET

Amaç: Bu çalışma α-tocopherol uygulamasının uterin endometrium vaskülarizasyonu arttırmada, uterin servik soksidatif stresinin hibe edilmesi ve sigara dumanına maruz kalan dişi sıçanlarda servikal çoğalmanın artırılabileceğinin mümkün olup olmadığını öğrenmeyi amaçlamıştır.

Materyal ve Metod: Yirmibeş adet, 8 haftalık dişi Wistar sıçanı (Rattusnorvegicus) kontrol grubu (maruz bırakılmamış grup) dahil 5 gruba (n=5) ayrılmıştır; sigara dumanına (CS) maruz bırakılmış sıçanlar; sigara dumanına maruz bırakılmış ve 100mg/kg/gün dozluk (CS+AT1) α-tocopherol'aile muamele edilmiş sıçanlar; sigara dumanına maruz bırakılmış ve 200mg/kg/gündozluk (CS+AT2) α-tocopherolile e muamele edilmiş sıçanlar; sigara dumanına maruz bırakılmış ve 400mg/kg/gündozluk (CS+AT3) α-tocopherolile e muamele edilmiş sıçanlar. Endometrial VEGF'nin ekspresyonu, anti-VEGF, primer antikor vebiyotinlenmiş genel sekonderantikor kullanılarak immunohistokimyasal boyama ile gözlenmiştir. MDA düzeyleri kolorimetrik olarak ölçülmüştür. Spiral arterlerin sayısı ve Rahim serviks epitel hücrelerin çoğalması hematoksiline ozin boyalarla histolojik olarak sayılmıştır. Bu araştırma Endonezya Malang'daki (No 477/EC/KEPK-S2-KB/10/2013) Brawijaya Üniversitesi Tıp Fakültesi'nin araştırma etik komitesi tarafından onaylanmıştır.

Bulgular: Sigara dumanı control grubuyla karşılaştırıldığında özellikle VEGF seviyesini, spiral arterlerin ve uterin serviks epitel hücrelerinin sayısını düşürmüştür (P<0.05). α-tocopherol'un farklı dozlarda uygulanması özellikle VEGF seviyesini, control grubundaki seviyesine ulaşmak için arttırmıştır (P>0.05).a-tocopherol'ün İkinci ve üçüncü dozları CS grubuna kıyasla (P<0.05) spiral arterlerin sayısını control grubuyla aynı seviyeye ulaşmasında önemli ölçüde arttırmıştır. (P>0.05). Sıçanlarda sigara dumanına maruz kalmanın serviks MDA düzeyini control grubuna(P <0.05) gore anlamlı olarak artırabilir. α-tocopherol'ün değişen dozlarının CS (P<0.05) MDA düzeyini önemli ölçüde azaltabildiği, fakat control grubunun seviyesine ulaşamadığı görülmüştür. α-tocopherol uygulanmış grup, CS grubuyla (P>0.05) karşılaştırıldığında epitel hücrelerin sayısında önemli derecede bir farklılık saptanmamıştır.

Sonuç: α-tocopherol'ün uygulanması sigara dumanına maruz kalmış sıçanlarda endometrial uterinde vaskülarizasyonu arttırabilmektedir. Bunun yanı sıra, α-tocopherol uterin serviks oksidatif stresinİ hibe etmektedir, fakat uterin serviks epithelial hücrelerin çoğalmasını indüklememektedir.

AnahtarKelimeler: Lipid çözünürantioksidan, oksidatif stres, anjiyogenez, üreme.

INTRODUCTION

Smoking is a habit that can lead to public health issue and becomes one of the largest causes of death in the world. Tobacco smoke contains about 4,000 toxic chemical compounds, including polycyclic aromatic hydrocarbons, nitrosamines, heavy metals and aromatic amines[1]. In addition, reactiveoxygenspecies produced by tobacco smoke can cause lipid peroxidation of cell membranes.

Smoking has a negative impact on all reproductive functions, among others, disorders in folliculogenesis, embryonic development, endometrial angiogenesis, streroidogenesis, amenorrhea, premature menopause, as well as the interruption of blood flow to the myometrial tissues[1,2]. Maternal smoking reduces endothelium-dependent nitric oxide–mediated relaxation in uterine small arteries[3]. Cigarette compounds also impair uterine and endometrial vascularisation and myometrial relaxation[4]. Tobacco smoke can reduce the synthesis of 17-β estradiol, in which during the process of proliferation of endometrial tissues.17-β estradiol plays a role in the regulation of the expression of vascular endothelial growth factor (VEGF)[5]. VEGF is a potential growth factor required in the process of endometrial proliferation, as well as plays a role in inducing increased permeability and angiogenesis in endometrial spiral arterioles[6]. In uterine cervix, smoking involved in the process of metaplasia and proliferation of uterine cervix cells[7].

α-tocopherol is a powerful fat-soluble antioxidant and therefore can protect cell membranes against oxidative damage that can prevent further damage to DNA and tissues. α-tocopherol can prevent lipid peroxidation due to can break the chains of lipid peroxyl rapidly through the provision of a hydrogen atom[8]. Previos studies showed that vitamin E, in single action or

combination with other compounds, have beneficial or detrimental effects on female reproductive system. α-tocopherol can improve blood supply to the granulose during ovarian follicles development thereby supporting the production of the hormone estrogen which plays a role in the thickening of the endometrium[9]. In combination with vitamin C, vitamin E decreased the frequency of litters, litter size, total number of offspring born and survival of male pups to weaning. This effect was associated with lower number of corpora lutea in the left ovary, decreased percentage of viable fetuses, and higher number of fetal resorptions in the left uterine horn when compared to the control group[10]. Besides, previous studies showed that smoking decreases tubal cilia numbers. Supplementation by vitamin E may treat or at least help to slow down the decrease in number of cilia caused by smoking[11]. Therefore, based on contradictive result aboved,this study aimed toinvestigate whether administration of α-tocopherolable to increase uterine endometrial vascularization, inhibit uterine cervix oxidative stress, and augment uterine cervix proliferation in female rats subchronically exposed to tobacco smoke.

MATERIAL andMETHODS

Animal

Twenty five, 8 weeks old female Wistar white rats (*Rattusnorvegicus*) were divided into 5 groups, each group consisted of 5 rats. Five groupsconsisting the control group (non exposure group); therats were exposed to tobacco smoke (CS); the rats were exposed to tobacco smoke and treated with α–tocopherol at dose of 100 mg/kg/day (CS + AT_1); rats were exposed to tobacco smoke and treated with α–tocopherol at dose of 200mg/kg/day (CS + AT_2); rats were exposed to tobacco smoke and treated with α–tocopherol at dose of 400 mg/kg/day (CS + AT_3). The dose α–tocopherol according previous study[12].

Tobaccosmoke

Exposure to tobacco smoke was conducted over 8 weeks with 2 cigarettes per day.One cigarette will exposed in the morning (10 a.m) after administration α–tocopherol. One cigarette will exposed in the mid-day (12 a.m). The brand of cigarette is Gudang Garam Merahwhich contains nicotine at 2.76 mg; 16.66 mg of carbon dioxide, and 45.77 mg of tar. The exposure was done using smoking pump to produces mainstream smoke which availbale in Pharmacology Laboratory, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia. This equipment is whole body exposure for 4 minutes in each exposure.

α–tocopherol

Administration of α–tocopherol were conducted over 8 weeks after prior cigarette smoke exposure in the morning (10 a.m). α–tocopherol was dissolved first with sesame oil and administered to the rats by oral gavage.

Tissue preparation

Uterine and cervical tissues were fixed in 10 % buffered formalin solution, and then put into the Tex Tissue Processor for 90 minutes. The tissues were then embedded with paraffin and cut with a thickness of ± 4 μm.

Hematoxylin-eosin staining

Histopathological profile of endometrial uterine and uterine cervix tissues was analyzed using hematoxylin-eosin (HE) staining. Sample was deparaffinized using xylene and dehydrated using alcohol. Slides were then washed with running water for 15 minutes and soaked in a hematoxylin solution for 10-15 minutes. Slides were then washed with running water for 15 minutes and dipped in acid alcohol 1%. The slides were then dipped in liquid ammonia and stained with eosin 1% for 10-15 minutes. Slides were dehydrated again with alcohol series (80%, 90%, and absolute alcohol) each for 3 minutes. Slides were then soaked with xylene for ± 2 hours and then dried. The results of staining were observed with a microscope XC 10 (Olympus) at 400 magnification.

Analysis of spiralisarterioles number

The numberis calculatedbased onthe number ofspiralisarteriolesbranchinginendometrialtissuepre parations. Observationwas performed in magnification of400timesineightfieldsof viewusing amicroscope.

Immunohistochemical staining

Immunohistochemical(IHC) staining was carried out to see the expression of VEGF protein in endometrial tissues. Slides were deparaffinized using xylene and dehydrated using alcohol series. The slides were immersed in citrate buffer of pH 6 and heated in a waterbath at temperature of 95ºC for 20 minutes. After slides were blocked using H2O2 3% in methanol for 15 min (endogenous blocking), they were then washed with PBS and blocked back with sniper and incubated for 60 min. Furthermore, the primary antibody (VEGF) was added in PBS + BSA 0.2 % and incubated overnight in 4ºC. Once the slides were washed with PBS, they were then incubated with *biotinylated universal secondary antibody* for 60 minutes at room temperature. Incubation of the enzyme SA-HRP (Streptavidin Horseradish Peroxidase) was performed for 40 minutes at room temperature, and then DAB (Diaminobenzidine) was added with ratio of DAB chromagen: DAB buffer = 1:50 for 10-20 minutes. After the slides were washed with PBS and distilled water, they were counterstained with Mayer's Hematoxilin for 5-10 minutes at room temperature. The slides were mounted and observed with a microscope at 400x magnification to count the number of cells expressing VEGF. VEGF expression in endometrial glandular epithelial cells was calculated based on the number of cells that showed cytoplasmic brown stained cells divided by the total number. Calculations performed at a magnification of 400 times in 8 fields of view.The results are expressed in percentage[13].

Malondialdehyde analysis

Levels of malondialdehyde(MDA) were measured by reaction with thiobarbituric acid (TBA) compound. Cervicaluterine tissues at 100 mg were pulverized in a cold mortar. Add 2 cc of phosphate buffer, 100μL TCA 10%, 250 μL HCl, 200 μL Na-Thio Barbiturates into the test tube and homogenized using a vortex. The test tube was then heated in a water bath at temperature of 105ºC and then centrifuged. The supernatant absorbance was read using a spectrophotometer at a wavelength of 532 nm.

Analysis of uterine cervix epithelial cell proliferation

All cells inthe epitheliumof the cervixina longitudinalcutsthat expressthe characteristics ofthe nucleusincludesthe proliferation oflarger anddark purple(hyperchromatic), and thecytoplasmpinkwillbe calculated. Attenvisual field,the total number ofproliferativecellsdivided bythe total number ofepithelialcellsinthevisual fieldof the cervix(percentage) with400xmagnificationiscervicalepithelialcellprolifer ationlevels.

Ethics

This research has been approved by research ethics committee Faculty of Medicine University of Brawijaya, Malang, Indonesia (Number 477/EC/KEPK-S2-KB/10/2013).

Statistics analysis

Data were presented as mean ± SD and differences between groups were analyzed using One - way ANOVA test with a significance level of 95 % (p value < 0.05).

RESULTS

Tobacco smoke significantly lowered VEGF level compared with control group ($P < 0.05$). Administration of α-tocopherol in different doses significantly increasethe VEGF level to reachs the level in the control group ($P > 0.05$), as showed in Table1.

Table 1. Expression of VEGF in exposure groups and control female rats

		Treatment groups			
Expression	Control	CS	CS + AT_1	CS + AT_2	CS + AT3
VEGF (%)	53.24 ± 12.91	17.79 ± 10.71[a]	38.96 ± 8.44[b]	53.06 ± 8.30[b]	47.48 ± 13.38b

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure) group; [b]p<0.05; in comparison with CS-exposure groups; CS: cigarette smoke; AT: alpha-tocopherol; VEGF: vascular endothelial growth factor; ng/100 mg: nanogram/100 miligram.

Tobacco smoke significantly reduced the number of spiralis arteriolesthan that control group (P < 0.05). Second and third doses of α-tocopherolsignificantly increased the number of spiralis arterioles compared with CS group (P < 0.05), to reached the similar level in the control group (P > 0.05), as given in Table2.

Table 2. The number of spiralis arterioles in exposure groups and control female rats

		Treatment groups			
Number	Control	CS	CS + AT1	CS + AT2	CS + AT3
Spiralis arterioles	89.60 ± 2.07	70.20 ± 9.20a	77.60 ± 9.39a	87.00 ± 8.57b	85.60 ± 2.70b

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure) group; [b]p<0.05; in comparison with CS-exposure groups; CS: cigarette smoke; AT: alpha-tocopherol.

Table3 shows the levels of uterine cervix MDA in the control group and CS group with or without the administration of α-tocopherol. Exposure to tobacco smoke in rats can significantly increase uterine cervix MDA level compared to the control (P< 0.05). The α-tocopherol in various doses cansignificantly reduce MDA level compared to the CS (P <0.05), but has not reached the levels of the control group.

Table 3. The level of malondialdehyde in exposure groups and control female rats

		Treatment groups			
Level	Control	CS	CS + AT1	CS + AT2	CS + AT3
MDA (ng/100 mg)	0.14 ± 0.01	0.56 ± 0.12a	0.42 ± 0.04ab	0.31 ± 0.06abc	0.30 ± 0.05abc

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure) group; [b]p<0.05; in comparison with CS-exposure groups; [c]p<0.05; in comparison with first dose alpha-tocopherol group; CS: cigarette smoke; AT: alpha-tocopherol; MDA: malondialdehyde; ng/100 mg: nanogram/100 miligram.

Exposure to tobacco smoke significantly reduce the number of epithelial cells in uterine cervix compared to the control (P <0.05).The number of epithelial cells in α-tocopherol-administered groupis not significantly different compared with CS group (P > 0.05) (Table 4).

Table 4. Level of epithelial cells proliferation in exposure groups and control female rats

		Treatment groups			
Level	Control	CS	CS + AT1	CS + AT2	CS + AT3
Proliferation (%)	43.60 ± 10.87	16.60 ± 7.66a	16.40 ± 7.47a	20.20 ± 6.97a	15.43 ± 3.57a

Note: values are presented as mean ± SD; [a]p<0.05; in comparison with control (non exposure) group; CS: cigarette smoke; AT: alpha-tocopherol.

DISCUSSION

Cigarette smoke may be inhaled directly by smokers (mainstream smoke), released from the burning end of cigarettes (sidestream smoke), or exhaled by smokers[14]. This study applied mainstream smoke inhaled to female rats. The cigarette that we used in this study contains nicotine at 2.76 mg; 16.66 mg of carbon dioxide, and 45.77 mg of tar. We found that exposurefor 8 weeks with 2 cigarettes per day significantly decrease the level of endometrial VEGF compared with control group ($P < 0.05$). This finding indicates that component of tobacco act together to modulate VEGF expression. Previous studies showed that chronic inhalation of 250 ppm CO will augments uterine blood flow and uteroplacental vascular growth[15]. In contrary, nicotine promotes endothelial cell migration, proliferation, survival, tube formation and nitric oxide (NO) production *in vitro*, mimicking the effect of other angiogenic growth factors[16]. Besides, smokers secrete significantly higher amounts of sVEGFR-1 than nonsmokers, which may result in decreased vascularization in female reproductive organ[17].

Human and animal studies have demonstrated that chronic supplementations with tocopherols have biphasic, proangiogenic and antiangiogenic therapeutic effects[18-22]. In this study, the administration of α–tocopherolsignificantly increase the expression of VEGF in endometrial tissues. This result is thought to be caused by the fact that α–tocopherol can induce the expression of VEGF promoter[23]. In addition, oral administration of α–tocopherol is known to induce increased expression of IL-6 as trigger of angiogenic activity through increased release of VEGF proteins[24,25].

Cigarette smoke inhibits processes that may hinder normal process of angiogenesis resulting in abnormal blood supply to tissues, decreased repair and remodeling[26].In endometrial tissues, tobacco smoke significantly decrease the number of spiralis arterioles compared with control ($P < 0.05$), the administration of α–tocopherol (second and third doses) able to reverse it. An increase in the number of spiral arterioles is may be associated with the increased expression of VEGF as a critical factor in the process of angiogenesis. Increased expression of VEGF as a result of the administration of α–tocopherol in rats exposed to tobacco smoke showed that the antioxidant activity of α–tocopherol is able to restore the angiogenic activity of VEGF. In the proliferative phase, the blood vessels will undergo angiogenic process through a formation of spiral arterioles. VEGF and its receptors (VEGFR-1 and VEGFR- 2), fibroblast growth factor-2 (FGF- 2) and its receptor (FGFR-1 and FGFR- 2), epidermal growth factor (EGF) and its receptor EGFR are known to be essential components in the angiogenic process and function of blood vessels in endometrium[7,27].

In this study the level of oxidative stress in cervical uterine was significantly higher in cigarette smoke group than that control group ($P < 0.05$). Reactive oxygenspecies produced by tobacco smoke can cause lipid peroxidation of membran cells in cervical uterine[5].The MDA levels may increase due to the metal content in cigarettes that can cause an increase in •OH through Fenton reaction. Administration of α–tocopherolable to significantly decrease MDA level compared with tobacco smoke group ($P < 0.05$). The α–tocopherol is fat-soluble makes it able to protect the cervical epithelial cell membranes against oxidative stress. Protection mechanism in the cell membrane due to donating a hydrogen atom that would break the chain reaction of oxidation on lipid peroxyl radical (LOO•), resulting in the decreased MDA levels of the uterine cervix. The results of this study are supported by prior studies that administration of α–tocopherol will decrease lipid peroxides and elevated glutathione level and increased activities of glutathione-S-transferase, glutathione peroxidase, catalase and superoxide dismutase

during carcinogenesis in uterine cervix of mice by chronic exposure to 20-methylcholanthrene[28].

Proliferation of epithelial cells was known to experience a decline in the group exposed to tobacco smoke and α–tocopherolthan that control group, but not reach sgnificantly different. The decrease in cell proliferation in CS group occurs allegedly due to destructive effects of oxidative stresson cell structure that induced cell apoptosis, confirmed as elevated MDA level. In addition, cigarette components can directly cause non-growth of ovarian follicles thereby inhibiting the secretion of the hormone estrogen which in turn leads to the absence of cell proliferation. In α–tocopherol-administered group,insignificantly result may be due to anti-proliferative activity of α–tocopherol[27].

In conclusion, administration of α–tocopherol can effectively increase the vascularization in endometrial uterine rats exposed tobacco smoke. Besides, α–tocopherolinhibit oxidative stress and act as anti-proliferative in epithelial cells of uterine cervix.

Declaration of interest

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

Acknowledgment

The authoracknowledged to all technician in Laboratory of Pharmacology, Biomedicine, and Pathology for helping this study.

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Geliştarihi/Received on :10.03.2014
Kabul tarihi/Accepted on:30.04.2014