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Effect of Pravastatin on eNOS and PECAM-1 Expression in the Placenta of Pre-Eclampsia Rat (*Rattus norvegicus*) Model

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KEYWORD
eNOS; PECAM-1; pre-eclampsia

ABSTRACT

Introduction: : Pre-eclampsia is caused by endothelial dysfunction, which is defined by a change in endothelial cell activity to a reduced ability to vasodilate (decreased eNOS) and prothrombotic conditions (decreased PECAM-1). Pravastatin, which limits placental transfer, can reduce endothelial dysfunction by targeting pleiotropic effects in pregnancy. The aim was to show the effects of pravastatin on the eNOS and PECAM-1 expression in the placenta of the pre-eclampsia rat model.

Methods: This is a true experimental study design. The sample is pre-eclamptic rat placental tissue given pravastatin. The study has five groups, each with five samples. Normal pregnant rats were the negative control group. The positive control group was a pregnant rat model of pre-eclampsia treated to L-NAME 125mg/kgBW/day. The treatment group was pre-eclampsia model rats given pravastatin dosages of 2, 4, and 8 mg/kgBW. eNOS and PECAM-1 expressions were examined.

Results: Pravastatin dosages 2, 4, and 8 mg/kg increased eNOS and PECAM in the treatment group compared to the positive control. The Shapiro-Wilk test result was significant (p>0.05). Annova tests on eNOS (p=0.000) and PECAM-1 expression (p=0.000) confirmed the hypothesis. The Tukey test showed significant differences in eNOS (sig. 0.001) and PECAM-1 (sig. 0.000) expression between normal and pre-eclampsia rats. The optimal dose of eNOS and PECAM-1 was in treatment 1, 2 mg/kgBW. Pravastatin dose considerably increased eNOS (p=0.015; r=0.536) and PECAM-1 (p=0.000; r=0.734) expression.

Conclusion: Pravastatin has been shown to increase eNOS and PECAM-1 expression in the placenta of pre-eclampsia rat model. The optimal dose of pravastatin in eNOS and PECAM-1 expression was 2 mg/kgBW.

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INTRODUCTION

Pre-eclampsia is a pregnancy complication characterized by hypertension in normotensive pregnant women after 20 weeks of gestation, with or without proteinuria and signs of damage to organ systems [1,2]. Pre-eclampsia can occur in 3-5% of pregnancies [3]. 24% of the causes of maternal death in Indonesia in 2020 are hypertension [4]. Pre-eclampsia can occur in 2 stages, namely because there are abnormalities in the placenta and abnormalities in the maternal circulation. In pre-eclampsia, abnormal trophoblast invasion causes decreased uteroplacental perfusion resulting in ischemia and hypoxia of the placenta. The occurrence of ischemia in the placenta causes endothelial cell dysfunction by stimulating the release of toxic substances into the endothelium [5,6].

Endothelial dysfunction is characterized by a change in endothelial cell action to a reduced ability to vasodilate (decreased eNOS), proinflammatory, and prothrombotic states (decreased PECAM-1). Endothelial dysfunction is often associated with decreased NO bioavailability through decreased synthesis or increased degradation, and altered NO

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metabolism may be a predisposing factor for pre-eclampsia. NO is synthesized by the endothelial enzyme Nitric Oxide Synthase (eNOS). eNOS is expressed primarily on the syncytiotrophoblast and endothelial cells of the placenta during pregnancy. A decrease in eNOS can trigger an increase in blood pressure [7–9].

Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) is a transmembrane glycoprotein that is expressed on endothelial cells, platelets, monocytes, and T lymphocytes. PECAM-1 is one of the factors for the success of trophoblast invasion in pregnancy, therefore failure of trophoblast invasion will lead to the occurrence of pre-eclampsia. In pre-eclampsia, PECAM-1 from the maternal and uteroplacental endothelium can induce neutrophil and platelet activity that increases vascular damage. Based on the research report, expression of PECAM-1 in the placenta was decreased in cases of pre-eclampsia [10,11].

Pravastatin is the most hydrophilic drug of the statin class and limits placental transfer. Statins besides being useful in lowering cholesterol also have a pleiotropic effect. Statins can reduce endothelial dysfunction, anti-inflammatory, angiogenesis, immune response, and antioxidant effects, by targeting pleiotropic effects in pre-eclampsia [12]. Several studies have shown the benefits of statin administration in reducing blood pressure, proteinuria, placental insufficiency, and the incidence of IUGR(13). The purpose of this study was to prove the effect of pravastatin on the expression of eNOS and PECAM-1 in placenta of pre-eclampsia rat (*Rattus norvegicus*) model.

MATERIAL AND METHODS

Study Design

This study was true experimental that only used post-tests with a control group design.

Sample Size

The sample in this study was stored biological material in the form of placental tissue from the pre-eclampsia rat (*Rattus norvegicus*) model which had been given various doses of Pravastatin. This study was divided into 5 groups and each with 5 samples, as follows:

- KN: Negative Control Group (No Treatment),
- KP: Positive Control Group (L-NAME 125mg/kg BW)
- P1 : L-NAME 125mg/kg BW + Pravastatin 2mg/kg BW
- P2 : L-NAME 125mg/kg BW + Pravastatin 4mg/kg BW
- P3 : L-NAME 125mg/kg BW + Pravastatin 8mg/kg BW

The parameters to be observed were the expression of eNOS and PECAM-1 in the placental tissue.

Study Site

The study was carried out in the "Institut BioSains" laboratory of Brawijaya University for the paraffin block and tissue sample process. An immunohistochemical process was carried out in the Biochemistry Laboratory of the Medical Faculty at Brawijaya University.

Material

Materials used for immunohistochemical were tissue cassette, cover glass, object-glass, sniper background, anti-CD31/PECAM-1 antibody (sc-376764), anti-NOS3 (sc-376751), absolute ethanol, H2O2, and methanol, 10% formalin buffer, acetone and xylol, distilled water, paraffin, phosphate buffer saline (PBS), Alcohol 90% and 80%, DAB Chromogen, Trecavidin HRP, Decloaking solution, Mayer Hematoxylin, Betazoid Dab buffer substrate.

Experimental Procedure

Experimental Animal Acclimatization

Female rats (*Rattus norvegicus*) were acclimatized in the laboratory for seven days. Mice were placed in 45 x 35 x 12 cm cages without treatment and provided ad libitum food and water. Each cage had four rats. Every morning, 40 g/day of palletized feed was given and 150 ml of water was given ad libitum to each animal.

Female Rat Mating (*Rattus norvegicus*)

Female rats were placed in the former male rat cage for 72 hours, whereby male rat urine containing pheromones induced estrus. Rats were mated 1:1 in one night. The following day, the vaginal plug was examined for signs of copulation. The appearance of a plug or considerable weight gain are indicators of pregnancy.

Preeclampsia Modeling in Experiment Animals

L-NAME dissolved in Phosphate Buffer Saline (PBS) was used to simulate preeclampsia in experimental animals. The solution was administered intraperitoneally to pregnant rats. The L-NAME dose is 125 mg/gBW/day.

Pravastatin Administration

Pravastatin doses of 2 mg/kgBW, 4 mg/kgBW, and 8 mg/kgBW can reduce blood pressure and proteinuria in a rat model of preeclampsia. The pravastatin used in this study was pravastatin tablets that were broken up into powder and dissolved. Pravastatin solution was administered orally to experimental animals via a probe from 13 until 19 days of gestation.

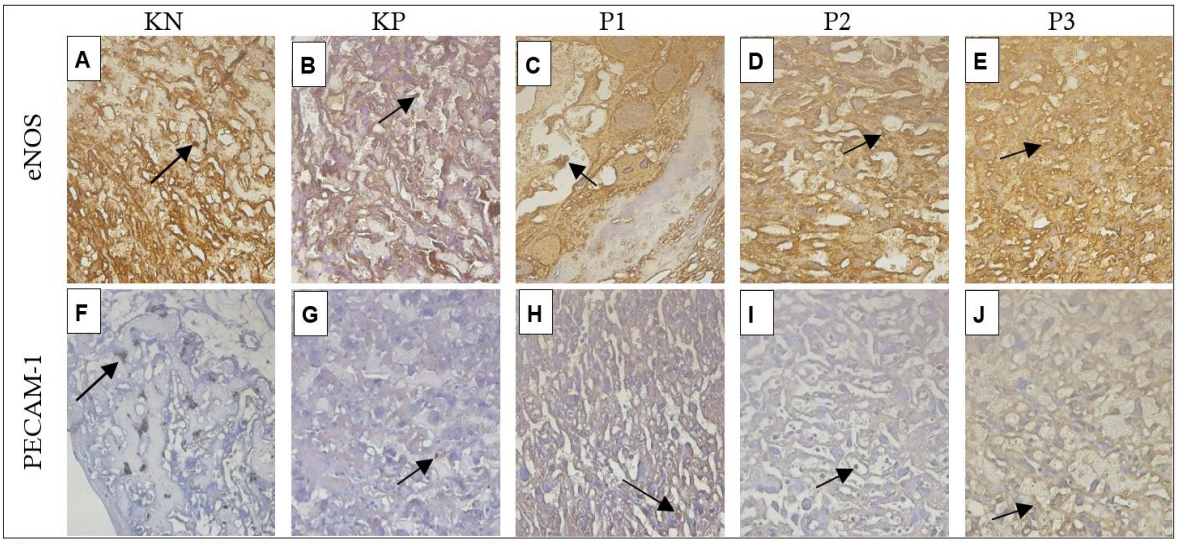

Fig. 1. Immunohistochemistry of eNOS and PECAM-1.
Images A-E show eNOS immunohistochemistry. Images F-J show PECAM immunohistochemistry. The arrows represent cells expressing brown antibodies as observed under a 400x magnification microscope. (A) negative control group with eNOS expression; (B) positive control group with eNOS expression; (C) treatment group 1 with pravastatin 2mg/kgBW/day showing eNOS expression; (D) treatment group 2 with pravastatin 4mg/kgBW/day showing eNOS expression; (E) treatment group 3 with pravastatin 8mg/kgBW/day showing eNOS expression; (F) negative control group with PECAM-1 expression; (G) positive control group with PECAM-1 expression; (H) treatment group 1 with pravastatin 2mg/kgBW/day showing PECAM-1 expression; (I) treatment group 2 with pravastatin 4mg/kgBW/day showing PECAM-1 expression; (J) treatment group 3 with pravastatin 8mg/kgBW/day showing PECAM-1 expression.

Placental Tissue Extraction and Surgery

Ketamine (10 mg/ml) was injected intraperitoneally at a dose of 40 mg/kgBW to anesthetize rats. Rats were placed on a rubber pad with their stomachs facing up, and needles were implanted into their feet. Laparotomy was performed on the rat by opening the abdominal wall. The uterus was opened on the anti-mesometrial side with the placenta attached. Placental tissue was extracted from both the right and left uterus. The placenta is cut transversely through the sagittal middle. To ensure the number of placentas and rats in a single section, a large incision was performed from the tip to the base of the left side of the rat's uterus. The umbilical cord and rat pups were used to separate the placenta. The placenta was fixed in 10% formaldehyde in PBS (pH 7.0).

Paraffin Block Process

Following 10% formalin fixation, tissue samples are cut to a thickness of 2-3 mm. The tissue is placed in a tissue cassette with a label and then sealed. Dehydrate and clear the tissues. The tissue is removed from the tissue cassette and paraffin is used to block it.

Tissue Sample Process

Using a microtome with a thickness of 2-3 m, placental tissue in a paraffin block was cut. The cutting results were placed in a warm water bath and then examined with an object-glass smeared with glycerin albumin. The selected pieces were dried and placed on a hot plate 38-400 to dry before being placed in an incubator at 38-400C for 24 hours.

The Immunohistochemical Process

With xylol I, II, and III, the slides were deparaffinized for ten minutes each. The samples were then immersed in 100% ethanol, 80% alcohol, and 70% alcohol for ten minutes each. For 60 minutes, samples were incubated with 3% H2O2 in methanol. After being incubated with 5 percent Blocking in a Moisture Chamber, the slides were then rinsed with phosphate buffer saline (PBS). Primary antibodies against eNOS and PECAM-1 from Santa Cruz Biotech were drip-coated onto the slides (USA). The slides received a DAB drip, were incubated (1 ml of Betazoid Dab Substrate Buffer and 1-2 drops of DAB Chromogen), and then were rinsed. Use Mayer's slide-in counterstain for hematoxylin. Slides were dehydrated using xylol I, II, and III as well as absolute alcohol, 80%, and 70% alcohol. The last step of immunohistochemical staining is mounting with Entellan, then the slides are dried.

ENOS and PECAM-1 Expression Analysis

An Olympus DP72 binocular microscope with a 400x magnification and a 5 m bar scale was used to examine the slides. The entire region of placental tissue was mapped. Slides were taken in 10 different fields of view at random. The expression of eNOS and PECAM-

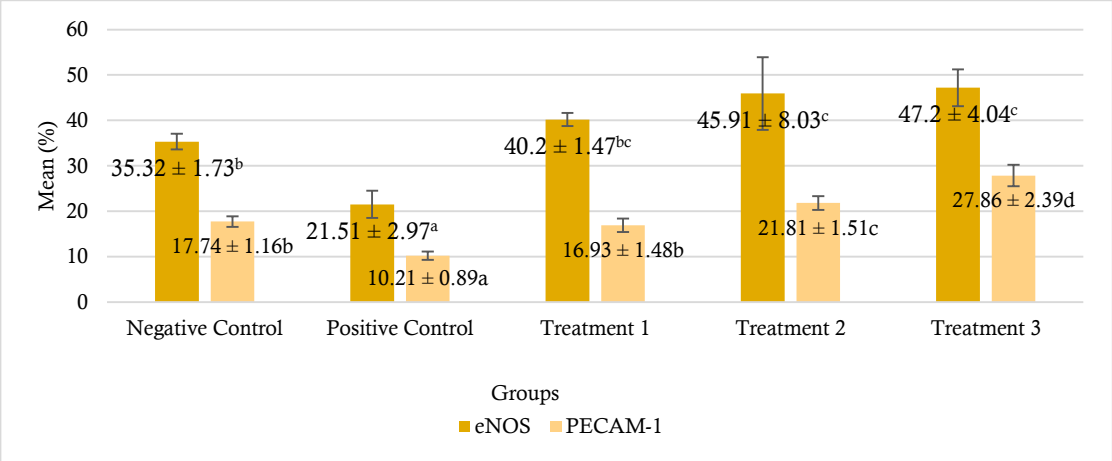

Fig. 2. Mean of eNOS and PECAM-1 expression in the study group.
Negative control group (no treatment); positive control group, pre-eclampsia rat model (induced by L-NAME); treatment 1, pre-eclampsia rat model with pravastatin 2mg/kgBW/day; treatment 2, pre-eclampsia rat model with pravastatin 4mg/kgBW/day; treatment 3, pre-eclampsia rat model with pravastatin 8mg/kgBW/day.

1, which were highlighted in brown on the pictures, was then examined using ImageJ 1.53c software.

Statistical analysis

Data analysis was performed using SPSS 22.0 software. The Shapiro-Wilk test was performed to determine whether the data distribution in this research was normal. We performed the Levene test to evaluate the homogeneity of variance. The analysis was carried out using the one-way Anova test and followed by the Post Hoc Tukey test. The correlation between the various doses of pravastatin with the expression of eNOS and PECAM-1 used the Pearson test. The p-value<0.05 was considered to indicate the statistical significance.

Ethics

A certificate of ethical exemption has been issued by the Health Research Ethics Commission, Faculty of Medicine, Universitas Brawijaya with number 71/EC/KEPK/03/2022.

RESULT

eNOS and PECAM-1 immunohistochemical Results

The result of immunohistochemical staining is shown in Fig. 1. The arrows indicate cells expressing eNOS (A-E) and PECAM-1 (F-J) antibodies in brown, as seen under a 400x magnification microscope.

Effect of Pravastatin on eNOS Expression

It is known from Figure 2 that the mean expression of eNOS in the negative control group is higher than in the positive control group, and conversely. The eNOS expression level increased on average in the pravastatin treatment group (P1, P2, and P3), with the highest percentage seen in the group receiving the highest dose of the medication (8 mg/kg BW/day).

The Shapiro-Wilk normality test was used to analyze the data, with the results p = 0.219 (p>0.05) indicating that the data are normally distributed. The Levene test was used to determine the homogeneity of the data, and the result was 0.517 (p>0.05), indicating that it is homogeneous. These results indicate that the data are homogeneous and normally distributed, allowing the One-Way ANOVA test, Tukey's post hoc test, and Pearson correlation test to be carried out.

Table 1. Multiple Correlation Test of eNOS and PECAM-1 Expression by Tukey

Group	Group	eNOS	PECAM-1
		p-value	p-value
Negative control	Positive control	0.001*	0.000*
	Treatment 1	0.417	0.923
	Treatment 2	0.008*	0.005*
	Treatment 3	0.003*	0.000*
Positive control	Treatment 1	0.000*	0.000*
	Treatment 2	0.000*	0.000*
	Treatment 3	0.000*	0.000*
Treatment 1	Treatment 2	0.269	0.001*
	Treatment 3	0.120	0.000*
Treatment 2	Treatment 3	0.989	0.000*

*the group that has a significant difference (p<0.05)

ANOVA analysis of eNOS expression showed a significant difference (p<0.05). The Post Hoc Tukey test was used to determine which groups were different. According to Table 1, the average value of eNOS expression in the negative control group was significantly different from the positive control, p = 0.001. The average value of eNOS expression at P1, P2, and P3 was significantly different from the positive control, p<0.05. As a result, pravastatin at doses of 2, 4,

Table 2. Correlation Test Results for eNOS and PECAM-1 by Pearson

Variables	Correlation coefficient (r)	Correlation direction	Interpretation	*p-value*
eNOS expression and pravastatin dosage	.536*	Positive	Moderate	.015
PECAM-1 expression and pravastatin dosage	.734**	Positive	Strong	.000

The Pearson correlation test results are significant if the p-value < 0.05. A positive correlation indicates a linear correlation, and conversely. A significant correlation at 0.05 is indicated by the sign (*), and a significant correlation at 0.01 is indicated by the sign (**).

and 8 mg/kgBW/day can increase the expression of eNOS in a pregnant rat model of pre-eclampsia. The optimal dose is the lowest dose that has a significant difference from the positive control group, that is treatment 1 with pravastatin 2mg/kgBW/day($p < 0.05$).

According to Table 2, the Pearson correlation test showed a significant relationship between pravastatin dose and eNOS expression in pre-eclampsia model rats ($p = 0.15$). The Pearson correlation coefficient ($r) = 0.54$, indicating that the higher the pravastatin dose, the higher the eNOS expression in preeclamptic rat placenta with moderate correlation strength.

Effect of Pravastatin on PECAM-1 Expression

Fig. 2 shows that the average expression of PECAM-1 in the negative control group is higher than in the positive control group, and vice versa. The average level of PECAM-1 expression increased in the pravastatin treatment groups (P1, P2, and P3), with the highest percentage seen in the group receiving the highest dose (8 mg/kg BW/day).

The Shapiro-Wilk test was used, with the results $p = 0.458$ ($p>0.05$) indicating that the data were normally distributed. It was determined to be homogeneous using Levene's test, which had a significance score of 0.956 ($p>0.05$). These results indicate that the data are homogeneous and normally distributed, allowing the One-Way ANOVA test, Tukey's post hoc test, and Pearson correlation test to be conducted.

The ANOVA test showed significant results, $p = 0.000$ ($p<0.05$). The Post Hoc Tukey test was used to determine which groups were different. According to table 1, the average value of PECAM-1 expression in the negative control group was significantly different from the positive control, $p = 0.001$. The expression of PECAM-1 at P1, P2, and P3 was considerably different from that of the positive control, different<0.05. As a result, administration of pravastatin at doses of 2, 4, and 8 mg/kgBW/day can increase the expression of PECAM-1 in a pregnant rat model of preeclampsia. The optimal dose is the lowest dose that has a significant difference from the positive control group, that is treatment 1 with pravastatin 2mg/kgBW/day($p < 0.05$).

According to Table 2, the Pearson correlation test showed a significant relationship between pravastatin dose and PECAM-1 expression in pre-eclampsia model rats ($p = 0.000$). The Pearson correlation coefficient ($r) = 0.734$ indicates that the higher the pravastatin dose, the higher the PECAM-1 expression in the placenta of preeclamptic rat models.

DISCUSSIONS

The expression of eNOS and PECAM-1 was lower in the positive control group (pre-eclampsia model pregnant rats) than in the negative control group in this study (normally pregnant rats). These findings indicate a significant difference between pre-eclampsia model rats and normal pregnant rats. A decrease in eNOS can reduce the bioavailability of NO, a potent vasodilator synthesized by NOS. Using L-NAME to inhibit NOS in pregnant rats results in elevated blood pressure, proteinuria, decreased glomerular filtration rate, thrombocytopenia, and IUGR, which resemble the symptoms of pre-eclampsia [14,15].

Pre-eclampsia can occur as a result of trophoblast invasion failure, which causes ischemia and hypoxia in the placenta. Oxidative stress brought on by uteroplacental ischemia will result in an inflammatory response and an unbalanced angiogenic response. As a result, toxic substances will be stimulated to be released into the endothelium, leading to the dysfunction of endothelial cells. Endothelial dysfunction causes changes in endothelial activity, one of which is reduced vasodilation, which is characterized by reduced NO as a result of decreased eNOS activation. Decreased eNOS levels can reduce NO bioavailability, requiring higher blood pressure to maintain blood flow to tissues [6,16–18].

According to the study's findings, pre-eclampsia model rats had lower eNOS expression than normal rats. This is consistent with the study by Du et al. (2017), which showed that the expression of eNOS in preeclamptic placentas was lower than in the control group with a p-value of 0.05 [9]. In addition to impaired vasodilation, which is indicated by decreased eNOS activation, endothelial dysfunction also entails a pro-inflammatory condition, indicated by reduced PECAM-1. Endothelial damage activates the coagulation and platelet systems. Platelets will adhere to the exposed basal membrane, causing platelet aggregation to form plaque around the wound and clot retraction to

completely close the wound. Compared to a normal pregnancy, pre-eclampsia causes a significant amount of platelet activation. This is characterized by a decrease in PECAM-1. PECAM-1 contributes to trophoblast invasion through its function in the transendothelial migration of leukocytes [18,19].

Endothelial cells typically produce relatively high levels of prostacyclin; otherwise, platelets would produce thromboxane. Thromboxane levels rise as a result of exposure to blood cells, particularly platelets, in the presence of endothelial cell dysfunction, while prostacyclin production declines. This causes platelet aggregation, increasing the vasoconstrictive effect and raising blood pressure [19,20]. According to the findings of this study, PECAM-1 expression was lower in preeclamptic rats than in normal rats. This is consistent with research by Erol (2012), who found that PECAM-1 expression was lower in preeclamptic placentas than in control placentas (normal endothelial cells) [21].

eNOS and PECAM-1 expression were higher in the treatment group compared to the positive control group at P1, P2, and P3 after receiving pravastatin at doses of 2, 4, and 8 mg/kg BW/day for 7 days. It is believed that giving pravastatin to pre-eclampsia rat models will increase the expression of eNOS and PECAM-1. The expression of eNOS and PECAM-1 in the placenta of preeclamptic rat models increased with increasing pravastatin dose. As shown in this study, a dosage of 2 mg/kgBW was the optimal dose since eNOS and PECAM-1 expression has a significant difference from the positive control group. In contrast to this study, pravastatin 5 mg/kg/day was able to reduce the arterial pressure and proteinuria that were clinical signs of pre-eclampsia in rat models. Pre-eclampsia rats model given pravastatin (L-NAME + pravastatin) had less placental sinus and loose tissue [22]. Additionally, Kräker's (2020) study shown that administering pravastatin at a dose of 5 mg/kgBW could reduce sflt-1 levels in preeclamptic rats [23]. Therefore, it was shown in this study that a dose of 2 mg/kgBW pravastatin was ideal for treating preeclampsia by regenerating placental endothelium cells.

Pravastatin is the most commonly used statin in animal model studies due to its hydrophilic and hepatoselective biochemical profile. By inhibiting HMG-CoA reductase, pravastatin can cause heme oxygenase-1 (HMOX-1) to produce carbon monoxide (CO). Hypoxia can be prevented by CO. As a result, CO production inhibits sFlt-1 and s-Eng. The inhibition of sFlt-1 and s-Eng causes an increase in VEGF. VEGF is crucial for angiogenesis, but it can also protect the integrity of endothelial cells by activating eNOS. PECAM-1 can rise in response to eNOS activation and NO production [24–27].

The limitation of this study is that the research was not carried out from the beginning of the rat pregnancy process because this is a continuation project with samples in the form of stored biological material, placental tissue. As a result, before placental tissue was obtained for this study, any interventions or changes in the conditions could not be made.

CONCLUSION

Pravastatin has been shown to increase the expression of eNOS and PECAM-1 in the placenta of the pre-eclampsia rat (*Rattus norvegicus*) model. The expression of eNOS and PECAM-1 in the placenta of the pre-eclampsia rat (*Rattus norvegicus*) model increased with increasing pravastatin dose. But the optimal dose of pravastatin in eNOS and PECAM-1 expression was 2 mg/kgBW.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest exist.

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