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The Effect of Extra Virgin Olive Oil (EVOO) on Expression of Vascular Endhothelial Growth Factor (VEGF) and Arteriole Number on Endometrium of Female *Rattus norvegicus* of Wistar Strain Exposed to Rhodamin B

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ABSTRACT

Rhodamin B is dangerous for body due to its ability to improve oxidative stress on various reproduction tissues on female. However, there is no significant scientific proof that Rhodamin B exposure influences VEGF and the number of arteriole on endometrium. VEGF and arteriole is crucial to angiogenesis process of endometrium. This study aimed at finding out the effect of EVOO on Expression of VEGF and Arteriole number of female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B. This was a true experimental study with Post-test Only Control Group Design. Independent variable was EVOO and dependent variable was VEGF expression and Arteriole number. Data were analysed using independent sample t- test, One Way Anova test, LSD test, Spearman rho test and linear regression test. The result showed a significant effect of Rhodamin B exposure to VEGF expression ($p=0.009<\alpha$) and arteriole number ($p=0.005<\alpha$) and a significant effect of EVOO administration on VEGF expression ($p=0.000<\alpha$) and arteriole number ($p=0.000<\alpha$) on endometrium of Rattus norvegicus of Wistar Strain exposed to Rhodamin B. EVOO might be alternative to prevent oxidative stress due to Rhodamin B exposure.

Keywords: Rhodamin B, Extra Virgin Olive Oil (EVOO), Vascular Endothelial Growth Factor (VEGF), Arteriole

INTRODUCTION

Background

In Indonesia, Rhodamin B are mostly used for many types of food-stuff such ice cream, chips, traditional cake, terasi and candies[(1)] and also for cosmetics[(2)]. Rhodamin B belongs to organochlorin xenobiotics group, which cannot be secreted well during its metabolism process. Therefore, once accumulated, it might cause cytotoxic and cell death[(3)]. It has quinone-like structure which is an highly redox active molecule and is able to form ROS causing oxidative stress which in turn result in peroxidation and in the end, causes damage to the cell, tissue or induces certain type of disease[(4,5)].

Based on data of previous study, it was found out that Rhodamin B exposure for 36 days with dosage of 4,5, 9 and 18 mg/200 g body weight might induce oxidative stress on cervix and improve cell proliferation on cervix epithelium[(6)]. In addition, on ovary tissue of rattus norvegicus exposed to Rhodamin B, we know that there was an improvement of Malondyaldehide content (MDA) as precursor of peroxidase[(5,7)]. The reduction of Superoxide Dismutase (SOD) content which is an endogenous antioxidant functions to neutrelaize excessive

oxydan inside out body. Besides, Rhodamin B exposure might reduce the number of primary follicle, secondary follicle and *Dee Graaf Follicle* and reduce the level of β-Estradiol[7].

The reduction of Estrogen level or 17 β-Estradiol influences normal development of female reproduction especially menstrual process. One of the important role of 17 β-Estradiol in menstrual cycle is to influence proliferation process on endometrium by regulating Vascular Endothelial Growth Factor (VEGF) functioning to induce the improvement of permeability of micro blood vessel and angiogenesis. Low 17 β-Estradiol also influences VEGF regulation which in turn, prevent proliferation process for the cycle in endometrium[8]. Estrogen induces transcription process of VEGF Gen A and stabilizes mRNA VEGF-A and prolongs half-transcript. VEGF plays crucial role in inducing permeability improvement of micro blood vessel and angiogenesis[9]. The development of blood vessel on proliferation phase occurs on all functionalist layer under the influence of estrogen. Arteriole spiralis also develops on fungisionalist layer[10].

Vascular Endothelial Growth Factor (VEGF) is one of the most important aniogenic factors and plays crucial role in physiology and pathology angiogenesis[11]. Artery spiralist functions to flow blood from two third of endometrium superficial tissue[12]. However, there has been no proof on the effect of Rhodamin B exposure on VEGF and arteriole number on endometrium.

Among various type of negative effect caused by oxidative stress resulting from Rhodamin B exposure, body need neutralizing compound to minimize negative effect resulting from imbalance number of prooxidant and antioxidant in our body and neutralizing excessive increase of ROS in our body. One of the natural material used as outside antioxidant and ROS neutralizer is Extra Virgin Olive OIL (EVOO).

In model study of in vitro and ex vivo, phenolat content in EVOO has antioxidant nature which is higher than that in E vitamin on lipid oxidase and DNA. It is also able to prevent dysfunction of endothel by reducing expression molecule of cell adhesive, increasing nitrate oxidase nitrate (NO) and inducing NO synthesis by removing free radicals of intracellular endothelium. Beside that, phenolic compound of olive oil impedes induction of platelet aggregate and improves mRNA transcription of antioxidant, anti glutathione peroxidase[13].

Other studies shows that phenolic content in olive oil gives beneficial effect to cope with oxidative stress which might be seen as sign of oxidative stress (F2-isopostan, Lipid peroxidase (LPO),oxidased gluthathione (GSSG), glutathione (GSH) and reduced gluthathione peroxidase (GSH-Px)[14]. Phenolate in olive oil is able to reduce production of reactive oxygen species (ROS) and produce significant reduction effect of free radicals[15].

Antioxidant function in pure olive oil is known from biological activities that is able to delay oxidation process. In this case, main antioxidant in Extra Virgin Olive Oil hindering oxidation process is phenol content in olive oil, functioning to interrupt the cycle by balancing radicals hydrogen on alchilperoxil radical, resulting from lipid oxidation and forming stable derivatives during reaction[16].

Purpose

This study aims at proving the effect of Extra Virgin Olive Oil (EVOO) on Expression of Vascular Endothelial Growth Factor (VEGF) and arteriole number on endometrium of female Rattus Norvegicus of Wistar strain exposed to Rhodamin B.

METHODS

Research design used was *true* experimental with *Posttest Only Control Group Design.* Sample used was 25 female *Rattus Norvegicus of Wistar Strain* which are divided into 5 groups consisting of 5 rats each. They were 1) negative control/NC (rats which is not exposed to Rhodamin B and given EVOO), 2) Positive Control/PC (rats exposed to Rhodamin B with dosage of 18 mg/200 g body weight/day without EVOOO, 3) treatment 1/P1 (rats exposed to Rhodamin B with dosage of 18 mg/200 g body weight/day + EVOO 1,5 ml/kg body weight/day, 4) treatment 2/P2 (rats exposed to Rhodamin B with dosage of 18 mg/200 g body weight/day+EVOO 3 ml/bodyweight/day, 5) treatment 3/P3 (rats exposed to Rhodamin B with dosage of 18 mg/200 g body weight/day + EVOO 4,5 ml/Kg Body weight/day.

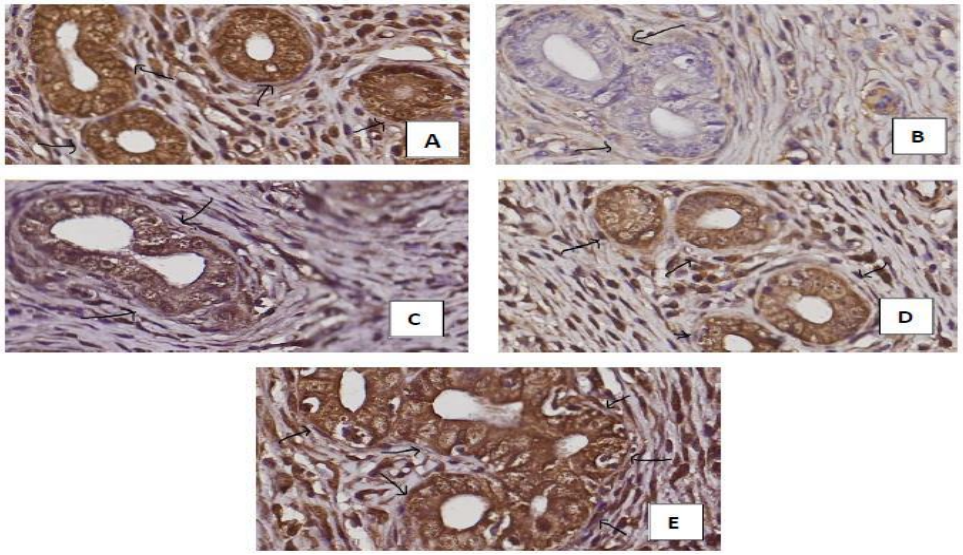
The treatment are given for 36 days and ended by synchronizing estruses phase before cutting open the rats. The cutting open of rat are conducted on proestruse phase. The independent variable used is administration of Extra Virgin Olive Oil (EVOO) and its dependent variable is expression of *Vascular Endothelial Growth Factor (VEGF)* and arteriole number. VEGF expression is seen on endometrium layer on uterus preparation cut longitudinally and inked IHK sing antobody of *Rabbit Anti-VEGF Polyclonal Antibody, Unconjugated* of Bioss of catalogue number bs-1665R and counted using Hsel software based on number of cell showing gland epithelium cytoplasm and brown-coloured stroma cell in 10 wide vista viewed under microscope of 400 times enlargement[17]. Arteriolee number are counted on endometrium tissue preparation cut longitudinally and HE colored with 400 enlargement in 10 wide vista using microscope. Counting scale of the two variables is ratio scale[17].

The data are analysed statistically with trust degree of 95% (p<0,05) using the following stages: (1) parametric requirement test which is normality test of sample data using *Shapiro-Wilk* test for ratio scale data and small scale sample and *Levene Test* to test data homogeneity, (2) independent Sample t Test (3) One Way Test (F test) (4) Multiple Comparison Test using LSD (*Least Significant Different*), (5) *Spearman's rho* correlation test and (6) Simple Linier Regression Test. All of them are conducted using *SPSS software for Windows 23.*

RESULTS

VEGF expression on Endometrium of Female *Rattus Norvegicus of Wistar Strain*

VEGF expression was counted using Hsel software based on the number of cell showing cytoplasm of gland epithelium celland brown-colored stromacell in 10 Wide vista and then the results were counted for average. VEGF expression is shown in Figure 1.



Note: (A) VEGF expression in healthy mice (NC), (B) VEGF expression appears negative in the exposure group Rhodamin B (PC), while (C) / P1, (D) / P2 and (E) / P3 respectively each showing a VEGF expression that looks higher than (B) / PC. VEGF expression on P3 is almost the same as NC.

Figure 1: VEGF expression on Endometrium of Female *Rattus Norvegicus* of Wistar Strain with IHK coloring and observed under microscope of 400 times enlargement

Average of VEGF expression for each group of study is shown in the following histogram (Figure 2).

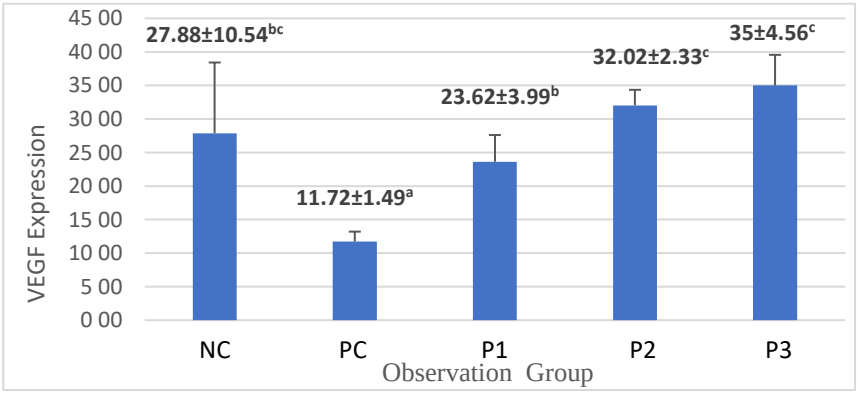


Figure 2: Average of VEGF expression

Based on data on figure 2, it was found out that the highest average of VEGF expression was on P3 group and the lowest was on Positive control Group (PC). Average of VEGF expression was increasing on P1, P2 and

P3 group compared to PC. The increase on VEGF expression occurs simultaneously with the increase of dosage of *Extra Virgin Olive Oil (EVOO)* given. In addition, EVOO dosage which was considered to be the best in increasing VEGF expression on endometrium was 3 ml/kg bodyweight/day.

In order to prove whether Rhodamin B exposure was able to influence VEGF expression in endometrium of female *Rattus Norvegicus of Wistar Strain* exposed to B Vitamin or not, we employed independent sample t test because it had fulfilled parametric requirement test (data were distributed normally and homogeneous). The comparison result of NC (negative control) and PC (positive control) is $p=0.009<\alpha$. It proved that Rhodamin B exposure gave significant effect on VEGF expression on Endometrium of Female Rattus Norvegicus of Wistar Strain.

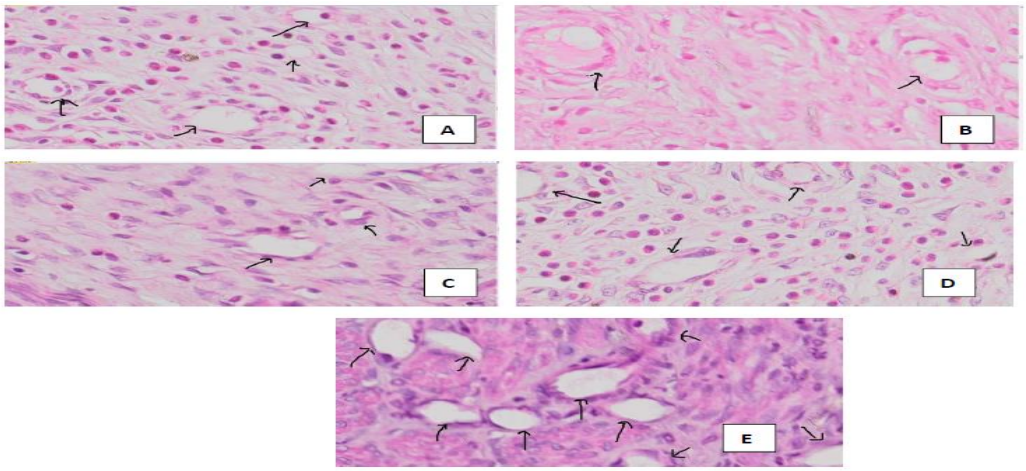
Then, we conduct analysis of EVOO effect on VEGF expression for four observation sample (CP, P1,P2 and P3) using Anova Test and the result was p-value = 0.000 <α. It proves that there was significant effect of EVOO administration on VEGF expression on Endometrium of Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B.

Figure 2 shows the result of multiple comparison test using Least Significant Difference (LSD). The finding shows that there was meaningful difference for average of VEGF expression on endometrium of Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B and given EVOO (P1, P2, P3) to those without EVOO (PC). It also proves that EVOO administration of 1.5 ml/kg bodyweight/day, 3 ml/Kg bodyweight/day and 4.5 ml/kg bodyweight/day on Female *Rattus norvegicus* of Wistar Strain exposed to Rhodamin B was significantly meaningful for improvement of VEGF expression. EVOO dosage with the most significant influence to VEGF expression on endometrium was dosage of 4.5 ml/kg bodyweight/day and the dosage was able to produce VEGF expression which was similar to VEGF expression for healthy rats (NC).

Then, we analyse the relation between EVOO dosages with VEGF expression on endometrium of Female *Rattus norvegicus* of Wistar Strain exposed to Rhodamin B using Spearman's rho test with p value < 0.05. This test was chosen because the dosage data were not distributed normally. The result of analysis shows that there was meaningful correlation between EVOO dosage with VEGF expression (p-value = 0.000<α) for rat group exposed to Rhodamin B and not given EVOO (n=15). The finding was based on high value of correlation coefficient of r = 0.814. Positive value on correlation coefficient of 0.814 indicated simultaneous correlation that the higher EVOO dosage, the higher VEGF expression on Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B. In addition, the lower dosage of EVOO, the lower VEGF expression.

Arteriole Number of Female Rattus Norvegicus of Wistar Strain

Arteriole is round with slight thick edge because it has tunika intima consisting of endothelium and interna elastica membrane. The arteriole is shown with arrows in the following figure.



Note: A (NC) shows distribution of more arteriole compared to B (PC). Meanwhile for C (P1), D (P2) and E (P3) shows increasing number of arteriole compared to B (PC).

Figure 3. Arteriole in endometrium of Female *Rattus norvegicus* of Wistar Strain exposed to Rhodamin B and given Extra Virgin Olive Oil (EVOO) observed under microsope with 400 times enlargement

Analysis of effect of Rhodamin B exposure on arteriole number on endometrium of Female *Rattus norvegicus* of Wistar Strain is conducted using independent sample t test. The result of p-value=0.005< α proves that the exposure of Rhodamin B gives significant influence on arteriolee number.

Based on the result of Anova test for arteriole number of sample, there was meaningful difference of arteriole number average on endometrium of four groups of observation sample shown by p-value = 0.000< α (table 1). Then, we conduct Multiple Comparison test using Least Significant Difference (LSD) shown in the following table.

Table 1. Comparison of arteriole number

Observation Group	Average ± SD	p-value
Control (+)	2.92±0.65a	0.000<∝
P1 (1.5 ml/KgBW/day)	3.56±0.11b	
P2 (3 ml/KgBW/day)	3.80±0.16b	
P3 (4.5 ml/KgBW/day)	4.86±0.54c	

The finding of study shows that there was meaningful difference of average of arteriole number on endometrium of Female *Rattus norvegicus* of Wistar Strain exposed to Rhodamin B and given EVOOO (P1, P2 and P3) to those without EVOO (PC). It is also proven that treatment of EVOO administration with dosage of 1.5 ml/kg body weight/day (P1), 3 ml/kg body weight/day (P2) and 4.5 ml/kg body weight/day (P3) on Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B gives meaningful effect on the increasing number of arteriole on endometrium. Dosage considered to be able to give significant effect on arteriole number on endometrium of Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B was 4.5 ml/kg bodyweight /day (P3) because it is the fastest to to increase arteriole number which is almost similar to arteriole number on health rat (NC).

The result of Spearman's rho test for data on EVOO dosage with arteriole number was p=0.000 and r value r=0.902. It indicates that there was a significant and simultaneous relation between EVOO dosage and arteriole number on endometrium of Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B.

Result of Regression Analysis of VEGF Expression Effect on Arteriole Number on Endometrium Female *Rattus norvegicus* of Wistar Strain Exposed to Rhodamin B and Given EVOO

Result of regression analysis showed that the effect of VEGF expression on arteriole number was statistically meaningful (p=0.018< α). The regression equation model was $\hat{y}$ = 2.095 + 0.065 x. It was found out that coefficient of effect of independent variable on dependent variable showed positive value which was 0.065. it means that there was a simultaneous effect between VEGF expression and arteriole number. If improvement of VEGF expression occurs, it will influence the increase of arteriole number. Vise versa, if VEGF expression decreases, then arteriole number decreases also.

We know the percentage of effect of VEGF expression on arteriole number from the determination of coefficient value of 36.3%. Therefore, the effect of VEGF expression on arteriole number was 36.3% and the number of arteriole is influenced by other variable which is not explored in this study with value of 63.7%.

DISCUSSION

Rhodamin B Influences the Decrease of VEGF Expression and Arteriole Number on Endometrium of Female *Rattus norvegicus* of Wistar Strain

Rhodamin B might decrease the VEGF expression and arteriole number on endometrium of Female Rattus Norvegicus of Wistar Strain because compound contained in Rhodamin B might increase ROS. Excessive ROS in body might induce oxidative stress which is able to damage endometrium organ.

Oxidative stress is an imbalance condition between prooxidant and antioxidant in body. It is caused by the increase of *Reactive Oxygen Species (*ROS) content and or *Reactive Nitrogen Species (*RNS) or it also might be caused by the decrease of antioxidant defence mechanism in our body[18]. Oxidative stress on female reproduction organ might cause *Poly Cystic Ovarian Syndrome (*PCOS), endometriosis, early menopause, and infertility with unknown cause, complication on pregnancy such as spontaneous abortus, abortus habitualis, pre ecclampsia and IUGR[18,19]and diabetes during pregnancy[20].

Rhodamin B content is xenobiotic compound which is able to activate enzyme system using cytochrome P450. It contains chlorine compound (C1) which is reactive hallogeneous in body. It has structure such as Quinone whch is highly redox active mollecule and it is a material containing alchilizing compound to produce ROS[21,22,23,3,4,5].

This study confirms the findngs of study conducted that Rhodamin B exposure is able to cause oxidative stress on ovary organ causing decreasing number of follicle on ovary and hormone 17β Estradiol[7,5]. Low level

of 17β Estradiol also influences VEGF expression regulation and therefore it impedes proliferation process on cycle in endometrium[8].

Oral exposure of Rhodamin B on white rats with dosage of 150 ppm, 300ppm, and 600 ppm during two cycles of estruses give significant effect in slowing down the estruses cycle on mature female white rat[24].

With the decrease of VEGF expression due to Rhodamin B exposure in endometrium, it will cause the decrease on arteriole number. It is because VEGF might influence artery spiralis angiogenesis process and it plays crucial role in forming process of new blood vessel during female reproduction cycle.

This confirms earlier research results suggest thatthe decreasing of VEGF expression in endometrium will influence artery spiralis angiogenesis process by lowering the number of arteriole spiralis in endometrium hindering tissue proliferation in endometrium[25,26]. Rhodamin B exposured during 36 days with dosage of 18 mg/kg body weight might increase oxidative stress resulting in the decrease of endometrium thickness[7].

Administration of Extra Virgin Olive Oil (EVOO) is Able to Improve the VEGF Expression and Arteriole Number on Endometrium of Female Rattus Norvegicus of Wistar Strain Exposed to Rhodamin B.

The administration of Extra Virgin Olive Oil (EVOO) is able to improve the expression of VEGF and arteriole number in endometrium because EVOO may function as antioxidant which is able to delay oxidation process in endometrium. EVOO compound might delay oxidation process because its phenol content might break radical ion chain by releasing radical hydrogen to alchilperoxil hydrogen produced by lipid oxidation in order to obtain stable derivatives. Besides that, EVOO is also able to lower bound with metal, improve endogenous antioxidant enzyme and improves transcription of mRNA GPx to minimize the increase of ROS which in turn, might damage reproduction organ especially endometrium.

It confirms finding stating that phenol in EVOO will react to free radicals by donating hydrogen ion which is able to change LOO radicals into hypo peroxide lipid (LOOH). Olive oil might function as antioxidant because phenol compound in olive oil is able to minimize ROS and reduce bound activity with metal and induce endogenous antioxidant enzyme[16].

Antioxidant from Extra Virgin Olive Oil contains polyphenol which is able to influence Keap 1. Keap 1 is inhibitor protein regulating Nrf2. Keap 1 releases Nrf2, Nrf2 is transcription factor with leusin basis functioning to maintain redox regulation key. Nrf2 will undergo translocation to nucleus and bound with antioxidant Respon element (ARE) along with protein of Small musculloponneurotic fibrosacoma (sMaf) to activate GSH. GSH is key component in regulating oxidation-reduction homeostasis. The increase of GSH help to fix oxidative stress caused by Rhodamin B. Phenolic compound contained in olive oil impedes the induction of platelet aggregate and it is reported that it is also able to improve transcription of mRNA from antioxidant enzyme glutathione peroxidase[27].

Hydrophilic and lipophilic content in Extra Virgin Olive Oil plays crucial role as antioxidant. Antioxidant obtained from phenol lipophilic compound such as tocoperol and tocotrinol. 90% of tocoperol in Extra Virgin Olive Oil is α-tocoperol. Antioxidant obtained from phenol hydrophilic compound which cannot be obtained from other oil and fats are phenolic acid, phenolic alcohol. Hydroxy-isochromans, flavonoid, lignan, and secoiridoid[16].

The relationship between administration of Extra Virgin Olive Oil (EVOO) with increase of VEGF expression and arteriole number on Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B is caused by compound content in EVOO functioning as antioxidant which is able to neutralize excessive antioxidant in our body that might damage reproduction organ.

Its role as antioxidant is marked by improvement of total activity of antioxidant plasma, GSH and GSH-Px. It is also marked by the decrease of oxidation from red blood cell, renal and gastrointestinal, oxidation, LDL, ROS, Fα-isoprostanes, and GSSG[15].

Extra Virgin Olive Oil might significantly reduce MDA content that is marker for peroxidation lipid on male Wistar rat exposed to cigarrete smoke[28]. Combination of 17-β estradiol, FGF-2 and VEGF-A might stimulate proliferation on endometrium cell of human and combination between progesterone and FGF-2 and VEGF-A might press proliferation of those cells.

Extra Virgin Olive Oil / EVOO contains big number of tocoferol (E vitamin) containing tocopherol alpha, beta, gama and delta. In addition, EVOO might improve body metabolism, revitalize immune system and improves body circulation resulting in strong defense for free radicals and it is similar to Rhodamin B that might reduce VEGF expression and arteriolee number in endometrium[30].

Meaningful correlation between the administration of Extra Virgin Olive Oil (EVOO) with the expression of *Vascular Endothelial Growth Factor (VEGF) and* arteriole number on endometrium of Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B is caused by high content of antioxidant containing in EVOO. The test result of antioxidant IC50 on EVOO supply of Borges conducted in Laboratory of Quality Test and Food Safety of Brawijaya University (No 0263/THP/LAB/2018) shows that result of antioxidant IC50 is 48,56.

A compound is considered as highly strong antioxidant if the IC5 score is less than 50, strong (50-100), and weak (151-200). The smaller IC50 value, the higher is antioxidant activity[31].

Positive Effect of VEGF expression on arteriole number of Female Rattus Norvegicus of Wistar Strain exposed to Rhodamin B

Other result obtained from this study is that there is simultaneous effect of VEGF expression on arteriole number. If there is an improvement on VEGF expression, then it will influence the improvement of arteriolee number. Vise versa, if VEGF expression decreases then arteriolee number decreases also. The effect of VEGF expression on arteriole number is 36,3%.

The decrease of VEGF expression in endometrium will influence the process of arteri spiralis angiogenesis by reducing the number of arteriolee spiralis in endometrium which in turn, disrupt the proliferation of endometrium tissue[25,26].

The amelioration of endometrium blood vessel and the forming of new branch of blood vessel of endometrium is crucial during menstrual period. After menstruation, the damage of blood vessel and gland damages stromal component. Angiogenesis process is needed after menstruation to minimize blood loss and rebuild endometrium function[10].

Hypoxia during or after menstruation on endometrium induces angiogenesis by stimulating VEGF-A expression and FGF-2 as potent angiogenic stimulator[28]. However, if damage on endometrium organ occurs such as reducing number of arteriole, the forming of new blood vessel and endometrium regeneration will be disrupted.

CONCLUSION

Rhodamin B is proven to be significant to VEGF expression and arteriole number on endometrium of Female Rattus Norvegicus of Wistar Strain. EVOO administration gives significant influence to improve the VEGF expression and arteriole number on Rattus Norvegicus exposed to Rhodamin B. EVOO might be alternative measure to prevent oxidative stress caused by Rhodamin B exposure because its antioxidant functions is proven to be able to neutralize free radicals caused by Rhodamin B exposure.

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