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Effect of Pravastatin on Vascular Endothelial Growth Factor (VEGF) and Endothelial Nitric Oxide Syntase (eNOS) Expression in Rat Liver (Rattus norvegicus) Preeclampsia Model

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ABSTRACT:

Introduction : Preeclampsia is an obstetric complication after 20 weeks of pregnancy with a new onset of hypertension and dysfunction of the mother's organs. A decrease in eNOS can trigger an increase in blood pressure as well as a decrease in NO. Vascular endothelial growth factor (VEGF) is not only pro-angiogenic but also important in maintaining endothelial and vascular health. Pravastatin can inhibit the activity of Hydroxymethylglutarate-Coa Reductase (HMG-CoA) thereby activating eNOS and producing Nitrite Oxcide (NO). Nitrite Oxcide (NO) serves as a vasodilator for angiogenic balance.

Materials and Methods : This research design uses true experimental design with posttest only control group design. The current study was conducted in vivo using stored biological material of liver organ tissue of pregnant rats (Rattus norvegicus) wistar strain model of preeclampsia with 3 different doses of pravastatin, namely 2 mg / KgBB, 4 mg / KgBB, and 8 mg / KgBB. VEGF expression, eNOS were observed in this study.

Result : Pravastatin doses of 2, 4, and 8 mg/kg increased VEGF and eNOS in the treatment group compared to positive controls. Anova's tests on VEGF (p=0.015) and eNOS (p=0.000) expressions confirmed the hypothesis. The correlation test showed a difference in VEGF expression (p-value 0.006; r = 0.594) and eNOS (p-value 0.000; r
= 0.856) between pravastatin dose and VEGF and eNOS expression there was a significant and positive direction, where the higher the dose of pravastatin, the expression of VEGF and eNOS increased. The optimal dose of VEGF and eNOS at a dose of 8 mg/kgBB.

Conclusion : Pravastatin has been shown to increase VEGF and eNOS expression in arat model of liver preeclampsia.

Keyword : Pravastatin, Preeclampsia, VEGF, eNOS

INTRODUCTION

Preeclampsia is a pregnancy disorder associated with new-onset hypertension, which most often occurs after 20 weeks gestation and is often approaching term. Although often accompanied by new-onset proteinuria, hypertension and other signs or symptoms of preeclampsia may appear in some women in the absence of proteinuria (Hypertension, 2020). Preeclampsia is an obstetric complication after 20 weeks of pregnancy with a new onset of hypertension and maternal organ dysfunction (Pragitara et al., 2020).

In Indonesia, preeclampsia ranks second in maternal deaths, with 128,273 cases per year, or 5.3% of all maternal deaths. Preeclampsia affects 3-8% of all pregnancies and is seven times more prevalent in developing countries compared to developed countries (Wulandari et al., 2021). The pathophysiology of preeclampsia is a condition consisting of two phases, with its specific etiology still unclear. The first phase is that abnormal placentation is often influenced by genetic, environmental, and immunological factors. The second phase includes angiogenesis imbalance, maternal endothelial dysfunction, oxidative stress, and increased placental inflammation (Smith & Costantine, 2022) (Rana et al., 2022).

Endothelial dysfunction is often associated with decreased NO bioacapability through decreased synthesis or increased degradation, and changes in NO metabolism may be factors influencing preeclampsia. Nitric oxide (NO) plays an important role in vascular homeostasis due to its vasodilated effects. NO is synthesized by nitric oxide synthase (NOS), from L arginine and molecular oxygen (O2). Nitric oxide (NO) is a powerful vasodilator and vascular smooth muscle relaxant. NO is synthesized by the endothelial enzyme Nitric Oxide Syntase (eNOS). eNOS is expressed mainly in syncytiophoblasts and endothelial cells during pregnancy. eNOS has been identified as one of the preeclampsia marker genes. A decrease in eNOS can trigger an increase in blood pressure as well as a decrease in NO (Shaheen et al., 2020; S-Vieira & A., 2018; Du et al., 2017; Guerby et al., 2021).

The right balance between pro and anti-angiogenic is necessary to regulate blood vessels, blood vessel formation and to maintain vascular function.

Vascular endothelial growth factor (VEGF) is not only pro-angiogenic but also important in maintaining endothelial and vascular health. This action is mediated through its receptor, VEGFR. Placental growth factor (PlGF) binds only to fms such as tyrosine kinase 1 (Flt-1, also known as VEGFR-1) and is thought to promote pathologic rather than physiological angiogenesis under inflammatory or hypoxic conditions. The main sources of VEGF are endothelial cells, stroma and hematopoietic cells. VEGF is expressed in cells above fenestrated and sinusoidal blood vessels such as renal podocytes and hepatocytes, and in tissues with high metabolism. Therefore, it is not surprising that the main feature of pre-eclampsia occurs in blood vessels with fenested endothelium. VEGF directly causes possible vasodilation through the production of nitric oxide and prostacycline. The effects of VEGF on the endothelium appear to be regulated primarily by VEGFR and antagonism of these receptors increases mean arterial pressure (Ngene & Moodley, 2017)(Flint et al., 2019).

Statins have a pleiotropic effect that does not depend on cholesterol. This cholesterol-free effect has therapeutic potential and is involved in angiogenesis, immunological response, anti-inflammatory response, and antioxidant benefits. Statins have also been linked to placental conditions, where the angiogenic effects of statins have potential in the treatment of preeclampsia (Kumasawa *et al*., 2020).

A type of natural statin with unique pharmacokinetic properties is pravastatin. Because pravastatin is hydrophilic, or water-soluble, it moves slowly through the placenta due to its slow molecular transfer. Therefore, pravastatin can only cross the placenta and cannot enter the developing fetus (Costantine *et al*., 2017). Pravastatin as an anti-inflammatory effect can inhibit the activity of Hydroxymethylglutarate-Coa Reductase (HMG-CoA) which causes Hmox-1 to catabolism with Carbon Monoxide (CO) so as to activate eNOS and produce Nitrite Oxcide (NO). Nitrite Oxcide (NO) serves as a vasodilator for angiogenic balance (Ives et al., 2020).

According to research (Rahardjo et al., 2022) Pravastatin has been shown to increase the expression of eNOS and PECAM-1 in the placenta of a mouse model of preeclampsia. The optimal dose of pravastatin in eNOS and PECAM-1 expression is 2 mg/kgBB. The purpose

of this study was to prove the effect of pravastatin on VEGF and eNOS expression in liver models of preeclampsia rats (Rattus norvegicus).

MATERIAL AND METHODS

Research Design

This study used a true experimental design, with a post-test only strategy with control group design Pregnant rats (Rattus norvegicus) from the Wistar strain that had been exposed to NG-nitro-L-arginine methyl ester (L-NAME) 125mg/kg/day and given pravastatin at different doses were used in this study, which was conducted in vivo using biological material that had been stored from rat liver.

Research Sample

The study samples were biological material that had been preserved at the Institute of Biosciences Universitas Brawijaya from the liver of pregnant rats (Rattus norvegicus) used as a model of preeclampsia. Using a direct random sampling approach, liver tissue was taken and divided into five treatment groups as follows:

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

The parameters observed were VEGF and eNOS expression in liver tissue.

Inclusion and Exclusion Criteria

Inclusion criteria in this study are tissue preparations from the liver organs of preeclampsia pregnant rats, paraffin blocks in good condition and quality so that immunohistochemical examination can be carried out, storage at room temperature. As for the exclusion criteria, damaged paraffin blocks cannot be cut, paraffin blocks are too dry, moldy, dyes can no longer color cells in stored biological material tissues.

Place and Time of Research

The research was conducted in the laboratory "Institute of Biosciences" Universitas Brawijaya for the process of sampling paraffin blocks and tissues. The immunohistochemical process is carried out at the Biochemistry Laboratory of the Faculty of Medicine, Universitas Brawijaya. The study runs from January to March 2024.

Research Materials and Tools

The materials used for

immunohistochemistry are rats liver tissue samples (vertical cutting method), primary antibodies, secondary antibodies (VEGF and eNOS), formalin buffer 10%, absolute ethanol, H2O2, methanol, Aceton, xylol, Paraffin, Aquades, Saline Buffer Phospate (PBS), DAB Chromogen, Alcohol 90% and 80%, Decloaking solution, Strepavidin-HRP, Betazoid Dab substrate buffer, Mayer Hematoxilen, tissue casset, object glass, cover glass, sniper background.

Research Variables

The variables in this study were Pravastatin with 3 doses as independent variables and expression of VEGF and eNOS in BBT liver of preeclampsia model bunting rats.

Paraffin Block Manufacturing Process

Liver tissue samples fixed with 10% formalin buffer were cut perfectly with a thickness of 2-3 μm. Then the piece of tissue is inserted in the tissue casset, labeled and closed. Tahap berikutnya yaitu dehidrasi, pembeningan (clearing), The next stage is dehydration, clearing, embedding, and blocking using paraffin.

Slide Creation Process

Tissue in the form of paraffin blocks is cut (sectioning) with microtomes with the result of cutting in the form of thin bands measuring 3-5 μm. Then the cutting results are put in a waterbath filled with warm water, taken with a glass object smeared with glycerin albumin. The selected pieces of tissue are dried and placed on a hot plate at a temperature of 36-40^{o}C until dry. The preparation is incubated overnight in an incubator temperature of 36-40^{o}C. The next stage is staining.

Immunohistochemical Staining Process

With xylol I, II, and III, the slides were deparafined for ten minutes each. The samples were then soaked in 100% ethanol, 80% alcohol, and 70% alcohol for ten minutes each. For 60 min, the sample was incubated with 3% H2O2 in methanol. After incubating with 5 percent blocking in the moisture chamber, the slide is then rinsed with phosphate buffer saline (PBS). Primary antibodies against VEGF and eNOS from Santa Cruz Biotech were funneled to the slide (US). The slides are given DAB droplets, incubated (1 ml of Betazoid Dab Substrate Buffer and 1-2 drops of DAB Chromogen), then rinsed. Use Mayer's sliding dye for hematoxylin. The slides were dehydrated using xylol I, II, and III as well as absolute alcohol, 80% alcohol,

and 70%. The last stage of immunohistochemical staining is pairing with Entellan, then the preparation is dried.

VEGF and eNOS Expression Analysis

The slide was observed using a binocular microscope brand olympus BX53 with a magnification of 400x and a scala bar of 5 μm. Then mapping was carried out on all areas of liver tissue. Slides in photos and taken 10 randomly selected fields. Analysis was performed on liver tissue expressing VEGF and eNOS at 400x magnification using ImageJ 1.53c software. The results obtained are in the form of the percentage of VEGF and eNOS expression shown in brown color in liver tissue.

Statistical Analysis

The data analysis used in this study was carried out in three stages, namely: Parametric Prerequisite Test, One Way ANOVA Test, Comparison Test. Posthoc and Correlation Test. All calculations are done using SPSS software for windows 19.

Ethics

This research has been approved by the Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia with a certificate.

RESULT

Endothelial Growth Factor (VEGF) and endothelial Nitric Oxida Syntase (eNOS) Expression in Rat Liver (Rattus norvegicus) Wistar Strain Model of Preeclampsia

The following are the results of VEGF and eNOS expression analysis using the immunohistochemical method using Image J 1.53c software.

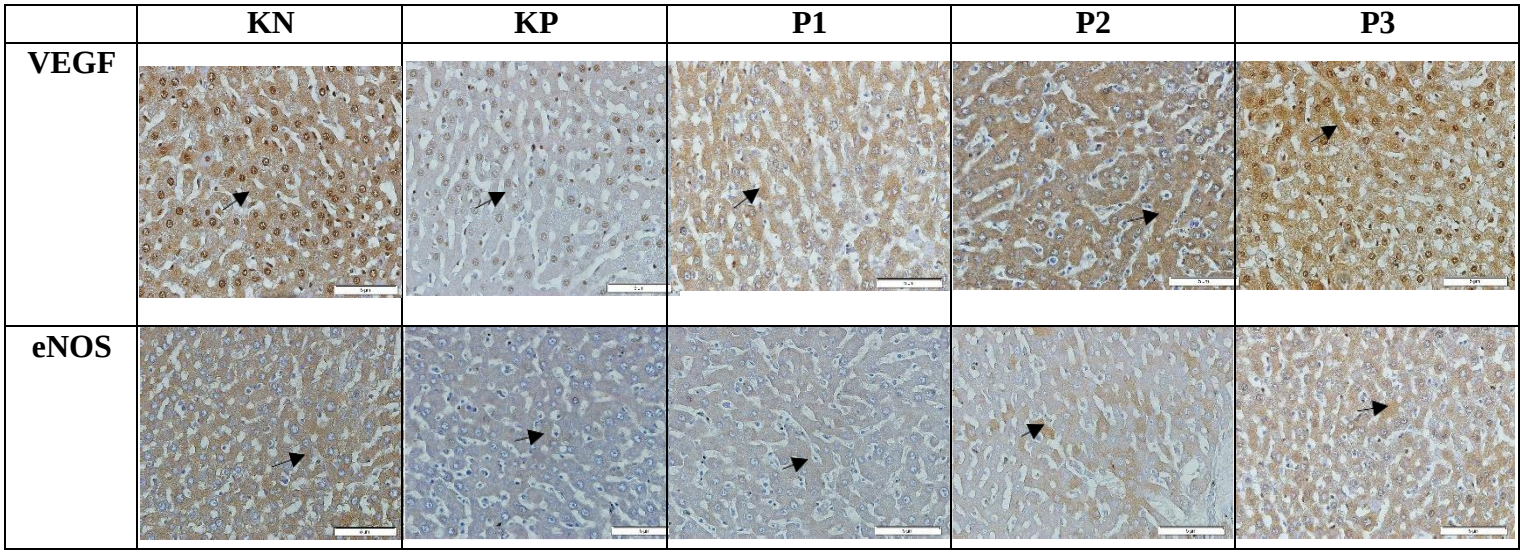

Figure 1. Immunohistochemistry of VEGF and eNOS Expression.
Differences in VEGF and eNOS expression in liver tissue between groups. The black arrow shows the expression of VEGF and eNOS of brown liver tissue seen from a microscope with a magnification of 400x and a scale bar of 5μm. (A) K (-) namely normal pregnant rat Liver; (B) K (+), namely the Liver of pregnant rat in the preeclampsia model; (C) P1, namely the Liver of a pregnant rat model of preeclampsia + pravastatin 2 mg/KgBW; (D) P2, namely the Liver of pregnant rats in the preeclampsia model + pravastatin 4 mg/KgBW; and (E) P3, namely the Liver of pregnant rats in the preeclampsia model + pravastatin 8 mg/KgBW.

Table 1. Effect of Pravastatin on VEGF Expression in the Liver of Rattus norvegicus Preeclampsia Model

Research Group	(n)	Mean ± SD (Percentage)	*p-value* (*One Way ANOVA*)
Negative Control	5	16.56 ± 3.20^{b}	0.015
Positive Control	5	5.50 ± 3.34^{a}	
Treatment 1	5	8.93 ± 8.36^{ab}	
Treatment 2	5	11.37 ± 5.82^{ab}	
Treatment 3	5	15.65 ± 2.78^{b}	

Table 2. Effect of Pravastatin on eNOS Expression in the Liver of Rattus norvegicus Preeclampsia Model

Research Group	(n)	Mean ± SD (Percentage)	*p-value* (*One Way ANOVA*)
Negative Control	5	19.78±2.50[b]	
Positive Control	5	1.43±0.26[a]	
Treatment 1	5	4.63±3.12[a]	0.000
Treatment 2	5	5.24±3.37[a]	
Treatment 3	5	15.10±3.93[b]	

Table 3. Pravastatin Dose Correlation

Variables	Correlation coefficient (r)	*P-value*
Dose of Pravastatin on VEGF expression	0.594	0.006
Dose of Pravastatin on eNOS expression	0.856	0.000

The Pearson correlation test showed that there was a significant correlation between the pravastatin dose variable and the variables VEGF (p-value 0.006; r = 0.594), eNOS expression (p-value 0.000; r = 0.856). The VEGF expression have a moderate correlation with a positive correlation towards pravastatin dose. The positive correlation between VEGF expression and pravastatin indicates that the higher the dose of pravastatin, the more VEGF expression increases. The positive correlation between eNOS with pravastatin indicates that the higher the dose of pravastatin, the more eNOS expression increases. eNOS has a strong correlation with a positive correlation direction to the dose of pravastatin. This means that the higher the dose, the more the expression increases.

DISCUSS

The expression of eNOS and VEGF was lower in the positive control group (pregnant rat model of preeclampsia) than in the negative control group in this study (normal pregnant rat). These findings showed significant differences between preeclampsia model mice and normal pregnant mice. The administration of NO inhibitors can be used in animal models of preeclampsia as a vasoconstriction model. A vasodilator called nitric oxide (NO) is produced when endothelial nitric oxide synthase (eNOS) is stimulated. The NO inhibition technique uses L-NAME (NG-nitro-L-arginine methyl ester) or L-NNA (NG-nitro-L-arginine) treatment. Because of the assumption that eNOS inhibition results in NO insufficiency, L-NAME was the first direct model, and it was this model that was used to study vascular disorders. Similar to signs of hypertensive disease in pregnancy, L-NAME produces long-term hypertension, hypovolemia, intrauterine growth retardation, proteinuria, thrombocytopenia, and altered renal morphology (Leo *et al*., 2015; Zhu *et al*., 2017).

Preeclampsia as the presence of hypertension and proteinuria that occurs after 20 weeks gestation in patients who were previously normotensive. Preeclampsia results from impaired invasion of spiral arterial trophoblasts by remodeling, leading to decreased placental perfusion and endothelial dysfunction. This leads to uteroplacental hypoxia, angiogenic and antiangiogenic protein imbalances, oxidative stress, maternal endothelial dysfunction, and increased systemic inflammation. Endothelial dysfunction is characterized by decreased bioavailability of NO through decreased production or increased consumption due to oxidative stress. NO is synthesized by the enzyme endothelial nitric oxide synthase (eNOS). eNOS is expressed in the endothelium and maintains vascular tone through the intrinsic synthesis of NO from the reduction of L-arginine to L-citruline. eNOS activity has been shown to decrease in preeclampsia compared to normal pregnancy (Shaheen et al., 2020)(Rana et al., 2019)

In this study proved that there were changes in the liver condition of pregnant female rats model of preeclampsia given L-NAME induction 125mg / KgBW. One of the changes that occurred was the expression of eNOS

detected in the liver using immunohistochemical methods. The value of eNOS expression in the liver in the positive control group (given L-NAME induction 125mg/KgBW) was shown to be lower when compared to the negative control group (pregnant rats were not given L-NAME induction).

Administration of L-NAME 125mg/KgBW was able to make mice model of preeclampsia, where the positive control group had lower eNOS expression in the liver than the negative control group with a p-value of 0.000. This shows that it is true that the condition of preeclampsia can reduce eNOS levels and affect other organs where one of them is expressed in the liver. This is in line with research (Shaheen et al., 2020) which states that decreased eNOS expression and oxidative stress can play a role in the pathology of preeclampsia in the maternal endothelium and research (Rahardjo et al., 2022) which states that endothelial dysfunction is characterized by changes in the action of endothelial cells on decreased vasodilating ability (decreased eNOS), pro-inflammatory states, and prothrombotic.

In addition to experiencing impaired vasodilation, which is characterized by decreased activation of eNOS, endothelial dysfunction also causes with a decrease in vascular endothelial growth factor VEGF. The resulting placental ischemia leads to an increase in angiogenic markers such as tyrosine kinase-1 (sFlt-1) such as soluble fms and soluble endoglin (sEng). sFlt-1 binds to and lowers levels of vascular endothelial growth factor (VEGF) and placental growth factor, which are important mediators of endothelial cell function, especially in fenestrated endothelium (brain, liver, glomeruli) (Ives et al., 2020)

A proper balance of pro and anti-angiogenic factors is necessary to regulate blood vessel formation and maintain vascular function. Vascular endothelial growth factor (VEGF) is not only pro-angiogenic but also important in maintaining endothelial and vascular health (Flint et al., 2019)

In this study, the expression of VEGF in positive controls decreased when compared to negative controls with a p-value of 0.022. So it can be concluded that giving L-NAME 125 mg/kgBW can cause preeclampsia conditions which result in changes in the liver in the form of decreased VEGF expression. Hal ini sejalan dengan penelitian yang This is in

line with research conducted by (Ives et al., 2020) found that decreased VEGF expression also causes liver dysfunction and thrombocytopenia and research (Guerby et al., 2021b) which states that free circulating levels of VEGF and PlGF decrease in preeclampsia patients, resulting in anti-angiogenic imbalance leading to maternal endothelial dysfunction.

Statins are one type of drug that can reduce LDL-c (Low-Density Lipoprotein cholesterol) levels in serum by inhibiting HMG-CoA (Hydroxymethylglutaryl-Coenzyme A), which plays an important role in the synthesis of endogenous cholesterol. Currently, there are two types of statins in use, namely natural and synthetic statins. Statins have a pleiotropic effect that does not depend on cholesterol. This cholesterol-free effect has therapeutic potential and is involved in angiogenesis, immunological response, anti-inflammatory response, and antioxidant benefits. Statins have also been linked to placental conditions, where the angiogenic effects of statins have potential in the treatment of preeclampsia (Kumasawa *et al*., 2020). Kemampuan statin untuk meningkatkan ekspresi eNOS, yang meningkatkan The ability of statins to increase the expression of eNOS, which increases the formation of NO and promotes vascular relaxation. Statins have also been shown to increase the expression of tissue-type plasminogen (t-PA) activators and reduce the expression of the strong vasoconstrictor ET-1, as well as restore eNOS activity in pathological states. Statins also promote the proliferation, migration, and survival of circulating endothelial progenitor cells, which are important for angiogenesis and endothelial recovery after injury (Smith & Costantine, 2022).

VEGF and eNOS expression was higher in the treatment group than in the positive control group in P1, P2, and P3 after receiving pravastatin doses of 2, 4, and 8 mg/kg body weight/day for 7 days. Administration of pravastatin in a mouse model of preeclampsia is believed to increase the expression of VEGF and eNOS. VEGF and eNOS expression in a mouse model of preeclampsia increased with increased dose of pravastatin. In this study, a dose of 8 mg/kgBB was the optimal dose because the expression of VEGF with a p-value of 0.015 and eNOS with a p-value of 0.000 had a significant difference compared to the positive control group. In line with research

(Smith & Costantine, 2022) which states that statins regulate eNOS, increasing the production of NO in blood vessels. Statins also increase the release of VEGF and PlGF, reduce sflt-1 and sEng concentrations, and regulate transcription and expression of HO-1 in endothelial smooth muscle and blood vessels and research by (Mészáros et al., 2023) mentions pravastatin increases microsomal arginine uptake thereby inducing NO synthesis which has a positive effect on microcirculation. There are lipophilic and hydrophilic statins. Pravastatin may correct the characteristics of insufficient supply of NO in preeclampsia. In the human trophoblast-like cell line (HUVEC), pravastatin increases endothelial NO-synthase expression and promotes eNOS activity by phosphorylating activating eNOS.

A limitation of this study is that researchers did not observe in the control group negatively with pravastatin administration. So researchers do not know the effect of giving pravastatin on the prevention of preeclampsia.

CONCLUSION

Pravastatin telah terbukti meningkatkan ekspresi VEGF dan eNOS pada liver model tikus Pravastatin has been shown to increase VEGF and eNOS expression in a mouse model of liver preeclampsia (Rattus norvegicus). VEGF and eNOS expression in a mouse model liver of preeclampsia (Rattus norvegicus) increased with increased dose of pravastatin. The optimal dose of pravastatin in VEGF and eNOS expression is 8 mg/kgBW.

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CONFLICT OF INTEREST

We as authors by stating that there is no potential conflict of interest.

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