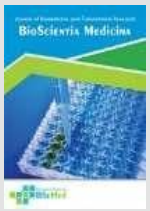
eISSN (Online): 2598-0580



Bioscientia Medicina: Journal of Biomedicine & Translational Research

Journal Homepage: www.bioscmed.com

The Effect of Pravastatin on Hypoxia Inducible Factor-1-Alpha (HIF-1-A) Expression on the Placenta of Preeclampsia Wistar Rats

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ARTICLE INFO

Keywords:
HIF-1-A
In vivo
Pravastatin
Preeclampsia
Pregnancy

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All authors have reviewed and approved the final version of the manuscript.

https://doi.org/10.37275/bsm.v6i14.658

A B S T R A C T

Background: Preeclampsia (PE) is hypertension in pregnancy that currently occurs starting from the time of placentation and is 1 of the five leading causes of maternal death. The main cause of preeclampsia is the placenta, which is hypoxic, and the spiral artery remodeling fails. Hypoxia-inducible factor-1-alpha (HIF-1-A), a marker in the nucleus, is overexpressed during tissue hypoxia. Pravastatin has a pleiotropic effect as the synthesis of nitric oxide (NO), a strong vasodilator to correct hypoxia. This study aims to explore the potential of pravastatin against functional abnormalities or placental hypoxia through in vivo HIF-1-A expression. **Methods:** This study is an experimental study using female Wistar rats (*Rattus norvegicus*) induced by preeclampsia. Assessment of HIF-1-A expression was carried out by immunohistochemistry. Data analysis was performed using SPSS, and then univariate and bivariate tests were performed. **Results:** Administration of pravastatin at a dose of 10 mg/kg BW showed the most optimal potential in reducing the expression of HIF-1-A protein, indicating tissue hypoxia. Pravastatin doses of 2.5 mg/kg BW and 5 mg/kg BW also could reduce HIF-1-A protein expression better than the K+ group who were not given pravastatin. **Conclusion:** Pravastatin can reduce the expression of HIF-1-A protein in pre-eclamptic rats, which indicates the potential of pravastatin to reduce the incidence of preeclampsia.

1. Introduction

Preeclampsia (PE) is defined as hypertension related to pregnancy accompanied with or without proteinuria with clinical manifestations of other organs.[1,2] PE occurs from week 20th of pregnancy to six weeks after delivery.[3,4] It is a high cause of maternal mortality. Ten million women develop preeclampsia each year worldwide. Worldwide, about 76,000 pregnant women die each year from preeclampsia.[5]

In preeclampsia, there is a decrease in vascular endothelial growth factor (VEGF) levels and an increase in circulating soluble VEGF receptors, namely soluble Fms-like tyrosine kinase-1 (sFLT-1).[6-8] VEGF is the main key used by hypoxic or oxygen-deprived cells to trigger the growth or remodeling of blood vessels.[9] Under conditions of placental hypoxia, it causes overexpression of hypoxia-inducible factor-1 (HIF-1) in placental tissue and blood plasma. HIF-1 is essential in regulating the transcription of various genes that arise under hypoxic conditions.[10] HIF-1 consists of two subunits expressed in hypoxia-inducible factor-1-beta (HIF-1β) and hypoxia-inducible factor-1-alpha (HIF-1α). HIF-1α or HIF-1-A plays an essential role in hypoxic signaling and

regulates trophoblast differentiation and the expression of transforming growth factor-3 (TGFβ3), which is involved in oxygen differentiation processes in placental development and various pregnancy disorders.[11,12] HIF-1-A and TGFβ3 are widely expressed in the placental tissue of patients with preeclampsia, so it is said that HIF-1-A expression is a good indicator of functional abnormalities or hypoxia in the placenta.[13]

Prevention and treatment of preeclampsia are being developed using various drugs, but not all are effective or have limitations. Recent in vitro studies have shown that pravastatin has the potential to be a preventive therapy that can be considered for use in preeclampsia.[14,15] pravastatin works as an enzyme 3-hydroxy-3methyl-glutaryl-coenzyme (HMG-CoA reductase) inhibitor in the liver, which will reduce LDL levels. However, pravastatin is also believed to have pleiotropic effects as anti-inflammatory, anti-oxidant, anti-thrombotic, pro-angiogenic, and protective of the endothelium.[16] This study aims as one of the initial studies to explore the potential of pravastatin against functional abnormalities or placental hypoxia through in vivo HIF-1-A expression.

2. Methods

This study is an experimental study with a post-test approach with a control group design. The research subjects were 30 female Wistar rats (*Rattus norvegicus*) and 30 male rats. Adult female rats (three months old, 200-250 g) were injected with 5 IU of Pregnant Mare Serum Gonadotropin (PMSG) hormone, and 48 hours later, 5 IU of Human Chorionic Gonadotropin (hCG) was injected. The female rats were put into a cage containing one male rat aged seven months weighing $\pm$60 grams heavier than the female. After 17 hours, an assessment of the presence of a copulatory plug (a plug that covers the rat's vagina from the cervix to the vulva) was assessed to determine the diagnosis of pregnancy. Pregnant female rats were grouped into five groups (every 6 rats per group). Group K- (a group of pregnant rats without induction and treatment); K+: a group of rats induced with preeclampsia, without pravastatin administration; P1, P2, and P3: a group of rats induced with preeclampsia and receiving pravastatin 2.5 mg/kg BW, 5 mg/day kg BW and 10 mg/kg BW. Preeclampsia was induced by injection of L-NAME (L-Arg-methyl Ester) in female rats for seven consecutive days (25 ηg/day; 7-18 gestation). Blood pressure assessment was carried out by Kent Scientific CODA using Volume Pressure Recording (VPR) tail cuff auto-pickup technique. This study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia.

On the 20th day of pregnancy, the rats which appeared the preeclampsia symptoms were terminated in all groups. The rats were then dissected and first anaesthetized using chloroform. After opening the abdominal cavity, the placenta was taken from the implantation side until both the fetal and maternal sides could be evaluated, put into a pot containing 10% Neutral Buffer Formalin, and euthanized the rats. Furthermore, the placenta was dehydrated in graded alcohol concentrations, namely 70%, 80%, and 90% alcohol absolute I, absolute II for 2 hours each. Then it was purified with xylol and printed using paraffin so that the preparation was printed in a paraffin block and stored in the refrigerator. The paraffin blocks were then cut into thin strips of 5-6 μm using a microtome. The object glass was washed with PBS pH 7.4 (to clean protein debris that might cover the material to be observed) twice for 5 minutes. Drops with endogenous peroxidase methanol H_2O_2 0.3% (to eliminate endogenous peroxidase activity) for 15 minutes, then rinse with running water for 5 minutes and wash again with distilled water for 5 minutes. Wash again using PBS for 2 x 5 minutes and drip with blocking serum. Drain, then drip with the prepared HIF 1-A monoclonal antibody. Incubate at 40^{o}C for 18 hours. Wash with PBS again for 2x5 minutes. Drop with secondary antibody for 10 minutes. Drop with streptavidin for 10 minutes, wash with PBS for 2x5 minutes, then give peroxidase enzyme substrate: diethyl amino benzene for 15 minutes. Wash with water for 15 minutes, drip with hematoxylin for 40 seconds, and wash with

running water for 10 minutes. Mounting, using entelan and cover with a cover glass. Observe on a light microscope. HIF-1-A expression was indicated by the golden brown colour of the chromatogen on the trophoblast. It was then examined under a Nikon Eclipse Ci light microscope equipped with a calibrated 12 Megapixel Optilab Plus Digital Camera and equipped with Image Raster 3 image processing software with 400x magnification. Expression of HIF 1-A in placental trophoblast cells was assessed semiquantitatively according to the HScore method.

Data were analyzed using SPSS (Software Package for Social Science) software. Statistical tests were carried out, namely the unpaired t-test if the data distribution was normal and the Mann-Whitney test if the data distribution was not normal. Statistical calculations in this study used a significance level of 0.05 (95% confidence interval) so that if the statistical test obtained p <0.05, it could be interpreted as significant.

3. Results

Table 1 shows the comparison of blood pressure between groups. Administration of pravastatin at a dose of 2.5 mg/kg BW showed an optimal decrease in systolic blood pressure of 29.03±1.87 compared to pravastatin at a dose of 5 mg/kg BW (26±1.88) and a dose of 10 mg/kg BW (26.5±1.76). Administration of pravastatin at a dose of 5 mg/kg BW reduced diastolic pressure by 9±0.72 the most optimally compared to pravastatin at a dose of 2.5 mg/kg BW (6±0.31) and a dose of 10 mg/kg BW (0.33±0.02). Administration of pravastatin at a dose of 5 mg/kg BW reduced MAP of 14.67±9.97 most optimally compared to pravastatin at a dose of 2.5 mg/kg BW (13.5±1.01) and a dose of 10 mg/kg BW (8.6±0.56).

Table 1. Comparison of blood pressure between groups.

	Group														
	K-			**K+**			**P1**			**P2**			**P3**		
	Pre	Post	δ	Pre	Post	δ	Pre	Post	δ	Pre	Post	δ	Pre	Post	δ
Systolic (mmHg)± SD	115,61± 3,98	130,65± 9,43	15,04± 1,78	131,12± 4,87	151,14± 8,87	20,02 ± 1,75	144,17± 9,87	115,14± 8,89	-29,03 ± 1,87*	142,83± 9,87	116,83± 9,65	-26± 1,88 *	142± 10,56	115,5 ± 4,18	-26,5± 1,76*
Diastolic (mmHg)± SD	79,5 ± 6,67	90± 8,23	10,5 ± 1,96	99,83± 6,65	119,5± 9,56	19,67 ± 1,12	80,83 ± 6,65	74,83 ± 5,44	-6± 0,31*	82,5± 5,73	73,5 ± 5,77	-9± 0,72 *	90,67± 6,34	91± 5,42	0,33± 0,02*
Mean arterial pressure (MAP) ±SD	91,56± 6,21	103,61± 8,85	12,05± 1,12	110,44± 9,77	130,11± 9,98	19,67 ± 1,21	101,94± 8,89	88,44 ± 5,42	-13,5± 1,01*	102,61± 7,76	87,94± 5,54	-14,67± 9,97 *	107,77± 8,81	99,17 ± 6,21	-8,6± 0,56*

* Independent t-test VS group K+, p<0,05.

Table 2 presents a comparison of the expression levels of HIF-1-A between groups. Administration of pravastatin at a dose of 10 mg/kg BW showed the most optimal potential in reducing the expression of HIF-1-A protein, indicating tissue hypoxia. Pravastatin doses of 2.5 mg/kg BW and 5 mg/kg BW also could reduce HIF-1-A protein expression better than the K+ group who did not receive pravastatin.

Table 2. Comparison of HIF-1-A levels between groups.

Group	HIF-1-A expression (Mean±SD)
K-	6,2±0,47
K+	9,6±0,87
P1	6,1±0,43*
P2	5,2±0,31*
P3	3,5±0,22*

* Independent t-test VS group K+, p<0,05.

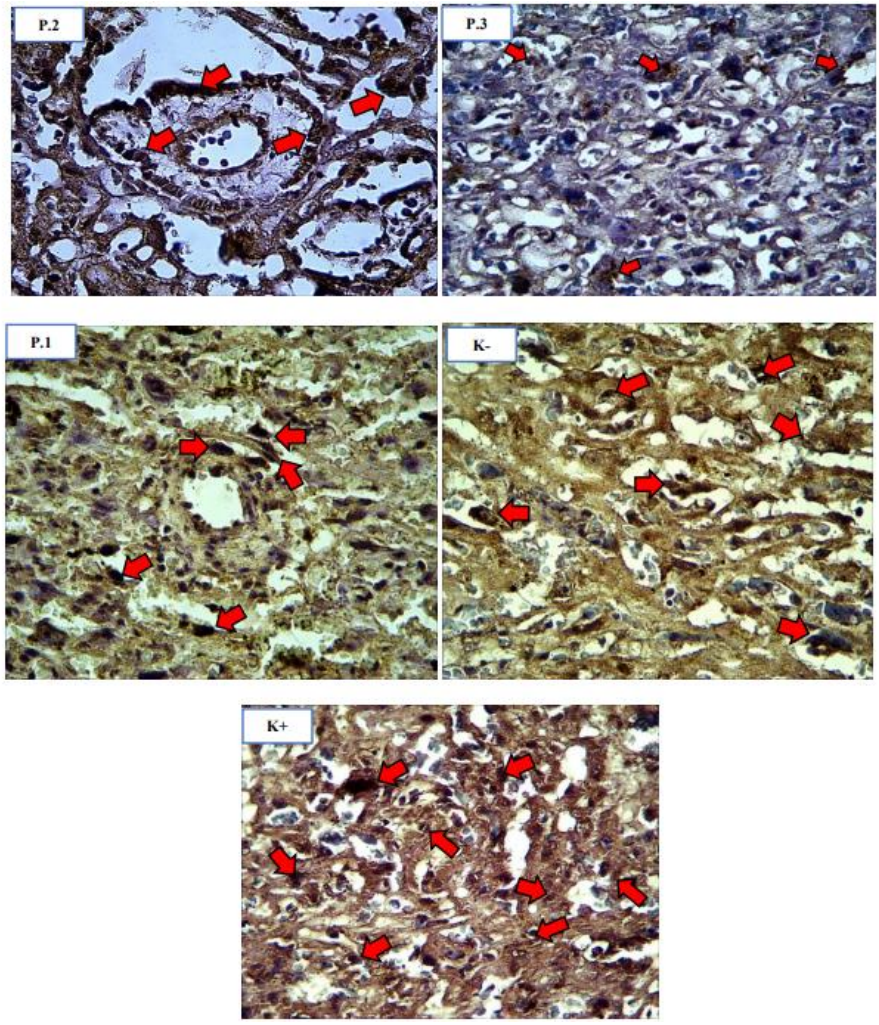

Figure 1. Immunohistochemistry of HIF-1-A protein expression (400x magnification). The red arrows indicate HIF-1-A protein expression.

4. Discussion

Nitric oxide (NO), a dissolved gas mediator, has a variety of physiological functions, including maintenance of vascular homeostasis and modulation of vascular tone.[17] During pregnancy, NO produced by eNOS (endothelial NO synthase) and iNOS (inducible NO synthase) regulates embryonic development, implantation, trophoblast invasion, development, and placental vascular function.[18] In preeclampsia conditions, ROS increases which can cause eNOS to be inhibited from producing NO, resulting in endothelial dysfunction.[19] The leading cause of increased blood pressure in pregnancy with hypertension is the lack of NO, which functions as a

vasodilator. Decreased NO levels will lead to the accumulation of HIF-1A expression. The increase in HIF-1A increases the production of sFLt-1 and sEng (soluble endoglin). Excessive production of sEng inhibits TGF- in blood vessels, inhibiting endothelial cell proliferation, angiogenesis, and vascular structure.[19,20] This condition causes endothelial dysfunction, as it is known that endothelial dysfunction is one of the early causes of preeclampsia. There is a crosstalk between HIF-1A and NO signaling.[21,22] Many cellular responses to NO are mediated by HIF-1A and vice versa. NO is also known to influence HIF-1A levels via the phosphatidylinositol 3-kinase (Pl3K) and mitogen-activated protein kinase (MAPK) signaling pathways. Statins are active substances that reduce cholesterol levels, namely low-density lipoprotein (LDL) in the blood, by competitively inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme reductase (HMG-CoA). HMG-CoA is an essential key enzyme in the synthesis process of endogenous cholesterol, so the enzyme heme oxygenase-1 (HMOx-1) is not inhibited. HMOx-1 plays a role in carbon monoxide (CO) catabolism and activates eNOS to produce NO, which as a strong vasodilator, can help maintain oxygen homeostasis in blood vessels.[20]

Statins have a positive pleiotropic effect on the NO pathway by increasing its expression activity and bioavailability through several mechanisms: stabilize cellular NO mRNA, reduce free radical oxidant formation, and reduce caveolin-1 levels in plasma membranes.[14] Statins also stimulate Akt activation (by inducing its translocation) to discrete sites, leading to the phosphorylation of eNOS and directly activating NO. Statins also induce Heat Shock Protein-90 (HSP-90), which interacts with Akt to increase NO activity. The better safety profile of pravastatin makes it the preferred statin for treatment in pregnant women.[23,24] Therefore, in this study, the experimental drug tested in preeclampsia rats was pravastatin. Pravastatin is one of the low-potency statins compared to other statins due to its hydrophilicity, so it has minimal penetration through lipophilic membranes of peripheral cells, increases the selectivity for hepatic tissue, and reduces side effects.[23]

5. Conclusion

Pravastatin can reduce the expression of HIF-1-A protein in pre-eclamptic white rats, which indicates the potential of pravastatin to reduce the incidence of preeclampsia.

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