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Submission date: 22-Mar-2022 02:55PM (UTC+0800)

Submission ID: 1789942739

File name: Omega-3_and_Vitamin_E_Supplementation_Effect.pdf (145.55K)

Word count: 4201

Character count: 22841

Omega-3 and Vitamin E Supplementation Effect to Rattus Norvegicus Placental Apoptosis Marker: Pre-Eclampsia Model

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ABSTRACT

Background: Pre-eclampsia has been associated with the increased of placental apoptosis caused by oxidative stress. Omega-3 fatty acids and vitamin E had beneficial function to maintain cell membranes, 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 BCL-2 and BAX on PE rats model.

Methods: This research has been worked 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. There were 35 pregnant rats were divided into 5 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 50mg/kg/day. At the 19th day of gestation, the pregnancy was terminated and the placental tissue was collected for examining BCL-2 and BAX levels by using ELISA kit. The data were analyzed by one way ANOVA and post hoc test LSD.

Results: The mean levels of BCL-2 in each group were K- =62.49 ng/mg, K+ =50.64 ng/mg, P1 =58.79 ng/mg, P2 =53.70 ng/mg and P3 =70.10 ng/mg. The mean levels of BAX were K- =72.06 ng/mg, K+ =78.61 ng/mg, P1 =73.71 ng/mg, P2 =74.31 ng/mg and P3 =71.63 ng/mg. The analysis results concluded that there

were significant differences between omega-3 and vitamin E supplements on BCL-2 levels ($p=0.000$) and BAX levels ($p=0.001$).

Conclusion: Omega-3 (DHA 120 mg, EPA 180 mg) and vitamin E (α -tocopherol 300 iu) supplement increased BCL-2 level and decreased BAX on pre-eclampsia rats model.

Keywords: Omega-3, Vitamin E, BCL-2, BAX, Pre-eclampsia

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. World Health Organization (WHO) estimated that pre-eclampsia rate were seven times higher in developing countries than developed countries. Pre-eclampsia prevalence in developed countries was 1.3% - 6%, while in developing countries three time higher (1.8% - 18%). The incidence of pre-eclampsia in Indonesia was 128,273/year or about 5.3% of the pregnancies (1). PE define as pregnancy syndrome that causing damage to multiorgan and diagnosed after 20th week of gestation. Clinical signs and symptoms of PE include increase or high blood pressure, protein urea, swelling, headache, visual disturbance and sudden weight gain (2). Pre-eclampsia is also

associated with poor fetal condition, including; fetal growth retardation, placental abruption, oligohydramnios and fetal death. Complications that can arise associated with PE are disseminated intravascular coagulation (DIC), cerebral hemorrhage, acute renal failure, liver damage, pulmonary edema, blindness, and seizures. PE is also closely related to cardiovascular diseases that can arise later in life such as hypertension, stroke, ischemic heart disease, and so on. The earlier the symptoms appear, the less the diagnosis is pure pre-eclampsia and have higher chance that the disease will persist into hypertension after delivery (2)(3).

Pre-eclampsia results from impaired trophoblast differentiation and invasion in early pregnancy resulting in the failure of trophoblast cells to destroy the muscularis layer of the spiral arterioles resulting in the development of a poorly perfused placenta and stimulation of a systemic inflammatory response. However, this reduced placental perfusion alone is not sufficient to cause the maternal syndrome of pre-eclampsia and it is thought that this process requires the influence of additional maternal factors including genetic make-up and environmental factors (such as obesity and diet), which together results in widespread endothelial dysfunction and hypertension (4). Placental ischemia and hypoxia produce highly toxic hydroxyl radicals. These hydroxyl radicals damage endothelial cell membranes which are rich of unsaturated fatty acids, converting them into lipid peroxides that the final product is malondialdehyde (MDA). The increase of MDA with hypoxia indicates oxidative stress (5) (6).

Oxidative damage in the placenta caused by impaired remodeling of the spiraled uterine arteries 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. These placental-

derived factors act on the maternal vascular endothelium, inducing oxidative stress and stimulating the production and secretion of pro-inflammatory cytokines, as well as vasoactive compounds (7). Oxidative stress also can induce apoptosis via extrinsic or intrinsic signals. The former are mediated by cell-surface death receptor Fas-induced caspase-8, while the latter are transmitted by mitochondria-mediated caspase-9 pathways. The two signaling pathways do not completely separate as they converge on the activation of caspase-3, resulting in apoptotic like chromatin condensation and cell shrinkage. Oxidative stress could also modify some key apoptotic regulators, such as proteins of the Bcl-2 family, p53, and components like apoptosis signal-regulating kinase-1 (ASK-1), c-JNK, and p38 MAPK (8).

The regulation of the apoptotic process varies. BCL-2 as an antiapoptotic agent will block cell death programs and maintain cell life. The increase of BCL-2 protects lymphocytes from apoptosis and allows these cells to survive for a long period. BCL-2 also has another function as an antioxidant to protect cells. The increase of BCL-2 Associated X protein (BAX) is always inversely proportional to BCL-2, which works to accelerate cell death. Anti- and proapoptotic ratios of BCL-2 family proteins determine the reaction to various apoptotic stimuli. The balance of BCL-2 and BAX also plays an important role in maintaining the balance of cell survival (9)(10).

Vitamin E, especially α -tocopherol has the main function as an antioxidant, thus acting as a chain breaking antioxidant that will capture peroxy radicals and provide phenolic hydrogen. Then, result less reactive products and stop the lipid peroxidation cycle (11). In a study to women who receive oral vitamin C in dose 500 mg and oral vitamin E in dose of 400 IU per day, there was a 46 % risk reduction in the incidence of preeclampsia in cases as compared to controls. The incidence of severe preeclampsia in cases was 2 % and in

controls was 7 %. This result showed 72 % reduced risk in the development of severe preeclampsia in cases as compared to controls (12).

Omega-3 PUFAs supplementation during pregnancy has shown 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 (13). There is a relationship between omega-3 fatty acids and apoptotic markers in the placenta of pregnant women. Women who were supplemented with docosahexaenoic acid (DHA) during pregnancy, reported, has lower placental caspase-3 levels compared to women who were not given the supplement (14).

METHOD

Design and sampling

This research was an experimental study, specifically post test only control group design. That comparing the experimental group with the control group. The sample that used for the treatment were Rattus Norvegicus wistar strain. There were 35 pregnant rats were divided into 5 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. Omega-3 (180 mg EPA and 120 mg DHA) that we

used were Omheart 300 mg from Mywell and vitamin E 300 iu from Sidomuncul that equivalent to 300 mg α -tocopherol.

Placental collection

At the 19th day of gestation, the pregnancy was terminated and the placental tissues were collected. The Placental tissues washed out with phosphate buffered saline (PBS) solution (0.02 mol, Ph 7.0-7.2) from remaining blood. The tissues were grinded out into small pieces and homogenized in PBS with a cold-temperature homogenizing glass. Amount 1.0 mg placental tissue homogenized with 9 μ l PBS solution (0.01 M, Ph=7.4). The suspension used for ultrasonication or two freeze-thaw cycles to break up the cell membrane. The suspension was centrifuged at 5000 x g for 5 minutes. Supernatants samples cell derived from placental trophoblasts stored at -20 $^{\circ}$ C before next step.

BCL-2 and BAX examination

All samples were examined by ELISA using Rat Apoptosis Bcl 2 Elisa Kit from BT-LAB catalog no E0037Ra for BCL-2 examination and using Rat Apoptosis Bcl 2-Associated X Protein Elisa Kit from BT-LAB catalog no E0034Ra for BAX. Each sample was made in duplicate to ensure the result better.

Data analyze

The data were analyzed by Shapiro-wilk, the result got was normal for normality test. Because the data got normally distributed (*p-value* = 0,252 for BCL-2 and 0,743 for BAX), so we proceed to one-way ANOVA analysis with 95% confidence degree ($\alpha=0.05$). Then analysis was continued with the LSD post hoc test.

RESULT

BCL-2 Levels

The mean levels of BCL-2 in the normal pregnant rats group (K-) was different from the group of pre-eclampsia model rats (K+). The mean level of the placental BCL-2 for normal pregnant rat

(K-) was 62.49 ng/mg, while in K+ was 50.64 ng/mg. This shows that L-NAME administration makes the BCL-2 level of the rat placenta lower.

Table 1 BCL-2 mean levels on each group based on treatment

Groups	Mean (ng/mg)	Std. Deviation	p-value
K -	62,4933	5,49184	0,000
K +	50,6417	5,35165	
P1	58,7950	5,10243	
P2	53,7017	3,49636	
P3	70,1050	3,26164	
Total	59,1473	8,17659	

The K+ group had the lowest BCL-2 mean level among the other groups, which was 50.64 ng/ml. The group of pre-eclampsia model mice that were given omega-3 (P1) appeared to have BCL-2 mean level was closed to the normal pregnancy group, while the P2 had BCL-2 mean levels not so different from the pre-eclampsia group (K+). The group that treatment with combination of omega-3 and vitamin E appeared to have the highest placental BCL-2 mean levels among the other groups (Table 1).

The results of the analysis of mean differences levels of BCL-2 placentas of pregnant rats in the treatment group and the control group obtained p value = 0.000 ($p < 0.05$). It can be concluded that there is a significant difference in BCL-2 levels of rat placenta between the treatment and control group.

BAX Levels

The mean level of placental BAX on normal pregnant rats was 72.06 ng/mg. The group that had the highest BAX mean level among the other groups was K+ group. This proves that the administration of L-NAME has an effect on the high levels of BAX in the rat placenta.

Table 2 BAX mean levels on each group based on treatment

Groups	Mean (ng/mg)	Std. Deviation	p-value
K -	72,0583	4,08301	0,001
K +	78,6067	3,05425	
P1	73,7117	1,78094	
P2	74,3150	2,27272	
P3	71,6300	1,68453	

The group of pre-eclampsia rats model that were given combination of omega-3 and vitamin E appeared to have the lowest mean BAX levels. Furthermore, the group of pre-eclampsia rat model that supplemented by omega-3 also had placental BAX mean levels that was almost close to the normal pregnant group (Table 2).

The one-way ANOVA analysis of the means difference on placental BAX levels obtained p value = 0.001. This result shown that at 5% alpha, it can be concluded that there was a significant difference between placental BAX levels between treatment and control group.

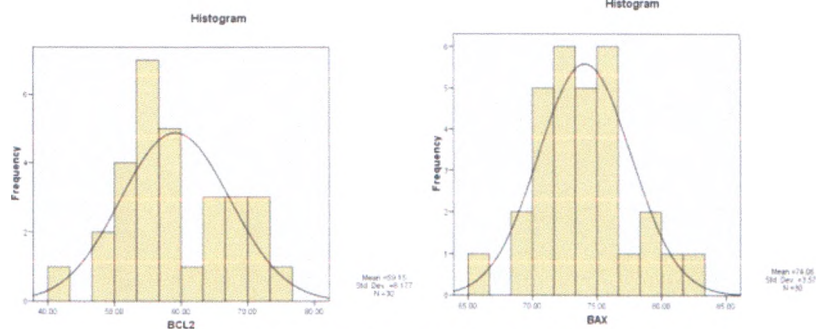


Figure 1. Frequency distribution and normal curves of BCL-2 and BAX

DISCUSSION

Programmed cell death (apoptosis) plays an important role during the development of the placenta and in the

regulation of its ageing during pregnancy. The regulation of programmed cell death is complex; both proapoptotic (stimulating programmed cell death) and antiapoptotic

(inhibiting programmed cell death) genes are involved. Members of the Bcl-2 (B cell lymphoma 2) protein family play a prominent role in this complex regulatory system (15). The B-cell lymphoma/leukemia (Bcl-2) is an antiapoptotic protein that regulates apoptosis by preventing cell death. PE is often accompanied by hypoxia of the placenta, and this condition induces apoptosis in trophoblastic cells. Hypoxia-induced apoptosis accompanied by decreased BCL-2 expression (16) BCL-2 from antiapoptotic group, and BAX from proapoptotic group had the strongest biological effects. BCL-2 is located on the outer mitochondrial membrane and inhibits the action of pro-apoptotic proteins such as BAX (16)(17).

The increased of BAX expression while the absence or decrease BCL-2 triggers an apoptotic cascade reaction. The cascade is initiated by the release of mitochondrial cytochrome-c into the cytosol and interacts with Apaf-1 to form an apoptosome that binds and activates procaspase-9. Activation of caspase-9 cleaves its effectors; caspase-3, caspase-6 and caspase-7. Effector caspases then cleave many substrates in cells and lead to characteristic morphological changes in apoptosis (18).

In this study, the BCL-2 mean level in pre-eclampsia rats model was lower or significantly different from normal pregnant rats. Omega-3 supplements (p-value = 0.005) and the combination of omega-3 and vitamin E (p-value = 0.000) were associated with higher levels of BCL-2 placentas in pre-eclamptic rat models compared to the control group. While the administration of vitamin E only, had no effect (p-value = 0.264). Omega-3 and vitamin E supplementation only, or in combination, had the same effect on rat placental BAX levels.

Omega-3 PUFAs has the potential effect to inhibit oxidative stress by increasing ROS binding capacity and limiting ROS regeneration. Omega-3

supplementation also increase mRNA expression and activity of major antioxidant enzymes in the placenta and reduced placental oxidative damage (13). Cardiolipin (CL) is a unique phospholipid found in the inner mitochondrial membrane (IMM), where CL associated with several enzymes required for oxidative phosphorylation, including cytochrome c. Since mitochondria is the main source of intracellular ROS, especially in complexes I and III, CL directly affected by relatively high ROS concentrations in mitochondrial stress. In addition, CL also contains four unsaturated fatty acyl chains, making it more susceptible to oxidation. CL-bound cytochrome c also serves as a cardiolipin-specific peroxidase that can oxidize CL in an H₂O₂-dependent manner. After oxidation, CL dissociates from enzymes in the mitochondrial inner membrane (IMM). On the other hand, tBID also targets the mitochondrial outer membrane due to its binding affinity to CL and induces BAX oligomerization which ultimately leads to pore formation and cytochrome c release (19). EPA supplementation not only efficient lengthened the Cardiolipin (CL) chain but also increased CL unsaturation (20).

Omega-3 fatty acids has anti-inflammatory and anti-apoptotic effects through the activity and regeneration of endogenous antioxidant mechanisms (21). In pre-eclampsia, the reduced ability of the antioxidant system to overcome lipid peroxidation products (oxidants; free radicals) causes 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 (22).

All vitamin E forms are potent antioxidants, as they scavenge lipid peroxyl radicals by donating hydrogen from the phenolic group on the chromanol ring (23). Vitamin E mainly protects polyunsaturated fatty acids (PUFA) in membrane phospholipids and plasma lipoproteins. Peroxyl radicals react 1000 times faster with

vitamin E than with PUFAs (24). Vitamin E also effective to intervened apoptosis through decreasing caspase-3 expression, upregulate Bcl-2 expression and alleviate DNA oxidative damage in bone marrow hemopoietic cells at the early stage of steroid-induced femoral head necrosis in rabbit models (25).

Omega-3 fatty acids are sensitive to oxidation, so they are likely to work better with vitamin E than on their own. Previous studies have shown that the combination of omega-3 fatty acids and vitamin E intake has a strong synergistic effect on glucose-related markers and fatty acid metabolism which may further work more effectively on biomarkers of oxidative stress and inflammation. Omega-3 fatty acids and vitamin E co-supplementation for 6 weeks in women with GDM had beneficial effects on plasma TAC, MDA and NO and on the incidence of the newborns hyperbilirubinemia (26).

It is important to consider, Rumbold et al., (2015) at their research said that there are no data showing that vitamin E supplementation or in combination with other supplements is beneficial for preventing stillbirth, neonatal death, premature birth, preeclampsia and PROM. There is no convincing evidence that vitamin E supplementation in combination with other supplements produces any other significant benefit or harm. In contrast, women who were given vitamin E supplements alone or in combination with other supplements had an increased risk of premature rupture of membranes (PROM) when compared to women given placebo controls. Further research is needed to elucidate the possible role of vitamin E in pregnancy (27).

CONCLUSION

Omega-3 (DHA 120 mg, EPA 180 mg) and vitamin E (α -tocopherol 300 iu) supplementation increased BCL-2 level and decreased BAX level on pre-eclampsia rats model.

ACKNOWLEDGMENT

Special thank for all examiner, Dr. Desmawati, M.Gizi, Dr. Andani Eka Putra, M.Sc. and Dr. Firdawati, M.Kes Ph.D. for their contribution to improve this research. Some of this research funding was provided by Human Resources development and Empowerment Agency, Indonesian Ministry of Health.

Conflict of Interest: None

Source of Funding: None

Ethical Approval: Approved

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Yesi Gusti et.al. Omega-3 and vitamin E supplementation effect to rattus norvegicus placental apoptosis marker: pre-eclampsia model.

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How to cite this article: Gusti Y, Amir A, Revilla G. Omega-3 and vitamin E supplementation effect to rattus norvegicus placental apoptosis marker: pre-eclampsia model. *International Journal of Science & Healthcare Research*. 2021; 6(4): 261-268. DOI: <https://doi.org/10.52403/ijshr.20211037>

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