Low-dose of Acetylsalicylic Acid Upregulates Expression of eNOS mRNA and Downregulates Interleukin-6 (IL-6) and Transforming Growth Gactor-β1 (TGF-β1) mRNA in Rat Kidney of Preeclampsia Model

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Abstract: Preeclampsia leads to endothelial dysfunction and kidney injury with inflammation and fibrosis. Low-dose acetylsalicylic acid (ASA) administration may decrease uterine artery resistance; however, its effect on the kidney has not been elucidated yet. We performed a preeclampsia model in pregnant female Wistar rats (PE group, n=5, 150-200 grams) using L-NAME 50mg/kg of BW intraperitoneal injection on day 1-18 of pregnancy. Low doses of ASA with dose 75 (PE+ASA75) and 125 (PE+ASA125) mg/kg body weight were administered in preeclampsia rats in the day 10-12 day of pregnancy. The Control group was normal pregnant rats with NaCl treatment (n=5). On day 18, rats were sacrificed; kidneys were harvested, then extracted for Reverse Transcriptase-PCR (RT-PCR) of eNOS, TGF-β1, and IL-6 mRNA expression measurements. Proteinuria and rat blood pressure were measured before termination. L-NAME injection-induced preeclampsia showed significantly higher systolic blood pressure and proteinuria in the PE group than in the control group ($p<0.05$). However, there were no changes in podocin and nephrin expression. In conclusion, the low dose of ASA 125mg/Kg BW ameliorates kidney inflammation and TGF-β1 expression in the rat preeclampsia model's kidney.

1 INTRODUCTION

The mortality of pregnant women still becomes a health problem, especially in developing countries. The major causes of maternal mortality during 2010-2013 are bleeding, hypertension during pregnancy, and abortion (Linggardini and Aprilina, 2016). In recent years preeclampsia and eclampsia are the leading causes of maternal mortality (Pellicer et al.,

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2011), with the number of preeclampsia in developing countries ranging from 2-8%. Preeclampsia is a condition in pregnancy characterized by hypertension (systolic pressure ≥140 mmHg and diastole ≥90 mmHg) and proteinuria ≥300 mg in24 hours. There is still no effective treatment for preeclampsia (Kemenkes RI, 2014; Rachmi and Sulistyono, 2016).

The pathogenesis of preeclampsia is related to trophoblast invasion of the spiral arteries of the uterus. This condition causes decreased uteroplacental blood flow and placental ischemia. Placental ischemia triggers tissue hypoxia, a release of oxidative stress, and anti-angiogenic factors' release into the maternal circulation, such as sFlt1. Increased levels of sFlt1 can cause a decrease in vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) levels. VEGF and PIGF play a role in maintaining the integrity of endothelial cells in the body. Decreased VEGF and PIGF in preeclampsia are believed to be the cause of systemic endothelial cell dysfunction and microangiopathy (Oparil et al., 2003).

One genetic predisposition that plays a role in triggering preeclampsia is an endothelial nitric oxide synthase (eNOS) gene disorder regulating nitric oxide activation. Nitric oxide (NO) is an endothelial vasodilator with functions as antithrombotic and atheroprotective. Under normal circumstances, the NO pathway is activated and increases the levels of NO in vessels. Increased NO levels are responsible for the maternal vasodilatation required to accommodate increased circulating volume during pregnancy without raising blood pressure. However, in preeclampsia, this process of adaptation is disrupted, an endothelial disorder occurs. Thus blood pressure increases, and proteinuria develops. The decrease in NO production is associated with polymorphisms in genes that regulate NO production, namely the endothelial nitric oxide synthase (eNOS) gene (Suharto et al., 2014).

Endothelial impairment in the kidneys caused by a decrease in VEGF will lead to glomerular capillary endothelins and proteinuria. Several studies have shown that interleukin-6 (IL-6) and transforming growth factor-β1 (TGF-β1) play a role in renal disease progression. IL-6 can cause kidney disease by increasing the tubular epithelial cell signal response to a pro-fibrotic growth factor such as TGF-β1. Increased expression of TGF-β1 mRNA may induce renal fibrosis by producing extracellular matrix (ECM). Fibrosis can lead to more severe tubulointerstitial damage resulting in a progressive decline in the nephron and renal function. The IL-6 and TGF-β1 role, both acutely and chronically in renal tubular damage in the preeclampsia model, has not been widely known (Munkhaugen and Vikse, 2009; Jones et al., 2015).

One of the therapeutic management in the case of preeclampsia is the administration of acetylsalicylic acid. Low-acetyl-salicylic acid administration may improve blood vessel circulation and prevent vasoconstriction, resulting in increased organ perfusion and preventable kidney damage. Acetyl-salicylic acid can also stimulate the activation of eNOS that catalyzes NO synthesis for hypertensive patients in preeclampsia (Danuyanti et al., 2018). Therefore, we need to observe the effect of low dose acetylsalicylic acid administration on renal function through eNOS, IL-6, TGF-β expression of mRNA on the preeclampsia model.

2 MATERIALS AND METHODS

2.1 Preparation of Experimental Animal

This research used Wistar strain female rat obtained from Integrated Research and Testing Laboratory (LPPT) Universitas Gadjah Mada with number 00104/04/LPPT/VIII/2017. Wistar strain female rats with age ± 12 weeks, and bodyweight 150-200 grams were used for the experiment. The rats were divided into 4 groups: Control group (normotensive pregnant rat), PE group (preeclamptic model rat induced with L-NAME of doses 50 mg/kg BW/day from the first day to 18th day of pregnancy), PE+ASA75 group (preeclamptic model and treatment of acetyl-salicylate acid of doses at 75 mg/Kg BW from the tenth day until the twelfth day of pregnancy), and PE+ASA125 group (preeclamptic model rad and treatment of acetyl-salicylate acid of doses at 125 mg/Kg BW). The doses of ASA was determined by rat bodyweight that was quantified daily.

2.2 Process of Impregnating Rat

The rats' conception was performed at the Faculty of Pharmacy Laboratory Unit V by placing one male rat and two female rats in one cage. The experimental animals were mixed in the afternoon at around 4 pm and then check for the vaginal plugs (copulation plug) on the following morning at 6 am. The presence of vaginal plugs (copulation plug) in animals was calculated as the 1st day of pregnancy (Han et al., 2015; Kaya et al., 2011).

2.3 Process of Measuring Blood Pressure, Proteinuria, and Preeclampsia Induction

Blood pressure measurement of an experimental animal was performed using a non-invasive sphygmomanometer. Blood pressure was measured five times during the study: a day before mating Day-0 (D-0), the 5th day of pregnancy (D-5), D-8, D-11, D-13, and before surgery (D-18). Examination of proteinuria using uriscan 3 GPH strips. Proteinuria was measured on the day before mating (D-0), the 6th day of pregnancy (D-6), D-9, D-12, D-14, and D-19. Preeclampsia induction was done by dissolving L-NAME 50 mg/kg BW/day with the gastric probe on the first day of pregnancy until the 18th day of pregnancy (Szalai et al., 2015). Each rat weighing 150-200 g was given L-NAME of 1.5 to 2 mL per rat.

2.4 Acetyl-salicylate Acid Therapy and Proteinuria Examination

Acetyl-salicylate acid therapy was performed by dissolving 75 mg and 125 mg tablets using aquadest first. Acetyl-salicylate acid was given orally with the probe to mice at 10 to 12 days of gestational age. Each preeclampsia mice with a weight range of 150-200 grams got acetyl-salicylate acid therapy that has been diluted as much as 1.35 to 1,8 mL/day in 10 to 12 days of gestational age. Proteinuria examinations using uriscan 3 GPH strips were performed at: days before mating (D-0), the 5th day of pregnancy (D-5), D-8, D-11, D-13 D-18.

2.5 Kidney Harvesting

After being treated for eighteen days, all groups were terminated 24 hours after the treatment. After anesthetized using ketamine (dose 60-100 mg/kg BW), rats were sacrificed with a lethal dose of ketamine. Rat's abdomen and thorax were opened, kidneys were harvested. The left kidney was kept in RNA later® for RNA extraction, and the right kidney was fixated in 4% PFA in PBS for paraffin making.

2.6 RNA Extraction, cDNA Making, and Reverse Transcription-PCR (RT-PCR)

Examination of eNOS, TGF-β1, IL-6, and GAPDH mRNA expression was done using RT-PCR (Transcription Reaction of Polymerase Chain Reactions). Kidney tissue was extracted using Trizol RNA solution (GENEzolTM; Cat. No. GZR100). RNA concentrations were quantified using nanodrop. The cDNA was synthesized using 5xRT-buffer (Toyobo, TRT-101), random primer (TAKARA®, 3801), dNTP (TAKARA®, 4030), ReverTra-Ace (Toyobo®; TRT-101).Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to examine the following cDNAs: eNOS 5’ -GTCCTGCAAACCGTGCAGAG-3’ (forward) and 5- TGGGTGCGCAATGTGAGTC-3 ’ (reverse); TGF- β 1 5-CCGTGGCTTC TAGTGCTGAC-3' (forward), and 5'-GGCGTTGTTGCG TTAGATAC-3' (reverse); IL-6 5-GCCCTTCAGGA ACAGCTATGA-3′ (forward) and 5′ -TGTCAA CAACATCAGTCCCAAAGA-3 ′ (reverse); and GAPDH 5’-CCCCCAATGTATCCGTTGTG-3’ (forward) and 5'TAGCCCAGGATGCCCTTGAGT-3'(reverse). Then a 35 cycle PCR was carried out with a denaturation condition of 94° C for 10 seconds, annealing at 60° C for 30 seconds and an extension of 72° C for the 1-minute final extension phase ending with a 72° C condition for 10 minutes.

2.7 Histopathological Examination

The kidney paraffin blocks were cut in 4 μm thickness for Periodic Acid Schiff (PAS) staining to assess glomerulosclerosis. The preparation was examined under a light microscope (Olympus CX22®), an image was captured using OptiLab software in 400× magnification with a random area.

2.8 Statistical Analysis

Data were analyzed using a one-way ANOVA test for normally distributed data and Kruskal-Wallis for data that were not normally distributed. The value of $p<0.05$ was considered statistically significant. Statistical analyses were accomplished using SPSS Software version 23 (SPSS Inc., Chicago).

2.9 PE Condition after L-NAME Treatment

L-NAME treatment in pregnant rats induced PE conditions as shown by higher proteinuria scores and higher systolic blood pressure in the PE group than the control group (Fig.1). However, both ASA treated groups did not demonstrate attenuation of blood pressure and proteinuria score.

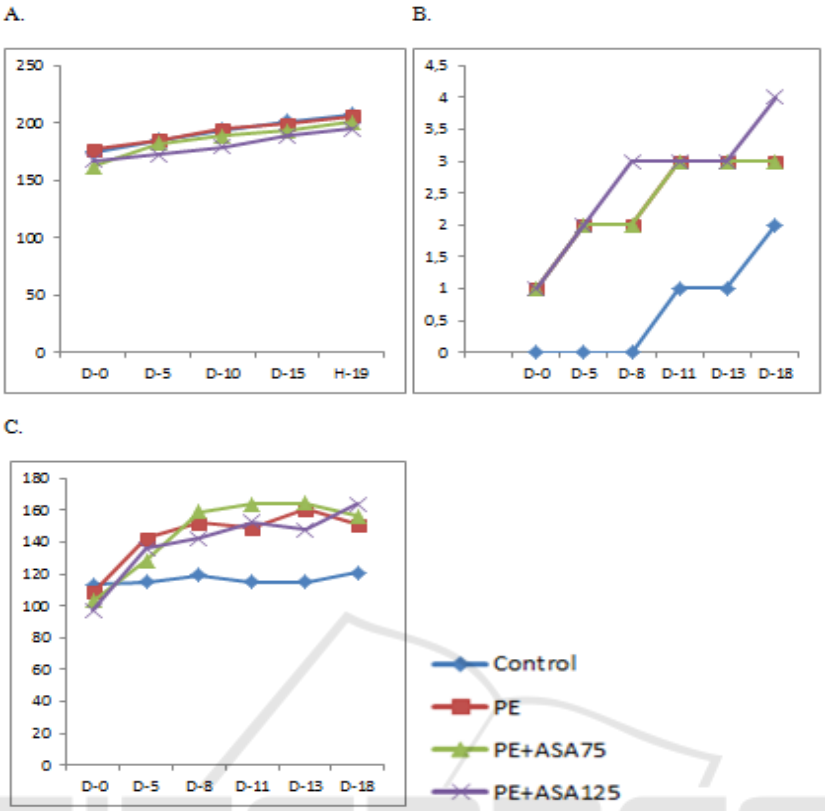

Figure 1: L-Name treatment demonstrated PE condition with increasing body weight, proteinuria, systolic blood pressure. The ASA treatment could not attenuate PE condition based on proteinuria score and systolic blood pressure. (A) Representative data of mean body weight from the groups. (B) Mean proteinuria of the groups. (C) Systolic blood pressure of the groups.

3 RESULTS

Low-dose of acetylsalicylic acid upregulates expression of eNOS and down-regulates interleukin-6 (IL-6) and transforming growth factor-β1 (TGF-β1).

The expression of IL-6 mRNA was described as the inflammatory condition of the kidney after PE. Meanwhile, TGF-β1 expression was represented chronic effect and pro-fibrotic substance. PE induction led to significantly higher IL-6 and TGF-β1 mRNA compared to the Control group. RT-PCR analysis was demonstrated the beneficial effects of ASA treatment. ASA treated groups was significantly lower IL-6 and TGF- β1 mRNA expression compared to the PE group. PE+ASA125 group was showed the lowest IL-6 and TGF- β1 mRNA expression. This group was significantly lower than the PE+ASA75 group.

4 DISCUSSION

This study revealed that a low dose of ASA treatment did not attenuate blood pressure and proteinuria in the PE model. However, low doses of ASA treatment had beneficial effects in inducing eNOS upregulation and reducing inflammation. ASA125 treatment induced higher eNOS mRNA expression based on this study. It seemed that acetylsalicylic acid might increase eNOS expression in endothelial dysfunction. Acetylsalicylic acid can stimulate the activation of eNOS, which can increase NO production for hypertensive patients in preeclampsia (Suharto et al., 2014). Nitric oxide can help oxygen transport by widening the blood vessel wall to facilitate the transfer of gas from the blood to the tissue. After NOS synthesizes NO from L-arginine, NO diffuses from endothelial cells to smooth muscle cells of the blood vessels and causes an increase in intracellular cyclic guanosine monophosphate (cGMP). Increased cGMP will trigger the relaxation of blood vessel muscles to

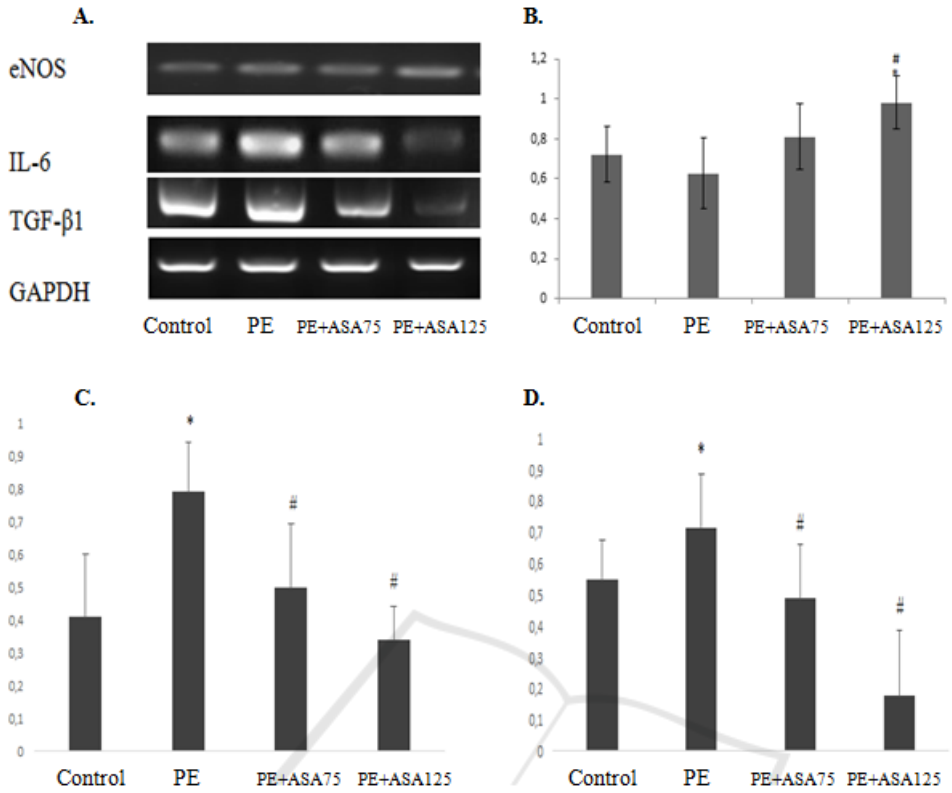

Figure 2. The effect of low-acetyl-salicylic acid on the increased expression of eNOS mRNA and decreased expression of IL-6 and TGF-β1 mRNA to renal failure in preeclampsia model animals. (A) Representative picture of endothelial function (eNOS), inflammation marker (IL-6), and fibrosis agent (TGF-β1) expression using RT-PCR. (B) Densitometry analysis of eNOS mRNA expression from PE kidney model (C) Densitometry analysis of IL-6 mRNA expression from PE kidney model PE. (D)Densitometry analysis of TGF-β1 mRNA expression from PE kidney model. (E) Representative picture of glomerulosclerosis based on Periodic Acid-Schiff (PAS) staining in 400× magnification.*:p<0.05 vs control; ##:p<0.01 vs. PE.***:p<.001 vs PE; ##:p<0.01 vs control.

become a vasodilator to suppress hypertension. Nitric oxide is known to have properties as an inhibitor of platelet activation (Jones et al., 2015, Burke et al., 2016)).

The results showed that preeclampsia's condition tends to increase the expression of IL-6 and TGF-β1 mRNA, which is often associated with inflammation and fibrosis. The low dose of ASA treatment in this study had beneficial effects in lowering IL-6 and TGF-β1 expression. IL-6 is a pleiotropic cytokine involved in the regulation of the immune response and inflammation. IL-6 has biological properties such as activating transducer signals and activation of transcription factor STAT3 in renal tubular cells, stimulation of expression of tissue factor, MCP-1, matrix degeneration enzyme, and low-density lipoprotein receptor on macrophage (Jones et al., 2015). The results of this study can be seen that IL-6 expression tends to increase in preeclamptic condition compared with the control group. This condition may be due to preeclampsia, the occurrence of placental ischemia may contribute to maternal endothelial cell dysfunction by increasing the synthesis of IL-6, TNF-α, and IL-8 (Creasy et al., 2004).

An imbalance between vasoconstrictor and vasodilator might induce kidney injury with inflammation and fibrosis. We found that upregulation of eNOS as vasodilator function in this study after ASA 125 mg/Kg BW treatment associated with attenuation of kidney inflammation and fibrotic marker. This phenomenon might relate to the function of ASA as anti-inflammatory without influencing vasodilator capacity in the kidney. A low acetyl-salicylate acid dose positively affects the balance between PGI2 as a vasodilator, TXA2 as a vasoconstrictor, and stimulant platelet aggregation. At a low dose, acetylsalicylic acid can inhibit TXA2 synthesis without affecting PGI2 synthesis in the vascular endothelium. According to Villa, research

states that a decrease in TXA2 production without a decrease in PGI2 production can prevent vasoconstriction and coagulation problems characteristic of preeclampsia (Villa et al., 2013).

We further examined the effect of low-dose acetylsalicylic acid administration on the expression of TGF-β1 as a pro-fibrotic factor in the renal model of preeclampsia. TGF-β1 is a pro-fibrotic growth factor that induces renal fibrosis by producing an extracellular matrix. In this study's results, it can be seen that TGF-β1 expression tends to increase in preeclamptic condition compared with the control group. This phenomenon may occur because, in preeclampsia conditions, high urinary protein content may cause pro-inflammatory and pro-fibrotic effects that contribute to tubulointerstitial damage and loss of renal function. The main pro-fibrotic factor involved in this process is TGF-β1 (Kuusniemi et al., 2005). Increased expression of TGF-β1 may induce renal fibrosis.

5 CONCLUSION

In conclusion, low doses of ASA treatment might attenuate inflammation and fibrosis in the kidney after PE induction. These effects might be associated with high eNOS mRNA expression.

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