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Short Communication

DOCA and L-NAME hydrochloride: Their impact on T regulatory cells, macrophage activity, and pro- and anti-inflammatory cytokine profiles in pre-eclampsia animal model

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Abstract

Deoxycorticosterone acetate (DOCA) and N-nitro-L-arginine methyl ester (L-NAME) hydrochloride have been well-reported as pre-eclampsia inducers due to their ability to mimic hypertension, endothelial dysfunction, and inflammatory response. However, no study has compared the two inducers in developing a mice model of preeclampsia characterized by proinflammatory and anti-inflammatory parameters. The aim of this study was to investigate the efficacy of DOCA and L-NAME hydrochloride in inducing pre-eclampsia in pregnant mice, focusing on the expression of regulatory T cells (Tregs), macrophages, anti-inflammatory cytokines TGF-β, and pro-inflammatory cytokines (IL-6 and IL-1β). Twenty-seven female BALB/c mice were grouped into three groups (n=9): healthy pregnant mice (NP), pregnant mice induced with DOCA (PD), and pregnant mice induced with L-NAME hydrochloride (PL). L-NAME hydrochloride was orally given to the pregnant mice at 4.464 mg/30 g body weight (BW) every day after five days of gestation. DOCA was injected subcutaneously in 0.1 mL of corn oil at 0.74 mg/30 g BW before mating and 0.38 mg/30 g BW once a week until dissection. Drinking water for PD and PL groups was replaced with 0.9% saline. On day 16 of pregnancy, the lymphocytes were isolated from the spleen to determine the profile of Tregs, macrophages, TGF-β, IL-6, and IL-1β using flow cytometry analysis. The results showed that administering L-NAME hydrochloride in pregnant mice exhibited a significant increase in the relative number of IL-1β and macrophages compared to DOCA (p<0.05). L-NAME hydrochloride significantly reduced the production of TGF-β compared to DOCA (p<0.05). Both DOCA and L-NAME hydrochloride could decrease Tregs and IL-6 levels. This study also found that L-NAME hydrochloride was more effective in inducing pre-eclampsia in pregnant BALB/c mice than DOCA indicated by the highest increase in pro-inflammatory cytokines and macrophage activity and a low anti-inflammatory cytokine. The present study provides a foundation for understanding the pathophysiological mechanisms of preeclampsia in the inflammatory pathway; however, further exploration of other mechanisms, markers, and target proteins can deepen insights into its development.

Keywords: Pre-eclampsia, eclampsia, inflammation, DOCA, L-NAME

Introduction

Pre-eclampsia is one of the pregnancy complications that becomes the primary cause of high maternal and child mortality rates in developing countries [1]. The prevalence of pre-eclampsia

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in pregnant women worldwide is around 3–5% with the second highest cause of death in pregnant women. In Indonesia, the incidence rate of pre-eclampsia reached approximately 24% in 2017 [2]. Pre-eclampsia is characterized by vascular endothelial dysfunction that occurs after 20 weeks of pregnancy, an increase in systolic blood pressure of 140 mmHg or diastolic pressure equal to or greater than 90 mmHg, and high proteinuria level (300 mg or more than 30 mg/dL per 24 hours) [3]. Pre-eclampsia is a complex disease with both maternal and fetal risks, and the underlying mechanisms and causes are not fully understood. During pregnancy, the maternal immune system undergoes significant changes [4]. Instead of attacking the fetus, which is considered foreign, the maternal immune system protects and tolerates it, this process is known as immune tolerance. Immune tolerance is essential to protect the fetus, which ensures the flow of nutrients and oxygen to the fetus and also prevents inflammation that is caused by excessive activation of the immune system that can harm the fetus and lead to pre-eclampsia [5]. Some factors that can trigger immune tolerance failure in pre-eclampsia include extensive invasion of trophoblast cells into the myometrial blood vessel layer and uterine spiral arteries; activation of the complement system failing to recognize trophoblast cells as self; endothelial dysfunction; and an imbalance between T helper 2 (Th2) cells and T helper 1 (Th1) cells [6].

Animal models have been useful in studying the pathophysiology, mediators, and even therapy options for various disorders, including preeclampsia. Previous studies have been conducted on pathophysiological mechanisms in preeclampsia to explore the best model of pre-eclampsia-like syndrome [7,8]. The induction of adenovirus expressing soluble fms-like tyrosine kinase-1 (sFlt-1) in pregnant rats caused hypertension, proteinuria, and glomerular endothelial swelling on the last day of pregnancy, which are the signs of pre-eclampsia-like syndrome [9]. The most common inducers to develop pre-eclampsia models are Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME hydrochloride) and deoxycorticosterone acetate (DOCA). The administration of DOCA and saline 0.9% as drinking water simulates disturbances in sodium excretion and excessive expansion of extracellular fluid volume by triggering increased sodium and water retention in the body which can increase blood volume as well as blood pressure [10]. This suggested that DOCA administration mimics the mechanism of hypertension induced by increased fluid volume that occurs in preeclampsia [11]. L-NAME hydrochloride can be used to decrease nitric oxide (NO) generation and then induce preeclampsia pathogenesis with early onset as well as late-onset [12].

Both inducers can decrease NO production in the body, leading to endothelial dysfunction [13]. L-NAME hydrochloride is an inhibitor of nitric oxide synthase enzyme, which produces NO, a molecule crucial in regulating blood vessel function and blood pressure [14]. Reduced NO can lead to endothelial dysfunction, hypertension, proteinuria, and placental abnormalities which are similar to the pathophysiology of pre-eclampsia [15]. DOCA is a precursor of aldosterone, a steroid hormone produced by the adrenal cortex [16]. This hormone plays a crucial role in regulating the body's electrolyte balance, particularly sodium and potassium levels, as well as blood pressure regulation [10]. Administration of DOCA to animal models can increase aldosterone levels [17]. Subsequently, aldosterone binds to mineralocorticoid receptors and attaches to specific DNA sequences within the cell nucleus that will trigger the production of collagen and fibronectin. The increase in these proteins leads to blood vessel stiffness [18]. Aldosterone can also enhance the activity of sodium channels in vascular endothelial cells and then lead to changes in the actin protein's shape, further reducing NO production [19,20]. The decrease in NO caused by aldosterone activation can lead to impaired blood circulation and endothelial dysfunction [20].

Excessive immune activation in pre-eclampsia progressively increases with elevated pro-inflammatory cytokines and anti-angiogenic factors in the intrauterine environment and endothelium, leading to placental complications and dysfunction [13]. Macrophages in pre-eclampsia conditions are generated by complement system activation and tend to differentiate into an M1 phenotype that functions to secrete interleukin-6 (IL-6) and interleukin-1β (IL-1β), contributing to a systemic inflammatory response [21]. Excessive IL-6 production leads to apoptosis and necrosis of trophoblast cells in the early stages of pre-eclampsia [22]. Overexpression of the IL-1β cytokine in pre-eclampsia causes structural and functional changes in endothelial cells, leading to oxidative stress and increased thromboxane levels, which lead to

trigger blood clotting and vasoconstriction [23]. The production of pro-inflammatory cytokines predominantly stimulates the maturation of Th1 lymphocytes and B cells, as well as the secretion of angiotensin II type I receptor antibodies (AT1-AA) [24]. AT1-AA functions to mediate the activation of endothelin I (ET-1), the oxidative stress pathway, anti-angiogenic factors (sFlt-1 and soluble endoglin), and increase sensitivity to angiotensin II. These factors can decrease transforming growth factor-β (TGF-β) and regulatory T cell (Treg) levels, positively correlating with endothelial dysfunction, placental ischemia, and hypertension during pregnancy [25].

Today, there is no definitive treatment for pre-eclampsia and its management mainly focuses on controlling symptoms and preventing complications during pregnancy until delivery. The aim of this study was to investigate the efficacy of DOCA and L-NAME hydrochloride in inducing pre-eclampsia in pregnant mice by highlighting the expression of Tregs, macrophages, anti-inflammatory cytokines TGF-β, and pro-inflammatory cytokines (IL-6 and IL-1β). This research is highly relevant for identifying the potential therapeutic targets of pre-eclampsia. By identifying new molecular targets or cellular mechanisms, this work could contribute to developing more effective treatments for pre-eclampsia and potentially other inflammatory-related disorders. The present study is expected could provide insights into better inducers for inducing pre-eclampsia by observing immune system dysregulation that plays a crucial role in the pathogenesis of pre-eclampsia.

Methods

Study design and setting

This study was true experimental research with a completely randomized design. The aim of this study was to compare the efficacy of DOCA and LNAME hydrochloride in inducing preeclampsia through the profile of pro-inflammatory cytokines (IL-6, IL-1β), anti-inflammatory cytokines (TGF-β), regulatory T cells, and macrophages. Pregnant mice were divided into three groups (nine mice in each group): (a) normal pregnant mice group (NP group) consisted of healthy pregnant mice without any intervention; (b) pregnant with L-NAME hydrochloride group (PL group) included healthy pregnant mice that were induced with L-NAME hydrochloride; and (c) pregnant mice with DOCA group (PD group) comprised healthy pregnant mice that were induced with DOCA.

Experimental animal

The present study used 27 male and 27 female BALB/c mice (*Mus musculus*) at the age of 5 and 8 weeks respectively, with the body weight of pregnant female mice ranging from 27–42 grams. Before treatment, BALB/c mice were acclimatized for 1 week with daily provision of food and water. If there were mice with hard lumps or poor physical condition, the mice were excluded from the study.

Pregnant mice preparation

Twenty-seven mice were divided into three treatment groups with nine replicates. Female and male mice were mated in a 1:1 ratio. The presence of a vaginal plug observed in female mice was checked twice daily. The first day of pregnancy was determined based on the presence of a vaginal plug in the female mice.

Pre-eclampsia mice model preparation

The pre-eclampsia mice model was obtained by induction with L-NAME hydrochloride and DOCA. L-NAME hydrochloride induction was performed according to a previous study [25] with modification of dose (from rats to mice) and the time of induction. The time of L-NAME hydrochloride was adjusted to early onset preeclampsia at the beginning of the third trimester of mice pregnancy. L-NAME hydrochloride (N5751-10G, Sigma-Aldrich, St. Louis, USA) was administered orally at a dose of 4.464 mg/30 g mice body weight (BW) starting from day 5 until day 15 of pregnant mice. DOCA (D7000-5G, Sigma-Aldrich, St. Louis, USA) was injected subcutaneously into the loose skin over the neck at a dose of 0.74 mg/30 g BW of mice before mating, and 0.38 mg/30 g BW of mice in the first week of pregnancy. Subcutaneous injections were then continued at a dose of 0.38 mg/30 g BW given once a week until dissection. DOCA was

dissolved in 0.1 mL corn oil (Mazola, MOI foods, Selangor Darul Ehsan, Malaysia). The drinking water of all mice except healthy pregnant mice was replaced with 0.9% saline [26].

Lymphocyte isolation

The protocol for lymphocyte isolation referred to a previous study [27]. The lymphocyte of each experimental group was isolated on day 16 of pregnancy. The mice were sacrificed by cervical dislocation and then dissected. The spleen was removed and washed with phosphate buffered saline (PBS). Subsequently, this organ was minced in a petri dish containing 5 mL of PBS in a clockwise direction. The cell suspension was centrifuged at 2500 rpm for 5 minutes at 10°C. The supernatant was discarded, and the pellet was suspended in 1 mL of PBS.

Expression of Tregs and macrophages

A total of 50 µL of the lymphocyte suspension was added with extracellular antibodies, including fluorescein isothiocyanate (FITC) anti-mouse CD4, phycoerythrin (PE) anti-mouse CD25, and FITC anti-mouse CD11b (clone: M1/70, BioLegend, San Diego, CA). The extracellular staining protocol followed the protocol from the previous studies [17,18]. The cell suspension containing the extracellular antibody was incubated for 20 min at 4°C in the dark. Then, it was added with 400 µL of PBS and transferred to a flow cytometry cuvette. The sample was analyzed using a flow cytometer (BD Biosciences FACSCalibur, Becton Dickinson and Company, San Jose, CA, USA). The relative number of Tregs ($CD4^+CD25^+$) and macrophages ($CD11b^+$) was analyzed using BD CellQuest Pro software (Becton Dickinson and Company, San Jose, CA) following the previous protocol [20].

Expression of anti-inflammatory cytokines TGF-β and pro-inflammatory cytokines (IL-6 and IL-1β)

The expression of TGF-β in Tregs and pro-inflammatory cytokines (IL-6 and IL-1β) were measured using flow cytometry analysis followed by extra and intracellular antibody staining. A total of 50 µL of the lymphocyte suspension was added with extracellular antibodies (fluorescein isothiocyanate (FITC) anti-mouse CD4, phycoerythrin (PE) anti-mouse CD25, and FITC anti-mouse CD11b) and incubated for 20 minutes at 4°C in the dark.

Then, intracellular staining was performed following the protocol from the previous study [30]. A total of 50 µL of Intracellular Fixation Buffer (Invitrogen, Thermo Fisher Scientific, Van Allen Way, Carlsbad, CA) was added to the cell suspension containing extracellular antibodies and incubated for 20 minutes at 4°C in the dark. After fixation, permeabilization was performed using Permeabilization Buffer 10X (Invitrogen, Thermo Fisher Scientific, Van Allen Way, Carlsbad, CA). Permeabilization buffer 10X was diluted with 10-fold in distilled water. A total of 500 µL diluted permeabilization buffer was added to the cell suspension and then centrifuged at 2500 rpm for 5 minutes at 10°C. The obtained pellet was stained with 50 µL of the following intracellular antibodies: PE-Cy5 anti-mouse IL-6, PE anti-mouse IL-1β, and PE-Cy5 anti-mouse TGF-β and incubated for 20 minutes at 4°C in the dark. The cell suspension was added with 400 µL of PBS and transferred to a flow cytometry cuvette. The sample was analyzed using a flow cytometer (BD Biosciences FACSCalibur, Becton Dickinson and Company, San Jose, CA). The relative number of each parameter was analyzed using BD CellQuest Pro software (Becton Dickinson and Company, San Jose, CA).

Statistical analysis

The relative number of each parameter that was obtained from the flow cytometry results was analyzed using a one-way analysis of variance (ANOVA), followed by a post-hoc Duncan test with statistical significance at $p<0.05$. All statistical analyses were performed using IBM SPSS Statistics for Windows ver. 22.0 (IBM, New York, USA).

Results

Effect of DOCA and L-NAME hydrochloride on regulatory T cells (Tregs)

The relative number of Tregs after DOCA and L-NAME hydrochloride administration was analyzed using the marker $CD4^+CD25^+$ T cells with flow cytometry (**Figure 1**). The

administration of both DOCA and L-NAME hydrochloride in pregnant mice caused a significant reduction in the relative number of Tregs compared to healthy pregnant mice (8.60% and 8.32%, respectively) compared to pregnant mice (10.61%) ($p<0.05$). The percentages (8.60%, 8.32%, and 10.61%) represented the proportion of $CD4^+CD25^+$ T cells relative to lymphocytes total. These results suggested that the induction of both inducers inhibited the recruitment of mature Tregs in pregnant mice.

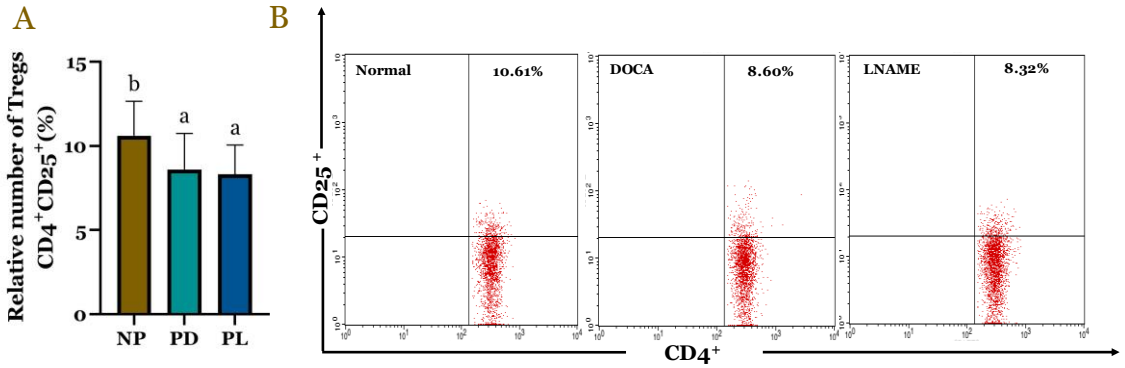

Figure 1. The relative number of Tregs ($CD4^+CD25^+$) in lymphocytes total of pregnant mice after being treated with DOCA and L-NAME hydrochloride. (A) The bar chart was obtained from the mean of the relative number of Tregs ± standard deviation (SD) in healthy pregnant mice (NP); pregnant mice with DOCA induction (PD); and pregnant mice with L-NAME hydrochloride (PL) induction (n=9). Data were analyzed using one-way ANOVA with post-hoc Duncan ($p<0.05$). Different superscripts indicate statistical differences. (B) Flow cytometry dot plot results of Tregs obtained from BD CellQuest Pro software from NP, PD, and PL groups.

Effect of DOCA and L-NAME hydrochloride on anti-inflammatory cytokines TGF-β

One of the major anti-inflammatory cytokines produced by Tregs was TGF-β. The relative number of this cytokine was analyzed from the expression of $CD4^+CD25^+TGF\text{-}\beta^+$ (**Figure 2**). The percentage of TGF-β produced by Tregs in pregnant mice after being treated with DOCA and L-NAME hydrochloride was not significantly ($p>0.05$) different from the healthy pregnant mice. However, the relative number of TGF-β to Treg total after L-NAME hydrochloride induction was significantly lower than DOCA (16.41% vs 18.82%).

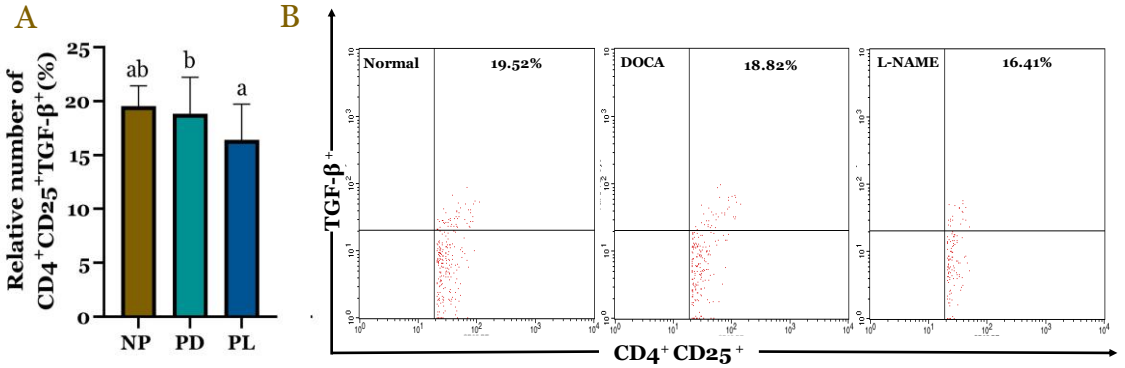

Figure 2. The relative number of TGF-β produced by Tregs ($CD4^+CD25^+TGF\text{-}\beta^+$) in pregnant mice after being treated with DOCA and L-NAME hydrochloride. (A) The bar chart was obtained from the mean of the relative number of TGF-β ± standard deviation (SD) in healthy pregnant mice (NP); pregnant mice with DOCA induction (PD); and pregnant mice with L-NAME hydrochloride (PL) induction (n=9). Data were analyzed using one-way ANOVA with post-hoc Duncan ($p<0.05$). Different superscripts indicate statistical differences. (B) Flow cytometry dot plot results of TGF-β obtained from BD CellQuest Pro software from NP, PD, and PL groups.

Effect of DOCA and L-NAME hydrochloride on CD11b expression

The expression of CD11b was highly expressed on the surface of mature macrophage cells. This study used CD11b as a marker to investigate the effect of both inducers in mature macrophage cells. The pre-eclampsia mice model with L-NAME hydrochloride induction has the highest

percentage of CD11b expression (62.85%) compared to DOCA (45.10%) and healthy pregnant mice (39.80%) (**Figure 3**). The percentages of CD11b represented the proportion of CD11b relative to granulocytes total analyzed via flow cytometry. These results indicated significant recruitment of mature macrophages after L-NAME hydrochloride treatment in pregnant conditions.

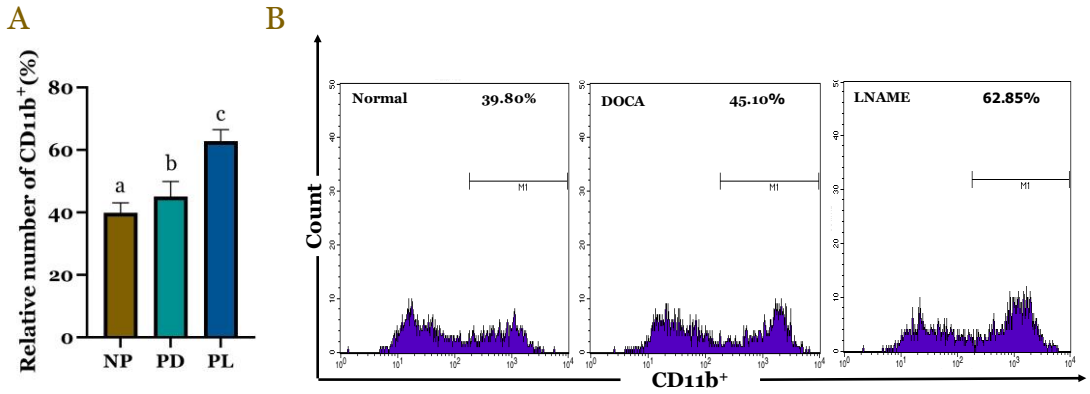

Figure 3. The relative number of macrophages (CD11b⁺) to granulocytes total in pregnant mice after being treated with DOCA and L-NAME hydrochloride. (A) The bar chart was obtained from the mean of the relative number of CD11b⁺ ± standard deviation (SD) in healthy pregnant mice (NP); pregnant mice with DOCA induction (PD); and pregnant mice with L-NAME hydrochloride (PL) induction (n=9). Data were analyzed using one-way ANOVA with post-hoc Duncan (P<0.05). Different superscripts indicate statistical differences. (B) Flow cytometry histogram of CD11b⁺ obtained from BD CellQuest Pro software from NP, PD, and PL groups.

Effect of DOCA and L-NAME hydrochloride on pro-inflammatory cytokine IL-6

Pro-inflammatory cytokines IL-6 were analyzed from the activated macrophage cells with the CD11b⁺IL-6⁺ marker (**Figure 4**). The flow cytometry analysis showed that pregnant mice after receiving L-NAME hydrochloride and DOCA had a significant (p<0.05) elevation of IL-6 (60.83% and 59.84%) compared to the healthy pregnant group (44.95%). The percentages of IL-6 represented the proportion of IL-6 relative to CD11b total analyzed via flow cytometry. These results indicated that both inducers could significantly trigger macrophage cells to secrete a high production of IL-6 and initiate the inflammatory response.

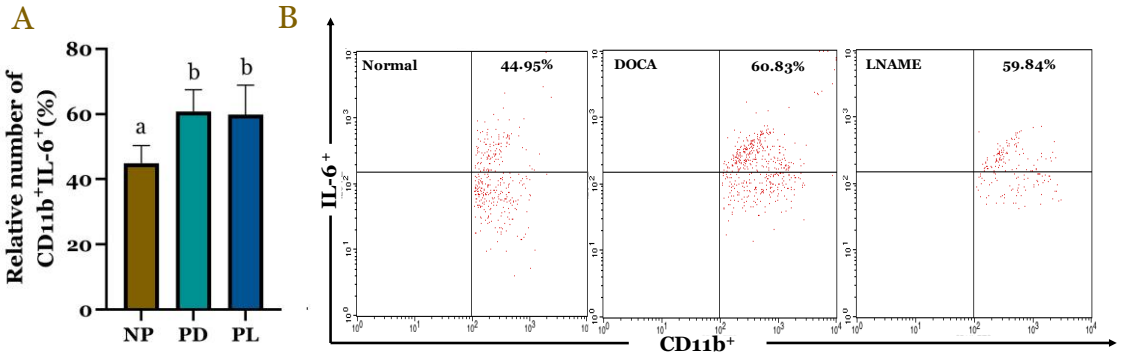

Figure 4. The relative number of IL-6 (CD11b⁺IL6⁺) in CD11b total of pregnant mice after being treated with DOCA and L-NAME hydrochloride. (A) The bar chart was obtained from the mean of the relative number of CD11b⁺ ± standard deviation (SD) in healthy pregnant mice (NP); pregnant mice with DOCA induction (PD); and pregnant mice with L-NAME hydrochloride (PL) induction (n=9). Data were analyzed using one-way ANOVA with post-hoc Duncan (p<0.05). Different superscripts indicate statistical differences. (B) Flow cytometry dot plot of CD11b⁺IL6⁺ obtained from BD CellQuest Pro software from NP, PD, and PL groups.

Effect of DOCA and L-NAME hydrochloride on pro-inflammatory cytokines IL-1β

The L-NAME hydrochloride administration group showed significantly superior results compared to the DOCA group in the analysis of the relative amount of IL-1β cytokine as shown in

Figure 5. In this case, the PL group had a relative amount percentage of IL-1β cytokine of 60.77%, which was significantly different from the NP (55.92%) and PD (52.76%) groups. The percentages of IL-1β represented the proportion of IL-1β relative to CD11b total analyzed via flow cytometry. The DOCA-injected pregnant mouse group had a lower relative amount of IL-1β cytokine compared to the normal pregnant group and was not significantly different.

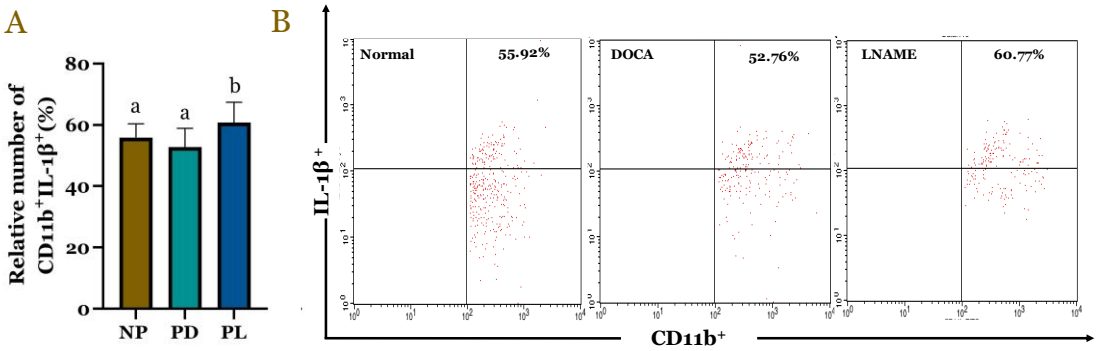

Figure 5. The relative number of macrophages ($CD11b^+IL\text{-}1\beta^+$) in pregnant mice after being treated with DOCA and L-NAME hydrochloride. (A) The bar chart was obtained from the mean of the relative number of $CD11b^+$ ± standard deviation (SD) in healthy pregnant mice (NP); pregnant mice with DOCA induction (PD); and pregnant mice with L-NAME hydrochloride (PL) induction (n=9). Data were analyzed using one-way ANOVA with post-hoc Duncan ($p<0.05$). Different superscripts indicate statistical differences. (B) Flow cytometry dot plot of $CD11b^+IL\text{-}1\beta^+$ obtained from BD CellQuest Pro software from NP, PD, and PL groups.

Discussion

Regulatory T cells are a critical subset of T cells for maintaining immune tolerance and preventing excessive inflammatory response, especially in pregnancy. The present study was the first evaluation of the comparison effect of L-NAME hydrochloride on the expression of Tregs, macrophages, anti-inflammatory cytokines TGF-β, and pro-inflammatory cytokines (IL-6 and IL-1β). These immune markers can be used to determine systemic inflammation, the main feature of pre-eclampsia. This study found both DOCA and L-NAME hydrochloride could decrease the relative number of Tregs in the spleen of pregnant mice. It was indicated that the recruitment of mature Treg in secondary lymphoid organs was dysregulated. Treg maintains immune tolerance in a normal pregnancy between the maternal and fetus. The inhibition of Treg recruitment suggested that maternal immune tolerance to the fetus is compromised, which could contribute to the inflammatory environment in pre-eclampsia [31]. This condition could lead to an immune attack on the placenta, resulting in the systemic inflammation characteristics of pre-eclampsia.

Both DOCA and L-NAME hydrochloride could induce a pre-eclampsia-like syndrome in mice, but the exact mechanism of disrupting Treg recruitment remains unclear. However, in this study, we tried to describe a potential mechanism of both inducers in disrupting Treg recruitment. Subcutaneous injection of DOCA and daily administration of 0.9% saline could activate mineralocorticoid receptors and increase salt intake, both of which have been shown to play a role in the development of hypertension and inflammation [32]. A previous study have shown decreased circulating Treg and increased blood pressure after DOCA administration [31]. In addition, angiotensin II causes a significant decrease in Foxp3+-expressing Treg cells in the renal cortex [16]. Angiotensin II induces an increase in systolic blood pressure, vasodilation reduction, remodeling of arterial blood vessels, and an increase in aortic macrophage infiltration [33]. DOCA could increase angiotensin II by activating the renin-angiotensin-aldosterone system [34]. Excess sodium retention inhibits the function of Tregs as immunosuppressive cells through the production of pro-inflammatory cytokines such as IFN-γ [35]. The imbalance of T regulatory cells triggers the initiation of angiotensin II-induced hypertension and organ damage [34]. In contrast to DOCA, L-NAME hydrochloride leads to uterine artery constriction mediated by ET-1 [36]. It is related to the role of T regulatory cells in suppressing ET-1 secretion by endothelial cells and other inflammatory cells [37]. Thus, the decreased T regulatory cells increase uterine artery resistance to blood flow and enhance the conversion of bET-1 into the active vasoconstrictor ET-

1 [36]. The decrease in T regulatory cells is also associated with increased reactive oxygen species (ROS) and elevated ROS levels can reduce the availability of NO molecules, which regulate blood pressure [38]. With reduced NO due to increased ROS, blood pressure in mice with decreased T regulatory cells becomes more difficult to maintain [36]. The present study also found that the level of TGF-β tends to decrease after L-NAME hydrochloride induction in pregnant mice, although the decrease was not significant. TGF-β1 can trigger ROS production and activate nuclear factor erythroid 2–related factor 2 (Nrf2) in certain cell, with increased Nrf2 expression can reduce ROS levels under hypoxic conditions [39]. Trophoblast cells have antigenic properties similar to tumor cells, and both trigger an immune response [40]. The role of TGF-β is known to be a tumor suppressor in the early stages of tumor growth and this may indicate that TGF-β can also inhibit trophoblast cell invasion during the first trimester of pregnancy [41].

Macrophages and monocytes also play vital roles during acute and chronic inflammation [42]. In this study, we used CD11b as a marker for mature monocytes or macrophages in secondary lymphoid organs. This marker is involved in macrophage adhesion, migration, chemotaxis, and accumulation during inflammation [43]. The present study found that there was a higher accumulation of CD11b expression in the spleen of pregnant mice after L-NAME hydrochloride induction compared to DOCA. The high level of macrophages in the pre-eclampsia condition indicated systemic inflammation after L-NAME hydrochloride induction. Monocytes not only become phenotypically activated and show functional changes, but they also produce an increase in free oxygen radicals [44]. Phenotypic activation of monocytes during pregnancy is evidenced by increased expression of activation markers CD11b, CD14, and CD64 on monocytes from pregnant women compared to monocytes from non-pregnant [44]. L-NAME hydrochloride administration decreases NO levels and increases blood pressure in pre-eclampsia animal models [14]. In response to L-NAME hydrochloride induction, the sympathetic nervous system is activated, triggering the release of hormones that increase blood pressure, including the stimulation of renin release [45]. The increase in renin due to L-NAME hydrochloride induction triggers the production of angiotensin I, which is then converted to angiotensin II, a hormone that causes vasoconstriction and increases Na^+ retention in the kidneys [45]. Previous studies have also shown that the CD11b molecule can recognize intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) (cell adhesion molecules play an important role in increasing blood pressure, vascular dysfunction, and heart damage), thereby increasing macrophage adhesion and migration [31, 38]. Angiotensin II-activated macrophages can cause inflammation and vascular damage, contributing to arterial hypertension [39]. In contrast, DOCA has a different mechanism to induce the activation of monocytes during pregnancy. DOCA can increase aldosterone, which stimulates macrophage infiltration, increases DNA binding activity, and influences transcription factors such as nuclear factor kappa B (NF-κB) and activator protein-1, leading to the expression of various genes involved in inflammation. Aldosterone can also activate innate and adaptive immune system cells by activating mineralocorticoid receptors, leading to the accumulation of immune cells, especially macrophages and T cells, in the kidneys, heart, and blood vessels [47]. Infiltration of these immune cells can damage cardiovascular organs and lead to hypertension or high blood pressure [33]. However, L-NAME hydrochloride is more effective in mimicking endothelial dysfunction, which is the main feature of pre-eclampsia.

NOS inhibitors, such as L-NAME hydrochloride, can decrease NO production [48]. NO plays an important role in normal pregnancy by increasing blood flow to the uterus, fetus, and placenta. Insufficient NO production in the fetus and placenta triggers fetal growth disturbances through decreased blood perfusion in the uterus [48]. This is one of the pathophysiological effects of pre-eclampsia that triggers an inflammatory response characterized by increased IL-6 circulation in the placenta. The inflammatory cytokine IL-6 is stimulated in response to placental ischemia and it plays an important role in forming ROS and the production of sFlt-1 [24]. Inflammatory cytokine production stimulates the maturation of T helper 1 lymphocytes and B cells and the secretion of AT1-AA and AT1-AA mediates the activation of ET-1 and sFlt-1 and increases sensitivity to angiotensin II, which is characterized by the occurrence of hypertension during pregnancy [11]. A significantly higher number of $CD11b^+IL\text{-}6^+$ was found in the DOCA and L-NAME hydrochloride groups compared to normal pregnant mice. DOCA acts as a direct

biosynthetic precursor of aldosterone in the adrenal cortex [16]. The intracellular pathways used by aldosterone to increase IL-6 expression in human umbilical vein endothelial cells are through the mineralocorticoid receptor (MR), phosphoinositide 3-kinase (PI3K), or protein kinase B (Akt)/NF-κB pathways. IL-6 trans-signaling plays a role in the expression of fibronectin and type I collagen induced by aldosterone [33]. Other studies have shown that increased type I collagen occurs in conditions of maternal hypoxia. Type I collagen and HIF-1α expression are both increased in hepatic stellate cells under hypoxic conditions [30, 41]. This is related to the condition that occurs in pre-eclampsia, namely the release of various mediators from liver endothelium and blood vessels, such as fibronectin, thrombomodulin, endothelin-1, and thromboxane, which cause vasoconstriction and liver hypoxia [50].

In pre-eclampsia, the circulating levels of IL-1β are increased in the placenta. IL-1β is a pro-inflammatory cytokine that contributes to endothelial dysfunction [51]. L-NAME reduces NO production, leading to increased inflammation and vasoconstriction, which could exacerbate IL-1β upregulation and worsen pre-eclampsia symptoms [52]. The relative number of $CD11b^{+}IL\text{-}1\beta^{+}$ in normal pregnant mice was lower than in the L-NAME hydrochloride group. In normal pregnancy, the cytokine IL-1β is required for the labor process and its concentration in humans increases in the myometrium, cervix, and fetal membranes [53]. Targeting IL-1β has shown promising therapeutic potential for hypertension and cardiovascular disease, as evidenced by a study on DOCA-salt-induced hypertensive mice, where IL-1β inhibition effectively lowered blood pressure [54]. This protective effect is attributed to the ability of IL-1β to promote the production of ROS. ROS, in turn, contributes to the development of vascular damage, a hallmark of cardiovascular diseases [55].

The present study highlighted the effectiveness of L-NAME hydrochloride in inducing pre-eclampsia, particularly in increasing the number of macrophages and their secreted cytokine, IL-1β, compared to DOCA. The lower IL-1β levels observed in the DOCA group relative to the L-NAME hydrochloride group may be due to the distinct mechanisms by which these agents affect the immune system. DOCA primarily induces hypertension through mineralocorticoid receptor activation, with minimal direct stimulation of inflammatory pathways. In contrast, L-NAME inhibits nitric oxide synthase, leading to endothelial dysfunction and a more pronounced inflammatory response, including higher IL-1β levels. Thus, L-NAME promotes a stronger pro-inflammatory state, whereas DOCA, despite inducing hypertension, may not elicit the same degree of inflammatory cytokine release. However, this study has certain limitations, including restricted immune cell profiling. It only focused on T regulatory cells and macrophages, without investigating other key immune players, such as dendritic cells or neutrophils.

Conclusion

L-NAME hydrochloride was more effective in inducing pre-eclampsia in pregnant BALB/c mice than DOCA, indicated by the highest increase in pro-inflammatory cytokines IL-1β and macrophage activity and a low production of anti-inflammatory cytokine. Both DOCA and L-NAME hydrochloride led to a substantial increase in IL-6 production and a decrease in regulatory T cells. Further studies are needed to explore the involvement of other immune cells, such as dendritic cells and neutrophils, and to assess whether these models accurately mimic the immune alterations observed in human pre-eclampsia.

Ethics approval

Animal handling and protocol were approved by the Animal Care and Use Committee at Brawijaya University with approval number 051-KEP-UB-2024.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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