



Omega-3 and Vitamin E Supplementation Effect on Reactive Oxygen Species and Placental Vascular Endothelial Growth Factor: Pre-eclampsia Model

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Abstract

BACKGROUND: Pre-eclampsia (PE) has been associated with oxidative stress and inflammation. Omega-3 fatty acids and vitamin E have beneficial function to maintain cell membrane, prevent oxidative stress, and inhibit the production of proinflammatory cytokines. The purpose of the study was to determine the effect of omega-3 and vitamin E supplement to Reactive Oxygen Species (ROS) and placental Vascular Endothelial Growth Factor (VEGF) on PE rats model.

AIM: This study aimed to investigate the effect of omega-3 and vitamin E supplementation on ROS and placental VEGF.

METHODS: This research has been carried out at animal house and Biomedical Laboratory of the Medical Faculty Andalas University. The design of this research was experimental study with post-test only control group design. Thirty pregnant rats were divided into five groups. Group K-without treatment, group K+ was given L-NAME, group P1 was given L-NAME + omega-3, group P2 was given L-NAME + vitamin E, and group P3 was given L-NAME + omega-3 + vitamin E. The L-NAME dose was 50 mg/kg/day. At the 19th day of gestation, the pregnancy was terminated and the blood serum was used for examination of ROS and placental tissue was collected for examination of VEGF using ELISA kit. The data were analyzed by one-way ANOVA and *post hoc* test Bonferoni.

RESULTS: The analysis results found that there were significant differences between omega-3 and vitamin E supplements on ROS levels and placental VEGF levels. Administration of omega-3 only or combination with vitamin E can effectively improve endothelial function by increasing the level of VEGF.

CONCLUSION: Omega-3 (DHA 120 mg, EPA 180 mg) and vitamin E (α -tocopherol 300 iu) supplement decreased ROS and increased placental VEGF on pre-eclampsia rats model.

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Introduction

Pre-eclampsia (PE) is a major complication in pregnancy and a major cause of maternal and perinatal mortality and morbidity. It was estimated that 50,000–60,000 maternal mortality caused by pre-eclampsia per year. The World Health Organization (WHO) estimated that pre-eclampsia rate was 7 times higher in developing countries than in developed countries. Pre-eclampsia prevalence in developed countries was 1.3–6%, while in developing countries were 3 times higher (1.8–18%). The incidence of pre-eclampsia in Indonesia was 128,273/year or about 5.3% of the pregnancies [1].

The pathophysiology of PE is not clearly understood, although it is believed to be of placental origin. It is known that the placental function is dependent on a proper vascular network. Over the last one decade, several reviews have highlighted the association between altered placental vascular development and PE. Vascular endothelial growth factor (VEGF) plays a key

role in vasculogenesis and angiogenesis, both of which are important in the development of the placenta [2]. Adequate angiogenesis and subsequent vascularization of a tissue are a vital step for cellular functions to satisfy energy requirements. Mammalian placentation requires extensive angiogenesis for the establishment of an appropriate vascular network to supply oxygen and nutrients to the growing fetus. VEGF is believed to play a critical role in development of normal placental vasculature by binding to its receptors VEGFR-1 and VEGFR-2. The decrease in circulating VEGF and concomitant increase in its soluble receptor of sVEGFR1 (sFlt-1) have been an established hallmark of PE [3].

Pre-eclampsia is also known to be associated with oxidative stress and inflammation [4]. This originates from a defect of placental implantation into the maternal uterine wall. The impaired remodeling of the spiral uterine arteries by the extravillous trophoblasts (EVT) leads to decrease placental perfusion. Consequently, intermittent arterial blood flow generates repeated ischemia/reperfusion episodes, thus creating a favorable environment for developing

oxidative stress. Oxidative damage in the placenta leads to inflammation, apoptosis, and the release of cellular debris into maternal circulation, along with several anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt1) and soluble Endoglin (sEng), cytokines, and oxidants [5].

Although the ischemic placenta in PE is known to induce ROS production. ROS is also produced in the systemic vasculature at the second stage of PE pathogenesis. In some studies, placental homogenates derived from patients with PE showed 39% higher hydrogen peroxide production than those derived from normal pregnant women. In addition, increased ROS concentrations in patients with PE have been proved by the increased levels of malondialdehyde, an index of lipid peroxidation [6].

Vitamin E is very potent in prevention of pre-eclampsia, because it is the most important fat-soluble antioxidant in preventing lipid peroxidation and has an antioxidant properties such as: preventing free radical formation, preventing apoptosis of placental tissue, inhibiting activation of the endothelium and leukocytes, and as anti-inflammatory agent, in which all can play a role in the prevention of pre-eclampsia [7]. Vitamin E is a proxyl radical scavenger and acts as a protector for polyunsaturated fatty acids (PUFA) within membrane phospholipids and in plasma lipoproteins. In addition, α -tocopherol has specific molecular functions such as inhibition of protein kinase C activity, which is involved in cell proliferation and differentiation of smooth muscle cells [8].

Omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to have anti-inflammatory effects [4]. Omega-3 PUFAs has the potential effect to inhibit oxidative stress by increasing ROS binding capacity and limiting ROS regeneration. Omega-3 supplementation also increased mRNA expression and activity of major antioxidant enzymes in the placenta and reduced placental oxidative damage [9]. Omega-3 PUFAs supplementation during pregnancy has shown to improve pregnancy quality and reduce pregnancy complications risk, although the exact mechanism that regulates this effect remains uncertain. Omega-3 PUFAs are involved in several physiological processes through anti-oxidative, anti-inflammatory, and pro-resolving pathways [9]. High dose n-3 PUFAs can suppress endothelial proliferation, migration, and VEGF production. This effect of n-3 PUFAs may benefit angiogenic diseases such as cancer, chronic inflammation, and diabetic retinopathy; it may be detrimental to artery angioplasty and vascular repair or regeneration [10].

LNAME or nitro-L-arginine methyl ester is well known as nitric oxide synthase inhibitor. Administration of L-NAME inhibits nitric oxide synthase activity, and therefore, biosynthesis occurs and causes hypertension. Administration of L-NAME to rats causes injury to the vascular endothelium, and this model is widely used to

study hypertension, as well as cardiovascular and renal diseases.

The aim of the study was to investigate the effect of omega-3 and vitamin E supplementation on ROS and placental VEGF.

Methods

Design and sampling

This research was an experimental study, specifically post-test only control group design, which compared the experimental group with the control group. The sample used for the treatment were *Rattus Norvegicus wistar* strain. Before treatment, rats were quarantined for 1 week with the aim of conditioning the rats in a labor atmosphere to relieve stress. Room temperature was maintained at $25 \pm 1^\circ\text{C}$ and humidity $50 \pm 10\%$. Food and drink were provided ad libitum. The food given was in the form of pellets mixed with aquadest. The average daily feed intake was 5 g/100 g BW, water needs was 8–11 ml/100 g BW.

Thirty pregnant rats were divided into five groups. The normal pregnant rats group without treatment (K-) as the negative control, the group given L-NAME (K+) as positive control, the group given L-NAME + omega-3 (P1), the group given L-NAME + vitamin E (P2), and the group given L-NAME + omega-3 + vitamin E (P3). This research has passed out an ethical clearance from the Ethics Committee of Medical Faculty, Andalas University with certificate No: 344/UN.16.2/KEP-FK/2021.

Treatment dose

L-NAME was used to create hypertensive state on pregnant rats like pre-eclampsia, given orally on the 10th to 19th day of gestation. The dose of L-NAME administration was 50 mg/kg/day for 10 days. Omega-3 (180 mg EPA and 120 mg DHA) used was Omeheart 300 mg from Mywell and vitamin E 300 iu from Sidomuncul equivalent to 300 mg α -tocopherol.

Blood and placental collection

At the 19th day of gestation, the pregnancy was terminated. Rats were anesthetized by inhalation method using chloroform. Then, they were dissected to take a tissue sample. After the tissue was removed, a cervical dislocation was performed.

Then, the blood sample was collected. The rats were pierced with a syringe to take the blood. The placental tissues were also collected. The placental tissues were washed with phosphate buffered saline

(PBS) solution (0.02 mol, Ph 7.0–7.2) from remaining blood. The tissues were ground out into small pieces and homogenized in PBS with a cold-temperature homogenizing glass. Each 1.0 mg placental tissue was homogenized with 9 μ l PBS solution (0.01 M, Ph = 7.4). The suspension was used for ultrasonication or two freeze-thaw cycles to break up the cell membrane. The suspension was centrifuged at 5000 \times g for 5 min. Supernatants samples cell derived from placental trophoblasts were stored at -20°C before next step.

ROS examination

Serum from blood samples was examined by ELISA using ROS ELISA kit from BT LAB catalog no. E0900Ra.

VEGF examination

Homogenate from placental samples was examined by ELISA using VEGF ELISA kit from BT LAB catalog no. E0659Ra. Each sample was made in duplicate to ensure the better result.

Data analyze

The data were analyzed by Shapiro–wilk. The result was normal for normality test, because it was normally distributed ($p = 0.252$ for ROS and 0,743 for VEGF). Thus, it was proceed to one-way ANOVA analysis with 95% confidence degree ($\alpha = 0.05$). Then, analysis was continued with the Bonferoni *post hoc* test [11].

Results

ROS levels

The mean level of ROS in the normal pregnant rat group (K-) was different from the group of pre-eclampsia model rats (K+). The mean level of ROS for normal pregnant rat (K-) was 121,683 ng/ml, while in K+ was 143,900 ng/ml. This result indicated that L-NAME administration increased the ROS level of the rat placenta (Table 1).

Table 1: ROS mean levels on each group based on treatment

Groups	Mean (ng/L)	SD	p-value
K -	121,683	1,8989	0.001
K +	143,900	2,7335	
P1	136,250	2,9263	
P2	132,433	3,5455	
P3	123,000	3,1483	
Total	131,453	8,8774	

The group of pre-eclampsia rats model of combination of omega-3 and vitamin E appeared to have

the lowest ROS mean level among other pre-eclampsia groups (Table 2). The one-way ANOVA analysis of the means difference in ROS levels revealed $p = 0.001$. This result showed that there was a significant difference on ROS levels between treated and control group.

Table 2: Placental VEGF mean levels on each group based on treatment

Groups	Mean (ng/mg)	SD	p-value
K -	382,6500	3,13225	0.000
K +	365,8167	2,72721	
P1	373,6500	1,34573	
P2	369,8667	2,53351	
P3	389,3667	2,14725	
Total	376,2700	9,03374	

Placental VEGF levels

The mean levels of VEGF in the normal pregnant rat group (K-) were different from the group of pre-eclampsia model rats (K+). The mean level of VEGF for normal pregnant rat (K-) was 382,6500 ng/mg, while in K+ was 365,8167 ng/mg. This result showed that L-NAME administration decreased the VEGF level of the rat placenta, as shown in Table 2.

The K+ group had the lowest VEGF mean level among the other groups, which was 365,817 ng/ml. The group of pre-eclampsia rat model given vitamin E and omega-3 (P3) appeared to have VEGF mean level closed to the normal pregnancy group, while the P2 had VEGF mean levels not so different from the pre-eclampsia group (K+). The treatment group of combination of omega-3 and vitamin E appeared to have the highest placental VEGF mean levels among the other groups (Table 2).

The analysis result of mean differences levels of placental VEGF of pregnant rats in the treated group and the control group obtained $p = 0.000$ ($p < 0.05$). It can be concluded that there were significant differences in VEGF levels of rat placenta between the treated and control group (Figure 1).

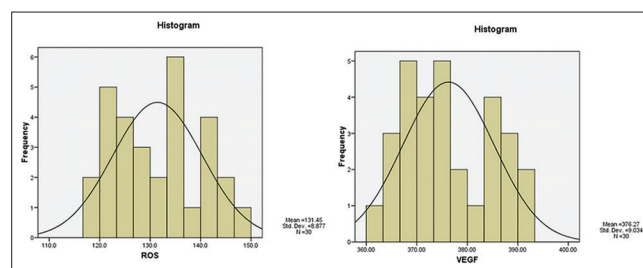


Figure 1: Frequency distribution and normal curves of ROS and placental VEGF

Discussion

Maternal vitamins and minerals have been shown to influence angiogenic factors in PE. In

pre-eclampsia, the reduced ability of the antioxidant system to overcome lipid peroxidation products (oxidants; free radicals) caused oxidative stress. In addition, the levels of protective antioxidants such as vitamin E decreased, while the amount of lipid peroxidation products in the patient's circulation increased [12].

Vitamin E also had effect on angiogenesis and vasculogenesis. This effect has been observed in several studies, and recently, it has been demonstrated in the placenta of pregnant ewes, possibly involving the stimulation of VEGF expression. The phosphorylated form of α T, α -tocopheryl phosphate (α TP), increases the expression of VEGF [13]. We propose that the stimulatory effect of α T on angiogenesis and vasculogenesis is potentiated by phosphorylation to α TP, which may act as a cofactor or active lipid mediator increasing VEGF expression. Increased VEGF expression and consequent enhanced angiogenesis and vasculogenesis induced by α TP may explain not only the essential roles of vitamin E on reproduction, but also its beneficial effects against pre-eclampsia, ischemia/reperfusion injury, and during wound healing [14].

Omega-3 and Vitamin E supplementation effect to reactive oxygen species (ROS)

In this study, the ROS mean level in pre-eclampsia rats model was higher or significantly different from normal pregnant rats. Omega-3 supplements ($p = 0.001$), vitamin E ($p = 0.000$), and the combination of omega-3 and vitamin E ($p = 0.000$) were associated with decreased levels of ROS in pre-eclamptic rat models compared to the control group. The most effective supplementation was combination of vitamin E and Omega-3, because the level approached was close to the normal pregnant rat levels.

This result was in accordance with Alcalá *et al.* (2017) study, which stated that vitamin E supplementation reduced the generation of ROS, the transcription of *Nox4*, and the levels of lipoperoxides (LPO) in obese mice [15]. Other study also suggested that EPA and DHA attenuate oxidative stress-induced DNA damage in vascular endothelial cells through upregulation of NRF2-mediated antioxidant response [16]. Sley *et al.* (2020) also found that third trimester n-3 FA intake was associated with lower concentrations of 8-iso-PGF and its metabolite, suggesting a decrease in maternal oxidative stress during pregnancy [17].

The central organ regulating pregnancy, the placenta, is a major source of ROS. During normal pregnancy, placental oxidative stress (OS) is presented during all three trimesters and is necessary to obtain normal cell function, including activation of redox-sensitive transcription factors and protein kinases. Although OS is a common necessary feature of normal pregnancy, augmented OS could give rise to different disease-states, such as pre-eclampsia [18]. Omega-3 fatty acids are

very susceptible to peroxidation due to the high number of double bonds in their structure. The combination of vitamin E with DHA will help reduce lipid peroxidation. Administration of omega 3 and vitamin E was suggested to reduce ROS and help to prevent PE [19].

Omega-3 and Vitamin E supplementation effect to vascular endothelial growth factor (VEGF)

Pre-eclampsia is a pregnancy complication characterized by impaired invasion of fetal trophoblasts, causing abnormal spiral artery remodeling, and leading to a decrease in the blood flow between the mother and fetus. This affects placental oxygen and transfer of nutrients to the fetus. To compensate for the blood flow deficiency, the mother develops hypertension and increases the blood flow. Thus, pre-eclampsia originates from the placenta resulting in maternal endothelial and vascular dysfunction [20].

VEGF is a potent angiogenic growth factor and acts as a key regulator of vascular development, implicated in the development of both pathological and physiological angiogenesis. In the placenta, it has been identified recently and considered as one of the important angiogenic growth factors in the development of the embryo [21].

The principle tissues expressing VEGF is surface of placental syncytiotrophoblast cells and invasive chorionic trophoblast cells during pregnancy with, in particular, expression at the vascular bed site during early pregnancy, when syncytiotrophoblast cells are abundant. Overexpression of VEGF is responsible for vascular endothelial proliferation which may culminate in endothelial damage in long term [22].

The decreased blood flow to the placenta mentioned above leads to a local hypoxic environment in the placenta. In response to hypoxia, VEGF is transcriptionally upregulated; this growth factor is important to maintain the health of existing vessels. Although VEGF can bind three receptors, its actions are mediated through two tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). VEGFR-1 is also known as Flt-1 (fms-like tyrosine kinase 1), and VEGFR-2 is commonly referred to as Flk-1 (fetal liver kinase 1). VEGFR-1 also has a splice variant sVEGFR-1/sFlt-1, which contains only the extracellular domain of the receptor, making it soluble in plasma. Because sFlt-1 contains the binding site for VEGF, it is still able to bind all isoforms of the growth factor, as well as its close relative placental growth factor (PlGF) [23].

Our results demonstrated that administration of omega-3 only ($p = 0.000$) or combination with vitamin E ($p = 0.000$) can effectively improve endothelial function by increasing the level of VEGF. This result was in line with study by Ahmadi (2014), which concluded that the administration of omega-3 can effectively improve

endothelial function in adolescents [24]. Kemse *et al.* (2016) also proved that omega-3 supplementation is beneficial in reducing inflammation and increasing angiogenesis in rat models of hypertension [2]. Further studies need to be tested on humans.

Conclusion

Omega-3 (DHA 120 mg, EPA 180 mg) and vitamin E (α -tocopherol 300 iu) supplementation decreased ROS level and increased placental VEGF level on pre-eclampsia rats model.

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