

ARTICLE

RELATIONSHIP BETWEEN BCL-2 EXPRESSION AND APOPTOSIS INDEX ON RAT (*RATTUS NORVEGICUS*) MODEL OF PREECLAMPSIA AFTER ADMINISTRATION OF EVOOIrianti Evi^{1*}, Ilyas Syafruddin¹, Rosidah², Salomo Hutahaean¹¹Departement of Biology, University of Sumatera Utara, Medan, INDONESIA²Department of Pharmacy, University of Sumatera Utara, Medan, INDONESIA

ABSTRACT

One cause of preeclampsia is due to an imbalance between antioxidants and free radicals, due to the failure of spiral artery remodeling, resulting in placenta ischemia and hypoxia. The occurring hypoxia mediates mir210 to induce the expression of the antiapoptotic Bcl-2 (B-cell lymphoma-2) target gene that may reduce excessive apoptosis. This study aims to prevent this imbalance by giving extra virgin olive oil (EVOO) rich in tocopherol content (vitamin E). The design of this study is pre- and post-test with control group design in the laboratory. Subject is a female rat, Sprague Dawley strain, weight \pm 200g. The control and treatment groups consisted of a total sample of 25 individuals. Bcl-2 expression increased significantly, especially in the treatment group that received EVOO (P value 0,009). Apoptosis index tended to decrease in treatment group that received low and moderate dose of EVOO, but there was no significance (P value 0.332). Conclusions: EVOO was able to increase Bcl-2 to prevent excessive apoptosis on preeclampsia. It is recommended to examine the expression of p53 or Bax protein to find out which protein regulates apoptotic.

INTRODUCTION

Maternal Mortality Rate (MMR) is an important indicator of public health status. Maternal mortality can be caused by several factors, including bleeding, preeclampsia, and infection [1]. In 2012, Indonesia is still one of the countries in Southeast Asia with the highest MMR, 359 / 100.000 live birth and about 27% of the cause is preeclampsia, about 13,500 persons per year¹. Preeclampsia is a syndrome in early pregnancy, characterized by gestational hypertension and proteinuria (although current diagnosis of preeclampsia does not depend on proteinuria), occurring after 20 weeks of gestation, and is one cause of maternal death [2-4].

One of the major causes of preeclampsia is an imbalance between antioxidants and free radicals, due to the failure of spiral artery remodeling. Failure of spiral artery remodeling results in the placenta having ischemia and hypoxia [5]. The occurring hypoxia mediates m RNA210 to induce the expression of the anti-apoptotic Bcl-2 (B-cell lymphoma-2) target gene to reduce excessive apoptosis [6]. In addition, placental hypoxia with preeclampsia can lead to apoptosis, especially through the intrinsic pathway of mitochondria [7]. Another study revealed, in preeclampsia, there was an increase in index apoptosis [8]. Preeclampsia causes excessive apoptosis and decreased expression of Bcl-2[7]. Another study revealed the increase in apoptosis followed by decreased expression of Bcl-2 and Bcl-xl proteins in severe preeclampsia pregnancies compared to normotensive pregnancies [9]. Therefore, it is necessary to prevent preeclampsia, and one method is by giving antioxidant [10]. One type of antioxidant known is extra virgin olive oil (EVOO), rich in antioxidant content of tocopherol [11].

Another benefit of EVOO is to protect hepatic tissue from damage by oxidation by preventing lipid peroxide activity by increasing the formation of MUFA (monounsaturated fatty acid) and maintaining serum marker enzymes, as well as the activity of hepatic antioxidant enzymes at concentrations close to normal. In addition, the hydrophilic fraction, part of the olive oil proved to be effective in reducing oxidative stress and, in this extra hydrophilic case, it potentially has a direct antioxidant effect on hepatic cells[11]. Other studies have found that eating EVOO three times a day can reduce oxidative stress in the pancreas [12].

Based on the above description, the benefits of EVOO as an antioxidant have been known, but we know of no research on cases of preeclampsia. Based on the consideration of ethics and safety of materials used on mothers and fetuses, difficulty getting volunteers, as well as external factors (e.g., nutrients) that are not easily controlled, it is necessary to model the experimental animals of white rats (*Rattus norvegicus*) in this study[13]. Therefore, we are interested to know the relationship between Bcl-2 expression and apoptosis on placenta cells. The purpose of this research is to know the description of Bcl-2 expression and apoptosis index, as well as the relationship between of them. Hypothesis: The study found an increase in mean expression of Bcl-2 and apoptosis index in placenta after administration of EVOO. Novelty of this research: As far as we know, there is no research about the effect of giving EVOO on Bcl-2 expression on white rat (*Rattus norvegicus*) and its contribution to prevention of oxidative stress occurring in preeclampsia, so it is expected to give knowledge about alternative prevention of preeclampsia.

KEY WORDS

EVOO, preeclampsia, Bcl-2, apoptotic index

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MATERIALS AND METHODS

The location of the research was conducted in the veterinary laboratory of pharmacology department and therapy of Medical Faculty of Padjadjaran University Bandung and Anatomy Pathology Laboratory of Medical Faculty University of Sumatera Utara Medan (permit and implementation of attached study). Before the research was conducted, a permission letter of ethical clearance from the animal research ethics committee of FMIPA USU was issued. The type of research is true experiment done in a laboratory with pre- and post-test design with control group design.

Materials and tools of research at preparation of white female rat (*Rattus norvegicus*) strain of Sparague Dawley counted 25 tails, eight male rats (1: 3 or 4 mating ratio), 8-11 weeks old and body weight \pm 200 gram (addition or a reduction in the weight range of about 10%), mice in healthy condition, marked with the presence of fluff (not standing); the movement is quite agile and does not show physical disability. Given standard AIN93-M food and enough drinks. The implementation of the study was performed by injecting NaCl 6% sterile 3cc daily, starting from 6th to 12th days of pregnancy in subcutaneous and intramuscular to obtain preeclampsia model animals in the treatment group, sleeve or paralon pipe (rat body size) for 30 minutes only once on the 19th day of pregnancy. The required material at the time of the research intervention was EVOO, and the tool used was a non-invasive sphygmomanometer to measure increase in blood pressure. If blood pressure increased after giving treatment, it means the animal's sustained preeclampsia.

The observation of apoptotic cells using DNA-fragmented techniques. The materials used are TUNEL kit, PBS solution pH 7.4, H₂O₂ 3%, DAB (Diamino Benzidine), Peroxidase solution, Mayer Hematoxilen. The tool used a light microscope. Observation of Bcl-2 expression of the required material Tris Buffered Saline (TBS) pH 7.4, Primary TissueTM, peroxide block, Bcl-2 primary antibody Monoclonal Mouse Antibody IgG2B Clone # 625509 (catalog # MAB8272) with species reactivity Human / Mouse / Rat from R & D SYSTEMSbiotechne, PolyVue PlusTM Enhancer, PolyVue PlusTM HRP (Horseradish-peroxidase) reagents, DAB / PlusTM, Mayer Hematoxylin. The tools used are PT Link Dako Epitope Retrieval, Pap pen, cover glass, and microscope. The experimental animals consisted of 5 groups, each group consisting of 5 white rats. The group was divided into the control group (P0), and the treatment consisted of P1: preeclampsia model, P2: preeclampsia model + dosage of EVOO: 0.38 mL / kg BW, P3: preeclampsia model + dosage of EVOO 0.76 mL / kg BW, P4: model preeclampsia + dosage of EVOO 1.52 mL / kg BW. The data collected were analyzed by Anova or Kruskal Wallis statistical test and Spearman correlation.

RESULTS

The results of Bcl-2 expression of placenta tissue can be seen in [Fig. 1] below:

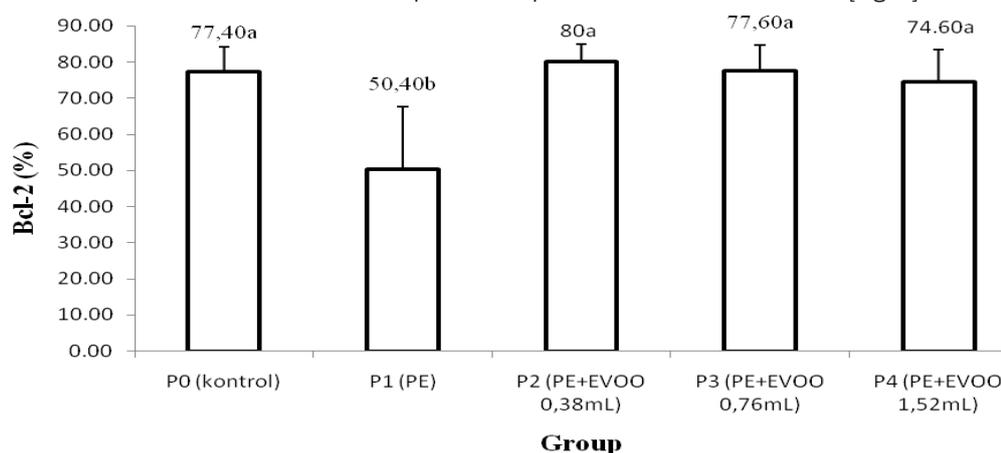


Fig.1: Effect of EVOO on protein expression of Bcl-2 placenta tissue of pregnant white rat. The same small letters show no significance ($P > 0.05$).

Based on [Fig.1], it is known that, after administration of EVOO, there is a significant difference in Bcl-2 protein expression between control and treatment groups (Kruskal Wallis test, $P = 0.020$). In this case, EVOO affects the increased expression of Bcl-2 protein in the treatment group that received it. Increased expression of Bcl-2 protein in the treated group with EVOO was similar to that of control, but not in P1 (group not given EVOO), which was lower. This is evident from Mann Whitney test results; the expression of Bcl-2 protein in P1 was significantly different from other groups. Interestingly, in this study, increased expression of Bcl-2 protein in the P2 group was quite high (strongly positive) in almost all placental tissues compared to the other groups. The P1 group of Bcl-2 protein expression was moderately positive.

Observation of apoptotic cells in the placenta tissue immunohistochemically using TUNEL kit. Apoptotic cells are calculated to determine the apoptosis index. The tissue used was the placenta stored in paraffin blocks. Calculation of apoptosis index was qualitative then converted to semi quantitative based on predetermined criterion [14], the result as shown in [Fig. 2] below:

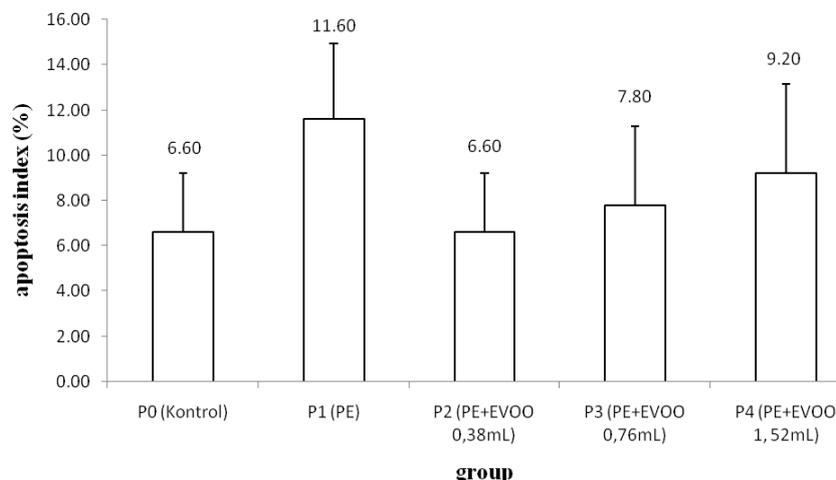


Fig. 2: The effect of EVOO on apoptosis of the placenta index of control and treatment groups.

DISCUSSION

Bcl-2 expression indicated significant difference in Bcl-2 protein expression in the normal group and preeclampsia. In Preeclampsia, there is decreased expression of Bcl-2 protein [8, 15]. Other researchers also reported that, in Preeclampsia, there was a decreased expression of Bcl-2 protein in placental cell syncytiotrophoblasts, resulting in delayed fetal growth. This is due to the failure of spiral artery remodeling that induces free radicals in the mitochondrial membranes of the placental cells, resulting in a decrease in the expression of Bcl-2 protein leading to apoptosis [7]. The expression of the Bcl-2 and Bcl-xl genes is lower in severe preeclampsia pregnancies than in normotensive pregnancies [9].

We suspect the α -tocopherol content in EVOO may suppress p53 activation. It is known that the increase in Bcl-2 expression is thought to be due to p53 inactivation, thus preventing cytochrome c release with Apaf1 and ATP to cause apoptosome not to occur. This causes caspase 9 not to be activated, resulting in apoptotic resistance [16, 17]. Other studies have shown the administration of α -tocopherol ointment on the back of mice after exposure to UV radiation can reduce 55% of the formation of cyclobutane pyrimidine p53 gene dimer that plays a role in the pathogenesis of squamous cell carcinoma [18]. In addition, other researchers stated the combination of vitamin E and exercise was able to suppress free radicals that can damage the DNA production of the p53 gene, so over expression can be suppressed in rat prostate gland tumors [19].

The results of observation on placental cells and the calculation of index apoptosis in this study are not different. Another study shows, in preeclampsia, apoptosis index is higher than the normal pregnancy¹⁵. Meanwhile apoptosis of placenta index of preeclampsia was higher than normal pregnancy, also causing complication of low birth weight of fetus about 14,3% [8].

The process of apoptosis in a normal pregnancy plays a role in the replacement of the cytotrophoblast and renewal of the syncytium surface from the corral villi. The process of apoptosis occurs in syncytiotrophoblast and the cytotrophoblast. This process of increase occurs as the age of pregnancy increases because of decreased expression of Bcl-2 protein that inhibits apoptosis. Therefore, if high levels of Bcl-2 protein expression in syncytiotrophoblast prevent apoptosis in trophoblastic [8], it is suspected the apoptosis index does not differ between groups because apoptotic cell count is performed at the end of pregnancy of rat.

Apoptosis in physiological conditions serves to regulate cell numbers, proliferation, and eliminate cells that are no longer useful as a normal development of cells, such as in embryogenesis, hormone-dependent involution in the menstrual cycle and follicular atresia in menopause, cell deletion in epithelial cell proliferation, excessive reactive cell lymphocyte elimination, cell death induced by cytotoxic T cells in viral infection and tumor progression [20]. Apoptosis is an important process in both normal tissue development and tissue homeostasis in adults, including immune system regulation, for example in T cell lymphocytes, which are cellular immune systems responsible for destroying damaged or infected cells in the body. T lymphocytes undergo maturation in the thymus gland, but before entering the bloodstream, it will be tested to ensure the cell is effective against reaction to normal cells. If there are ineffective or self-reactive T lymphocytes, they will be excluded by apoptosis [21].

However, in pre-eclampsia, immunologic adaptation fails, so the conception persists, but the trophoblast cells are unable to invade the spiral artery to dilate, so the blood vessel tone remains high and

vasoconstriction occurs. This condition causes maternal blood vessels to be unable to produce adequate of blood circulation, so that ischemia will occur and stimulate the occurrence of apoptosis placenta⁸. However, apoptosis is not only due to ischemic placenta, but hypoxia can also be the trigger of apoptosis through the mechanism of cytokines, such as TNF α or Fas ligand, which will activate caspase 8 and caspase 9 as initiators of apoptosis. Caspase 3 and caspase 6 are executors. It is known that free radicals often play a role in the occurrence of preeclampsia but does not entirely cause an increase in apoptosis[22]

We suspect the increase in apoptosis is strong in the P1 group because of the involvement of oxidation reactions that occur due to preeclampsia. This is evidenced by the results of plasma MDA levels at the end of pregnancy in the P1 group, which is higher than other groups. MDA is the end product of lipid peroxide, a free radical. These free radicals will attack cell growth, including DNA, unsaturated fatty acids (PUFAs). When free radicals react with PUFA in mitochondrial cell membrane, the structure and function become damaged. The researchers' allegations are consistent with the opinion that mitochondria play a role in regulating apoptotic processes in preeclampsia [15]. Apoptosis may occur in trophoblast cells involving mitochondria via one of the apoptotic pathways (CD95) or Fas receptor (incorporated in TNF Receptor family) and Fas ligand, referred to as TRAIL (TNF Receptor Apoptosis Inducing Ligand) apoptosis and is called the Extrinsic pathway or the Death Receptor Pathway, but this is not part of the Mitochondrial pathway[15].

Different opinions describe that apoptosis in PE occurs as a result of increased expression of Bax (Bcl-2 family) proteins in intracellular trophoblast cells, which is a proapoptosis protein that interacts with Bcl-xl or binds directly to the outer membrane of the mitochondria, resulting in cytochrome-c release, which will coincide with Apaf-1, procaspase-9, and ATP form the apoptosome, which will activate the path of internal apoptosis [20]. It is known that Bax activated by Bid causes Permeability Transition Pore (PTP) in the outer membrane of mitochondria to become open so that cytochrome-c exits (leaks) resulting in apoptosis. Proteins (Bcl-2 and Bcl-xl) prevent this PTP from occurring, suggesting increased expression of Bax proteins in apoptosis due to preeclampsia provides evidence of mitochondrial involvement in apoptosis¹⁵. Another opinion [15] is that Bax protein activation only does not induce apoptosis, but in this case, p53 also plays a major role in heavy preeclampsia in some trophoblast cells, so the apoptosis index is higher than that of normal pregnancy. However, in this study, there is no known expression of p53 and Bax, so the expression of proteins that play a role in inducing apoptosis is not clearly known [15].

In addition, α tocopherol in EVOO is significantly capable of inhibiting MGO (methyl glyoxal), whose reaction is very high in glucose metabolism and has been known to cause damage and induce apoptosis in endothelial cells. This inhibition of MGO is able to change the production of ROS in intracellular, resulting in increased expression of Bcl-2 protein and decreased Bax protein expression, which can prevent cell apoptosis from widening [23]. This is evidenced in [Fig. 3] above that there is a strong relationship between Bcl-2 protein expression and apoptosis. In addition, α tocopherol in EVOO can attack lipid peroxide, resulting from the reaction between lipids and free radicals in mitochondrial cell membranes that will protect and prevent further damage to the placental endothelial cells.

CONCLUSION

Administration of EVOO may increase Bcl-2 expression in the preeclampsia model. In addition, the apoptotic index of white rat in the preeclampsia model group was in grade III (++) , stronger / higher when compared with treatment group not given EVOO. It is recommended to examine the expression of p53 or Bax protein to find out which protein regulates apoptosis.

CONFLICT OF INTEREST

No conflict of interest

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REFERENCES

- [1] Kemenkes RI. [2013] Profil Kesehatan Indonesia 2012. Pusat Data dan Informasi Kemenkes RI, Jakarta.
- [2] Robert JM, Judith L, Lisa MB, Jose MB, Eduardo B, Anibal M. [2003] Nutrient Involvement in Preeclampsia. *The Journal of Nutrition*. 133:1684-1692.
- [3] Yusniar. [2004] Faktor Risiko Kejadian Preeklampsia dan Eklampsia di RSUD Labuan Baji Makassar. Skripsi. Universitas Hasanuddin, Makassar.
- [4] Matsubara K, Higaki T, Yuko M, Akihiro N. [2015] Nitric Oxide and Reactive Oxygen Species in the Pathogenesis of Preeclampsia. *Int J Mol Sci*. 16: 4601- 4614.
- [5] Chen bao D, Wang W. [2013] Human placental microRNAs and preeclampsia. Mini review. BOR Papers in Press. Vol.10.
- [6] Chio CC, Lin JW, Cheng HA, et al. [2013] MicroRNA-210 targets antiapoptotic Bcl-2 expression and mediates

- hypoxia-induced apoptosis of neuroblastoma cells. *Archives of Toxicology*. 87:458-468.
- [7] Ishihara N, Matsno H, Murakoshi H, Fernandez JB, Sannoto T, Maruo T. [2002] Increased apoptosis in syncytiotrophoblast in human term placenta complicated by either preeclampsia or intrauterine growth retardation. *Am J Obstet Gynecol*. 186:158-166.
- [8] Teguh M, C Johannes Mose, Jusuf SE, Betty SH. [2010] Perbedaan indeks apoptosis plasenta antara preeklampsia dan kehamilan normal serta hubungannya dengan berat badan lahir dan tekanan darah ibu, *Majalah Kedokteran Bandung*, 42(1): 1-5.
- [9] Arianto B, Rumekti HD, Detty SN. [2015] Perbandingan rerata ekspresi Bcl-2 dan Bcl-xl pada preeklampsia berat dan kehamilan normotensi. *IPAKESPRO*, 2:122-126.
- [10] Takiuti NH, Kahhale S, Zugaib M. Stress in pregnancy: a new wistar rat model for human preeclampsia. *Am J Obstet Gynecol*. 3: 544-550
- [11] Nakbi A, Tayeb W, Abir G, et.al. [2010] Effects of olive oil and its fractions on oxidative stress and the livers fatty acid composition in 2,4 Dichlorophenoxyacetic acid-treated rats. *BioMed Central*. 7: 1-11.
- [12] Lopez MJ0, Berna G, Everado MC, Hermina LG, Franz M M Carmen L.[2008] An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in Of1 Mice. *The Journal of Nutrition*, 138:1074-1078.
- [13] Kusumawati D. [2004] *Bersahabat Dengan Hewan Coba* (:8-50). Gadjah University Press, Yogyakarta.
- [14] Kokawa K, Shikone T, Otani T, et al.[2001] Apoptosis and the expression of Bax and Bcl-2 In hyperplasia and adenocarcinoma of the uterine endometrium. *Hum Reprod* 16:2211-2218
- [15] Keman K, Prasetyorini N, Langgar MJ. [2015] Perbandingan eskpresi p53, Bcl-2 dan indeks apoptosis trofoblas pada preeklampsia/eclampsia dan kehamilan normal. *Maj Obstet Ginekol Indones*, 33:151-159.
- [16] Kirkin S, Joos M, Zornig. [2004] The role of Bcl-2 family members in tumorigenesis. *Biochim Biophys Act*. 1644: 229-249.
- [17] Skommer JT, Brittain S, Rayhaudhuri. [2010] Bcl-2 inhibits apoptosis by increasing the time-to-death and intrinsic cell -to-cell bariations in the mitochondrial pathway of cell death. *Apoptosis*. 15:1223-1233.
- [18] Weixing Chen, Margaret B, Jessie M, David A and Helen L Gensler. [2009] Inhibitions of cyclobutane pyrimidine dimer formation in epidermal P53 gene of UV- irradiated mice by α tocopherol. *Journal nutrition of cancer*. 29(3): 205-211.
- [19] Dashtiyani Amin A, Sepehrimanesh M, Nader T, Mohammad EA. [2017] The effect of endurance training with and without vitamin E on expression of p53 and PTEN tumor supressing genes in prostate glands of male rats. *Biochimic open*. 4 : 112-118.
- [20] Sujatmiko T, Rumekti H Diah, Betty SN. [2015] Perbandingan rerata ekspresi protein Bax dan Bak pada preeklampsia berat dan kehamilan normotensi, *jurnal kesehatan reproduksi (IPAKESPRO)*, Vol:2.3.
- [21] Hermawan AG. [2012] Mekanisme apoptosis pada sepsis. *Majalah Kedokteran Terapi Intensif*. 02 : 26-32
- [22] Levy R. [2005] The role of apoptosis in preeklampsia. *IMAJ*. 7:178-181.
- [23] Moon ho Do, Su nam Kim, Seung-Yong Seo, Eui-Ju Yeo, Sun Yeou Kim. [2015] δ - Tochoferol prevents methylglyoxal-induced apoptosis by reducing ROS generation and inhibiting apoptotic signaling cascades in human umbilical vein endothelial cells, *Food Funct*. 6:1568-1577.