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Effect of Curcumin on Nitric Oxide and Endothelin-1 Levels in L-NAME-Induced Preeclamptic Wistar Rat

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Abstract

Preeclampsia is a pregnancy disorder marked by the onset of hypertension after the 20th week, posing risks such as cardiovascular disease. Curcumin, a commonly consumed herbal medicine, has been investigated as a potential antihypertensive agent in mouse models of preeclampsia. This study took place in the Bioscience Laboratory of Universitas Brawijaya from March to August 2023, employing a true experimental design with various groups of mice receiving different treatments. Nitric oxide (NO) and endothelin-1 (ET-1) levels were measured using calorimetry and ELISA. The rats were divided into five groups: positive control, negative control and P1, P2, and P3 as the treatment groups. Treatment grups received fifferent curcumin doses of 30 mg/kgBW/day, 50mg/kgBW/day, and 100mg/kgBW/day for P1, P2, and P3, respectively. Data analysis using the One-Way ANOVA and Post Hoc LSD revealed that curcumin at 100mg/kgBW/day significantly increased the NO level of 47.75±22.6 and decreased the ET-1 level of 67.03±24.47 when compared to the positive control ($p<0.05$). However, the 30mg/kgBW/day and 50mg/kgBW/day doses did not significantly affect the NO and ET levels. In conclusion, curcumin supplementation shows positive effects on NO and ET-1 levels in L-NAME-induced preeclamptic Wistar rats, highlighting its potential as an effective intervention for managing this pregnancy-related disorder.

Keywords: Curcumin, endothelin-1, nitric oxide, preeclampsia

Introduction

Preeclampsia is a pregnancy disorder associated with new-onset hypertension that occurs after 20 weeks of gestation,[1] with a global incidence ranging from 3-10% of all pregnancies.[2] In Indonesia, this obstetric issue has a prevalence of approximately 2.4 million cases. Preeclampsia poses a risk not only to the current health of pregnant mothers but also to long-term complications such as cardiovascular disease, chronic kidney failure, cerebrovascular disease, and venous thromboembolism.[3]

In the condition of preeclampsia, there is an increase in pro-inflammatory cytokines such as IL-1α, IL-6, and TNF-α,[4,5] leading to an imbalance in the function of endothelium. Endothelial function tends to undergo vasoconstriction, leukocyte adhesion, mitogenesis, prooxidation, and vascular inflammation. Endothelial dysfunction is characterized by a decrease in normal Nitric Oxide (NO) concentrations, and an increase in Endothelin-1 (ET-1) that mediates vasoconstriction, contributing to the occurrence of preeclampsia.[5] Some alternative treatments, which have been widely developed, include herbal medicine to prevent preeclampsia.

It is noteworthy that the use of herbal medicines is widespread among pregnant women in Indonesia, where the legislation for the distribution and purchase of herbal remedies is not as stringent as it is for conventional medicines. Turmeric (*Curcuma longa*) is a readily available herb commonly consumed and recognized by the Indonesian community. This herb is known for its antioxidant, anti-inflammatory, antibacterial, and anticancer effects.[6-9] Turmeric contains Curcumin, a

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lipophilic polyphenol.[10] Curcumin stimulates antioxidant enzymes such as Glutathione (GSH), catalase, and Superoxide Dismutase (SOD), as well as inhibits ROS-generating enzymes like cyclooxygenase and xanthine oxidase (Hewlings and Kalman, 2017). Recognized for its antioxidant properties, Curcumin can reduce the production of ROS that damage placental endothelial cells in some conditions such as preeclampsia.[11] Research by Zhou *et al.* indicates that curcumin administration inhibits pro-inflammatory factors and macrophage infiltration in the placenta through Akt phosphorylation signaling in rat.[12]

Wistar rats are commonly used experimental animals in studying hypertension models due to their physiological system's similarity to humans. N(ω)-nitro-L-arginine methyl ester (L-NAME) is a hypertension-inducing compound that, physiologically, is hydrolyzed by cellular esterase into bioactive NG-nitro-L-arginine in vascular endothelium. It inhibits NOS activity by binding to the enzyme and replacing its normal substrate, L-arginine.[13,14] The use of L-NAME in preeclamptic animal models triggers an increase in ROS and a decrease in NO bioavailability.[15] A study by Rahardjo et al. showed that administering L-NAME injection at 125 mg/kg/day to rats induces an increase in blood pressure, reflecting a condition similar to preeclampsia. Another study also mentions that pregnant rats given L-NAME experience changes resembling preeclampsia symptoms.[16]

This research focused on exploring the endothelial dysfunction pathway with the aim of increasing Nitric Oxide (NO) levels and reducing the concentration of Endothelin-1 (ET-1) as a potential preventive therapy for preeclampsia. The researchers sought to investigate the impact of curcumin administration on NO and ET-1 levels in the serum of preeclamptic rat models. The experimental study aimed to examine the role of curcumin supplementation in preeclampsia management, specifically in enhancing NO levels and reducing ET-1 in animal models of preeclampsia. While larger-scale randomized controlled trials are needed for further validation, this study is expected to contribute additional literature to support the understanding of potential therapies for preeclampsia, particularly those involving the regulation of NO and ET-1.

Methods

This research used a true experimental design in a laboratory setting with in vivo methods and a posttest-only controlled group design. Preeclampsia was induced in rats by administering L-NAME, and the rats were subjected to different doses of curcumin (30 mg, 50 mg, 100 mg/kg/day), selected based on a prior study by Longobardi et al.[16] This research received ethical approval from the Health Research Ethics Commission of General Hospital Dr. Saiful Anwar (No. 400/063/K.3/102.7/2023).

The experimental animals were female Wistar rats which were healthy, approximately 10-12 weeks old, and weighed around 180–220 grams. This age range was selected because it is considered adult for rats. The sample consisted of 25 pregnant and preeclamptic Wistar rats, selected randomly. The research subjects were divided into five treatment groups. The number of research subjects in each group was calculated based on the formula p (n-1) ≥1.[7] Additionally, a 10% surplus of the experimental animals was added as a reserve to prevent a reduction in the sample size during the study, resulting in 1 extra rat in each group. The researchers treated these 5 groups of rat the same on days 1–12, and to conclude that the rat had developed preeclampsia, there was a positive control group and a negative control group, where the positive control group given L-NAME showed high blood pressure and the presence of urine protein compared to negative controls. Therefore limitation of this study is that there are no studies comparing the effectiveness of curcumin therapy with other standard preeclampsia treatments.[7]

The acclimatization and treatment of the rats, including the induction of L-NAME and administration of Curcumin, were conducted at the Bioscience and Physiology Laboratory, Faculty of Medicine, Brawijaya University. The research spanned a period of 4 months, from March 2023 to June 2023. The study commenced with a 7-day acclimatization period for the rat, followed by random assignment into five groups. The negative control group consisted of normal pregnant rat, while the positive control group received subcutaneous injections of L-NAME (125mg/kgBW/day) from gestation day 13 to day 19. Treatment group 1 included rat that received subcutaneous injections of L-NAME (125mg/kgBW/day) from gestation day 13 to day 19, with an additional administration of Curcumin at a dose of 30 mg/kg/day from gestation day 13 to day 19. Treatment group 2 comprised rat that received subcutaneous injections of L-NAME (125mg/kgBW/day) from gestation day 13 to day 19, along with an

additional administration of Curcumin at a dose of 50 mg/kg/day from gestation day 13 to day 19. Treatment group 3 involved rat that received subcutaneous injections of L-NAME (125mg/kgBW/day) from gestation day 13 to day 19, with an additional administration of Curcumin at a dose of 100 mg/kg/day from gestation day 13 to day 19.

The Wistar rats were obtained from the Bioscience Laboratory of Brawijaya University. Before the treatment, all the rats were weighed, and randomization was performed. During the acclimatization period, the weight of the rats was recorded at the beginning and after the acclimatization to monitor that the rats' body weight did not decrease and remained in good condition. The rat cages were labeled according to the treatment, each housing 8 rats. The cages were covered with wire mesh to allow sufficient air ventilation, and they were placed at room temperature between 25-28°C with air humidity of 50-70%. The cages were lined with a sufficient thickness of rice husks, and the husks were changed every 3 days. The rats were placed in plastic cages with wire lids framed with wood and acclimatized for 7 days, given a normal diet to allow them to adapt to the environmental changes and feeding schedule.

The mating process involved placing male and female rats in the same cage. Mating typically occurred during the night. In the morning after mating, a vaginal swab was performed on the female rats, and the swab was observed under a microscope. The presence of sperm on the vaginal swab indicated day 0 of pregnancy (Shu *et al*., 2018). The rats were provided with distilled water every day, placed in 100 mL drinking bottles equipped with a ball valve for water dispensing. This water dispenser was positioned above the wire cage cover. The rats were fed with a diet consisting of BR1 Comfeed concentrate and wheat flour in a ratio of 3:1, mixed with 10 cc of water for every 40 grams for each rat.[16] The female rats were placed in the cages with the male rats for 72 hours to induce pheromone effects. Subsequently, they were paired with the male rats at a 1:1 ratio overnight (starting at 16:00). Vaginal swab checks were conducted at 05:00 the next day as a sign of copulation. Preeclampsia conditions were confirmed if blood pressure was ≥140 mmHg and/or urine protein was ≥+1 from metabolites collected over 24 hours.[16]

The preeclamptic model was established by dissolving L-NAME (N0661 Tokyo Chemical Industry, Gamma Scientific Biolab) diluted with PBS and injected intraperitoneally from gestation day 13-19. The L-NAME dose used was 125 mg/kgBW/day based on Rahardjo et al., 2021. The daily intraperitoneal administration was calculated to be a maximum of 0.2-5 cc per rat. L-NAME was administered intraperitoneally as a single daily dose for 7 days.[16]

The dose calculation for L-NAME per rat with an estimated weight of 200 grams is calculated as follows: 125 mg/kgBW/day = 125/1000 x 200 = 25 mg/rat. L-NAME was dissolved in PBS, and the maximum intraperitoneal volume was 0.2-5 cc. Thus, 3.2 cc of the PBS solution was needed for 16 rats (2 test groups). The L-NAME solution is calculated as follows: 4 cc PBS solution + 400 mg L-NAME = 4 cc L-NAME solution for 16 rats/day = 0.25 cc/rat/day.

Blood pressure measurements were conducted indirectly through non-invasive blood pressure assessment using the CODA® instrument on the tail of the rats. The measurement method employed the Volume Pressure Recording (VPR) tail cuff technique. The type of pressure perceived by this tool was systolic blood pressure, expressed in millimeters of mercury (mmHg). Blood pressure assessments were carried out on gestation days 13 and 19. After the 19th day, the rat had blood samples taken from the heart and were killed while pregnant.

The method of urine sample collection used a metabolic cage, specifically designed to collect rat metabolites such as urine. Urinalysis for protein assessment was performed using urinalysis reagent test strips or dipstick tests. The obtained values from color readings (qualitative) on the provided reading chart (which includes concentration values for each color) were then converted into semi-quantitative results. The results were interpreted as follows: + corresponds to > 0.3 g/L, ++ corresponds to > 2 g/L, +++ corresponds to > 3 g/L, and >+++++ corresponds to > 10 g/L.[18]

In this study, the obtained results were based on the qualitative data from the dipstick readings, which were then converted into numerical values (semi-quantitative) according to Ogi *et.al*.[18] The numerical data, after conversion, were tabulated and averaged based on the number of subjects in each observation group, resulting in the average urine protein values for each observation group. Urine protein assessments were conducted on gestation days 13 and 19.

Curcumin from TCI (Tokyo Chemical Industry), with code C0434, was initially dissolved in distilled water by adding 100mg of

Curcumin to 100mL of distilled water, resulting in a concentration of 100mg/mL. The rats were then weighed daily to determine the appropriate Curcumin doses (30mg/kg, 50mg/kg, 100mg/kg) based on their body weights. The Curcumin solution was subsequently administered orally to the treatment group of rats.

The procedure for assessing ET-1 levels utilized the ELISA (Enzyme Linked Immunosorbent Assay) method with the Rat ET-1 ELISA Kit (No E0462Ra) from Bioassay Technology Laboratory. The procedure for assessing NO levels involved using the Colorimetric Assay method with the Rat NO EBCK035S Kit from Bioassay Technology Laboratory.

The data from the calculations of NO and ET-1 levels for each group were tabulated. Based on this tabulation, statistical analysis was performed using SPSS 25.0. The data analysis involved two sequential stages: (1) testing the parametric preconditions, including normality testing using the Shapiro-Wilk test and homogeneity testing. Upon obtaining normally distributed and homogeneous data, (2) the One-Way ANOVA test was conducted, followed by post-hoc testing using the Least Significant Difference (LSD) test. Statistical analysis was performed at a 95% confidence level with α=0.05. Results with $p < 0.05$ were considered statistically significant.

Results

The study was conducted on preeclamptic rats to investigate the effects of oral administration of curcumin at doses of 30, 50, and 100 mg/kgBW/day from gestational day 13 to day 19 on the levels of NO and ET-1 in the preeclamptic model. Blood pressure and urine protein examinations were performed at two time points, specifically on gestational day 13 (G13). The first examination on gestational day 13 served as a screening for the presence of preeclampsia (hypertension and positive urine protein) before the intervention. The second examination on gestational day 19 was conducted to evaluate the effects of L-NAME and curcumin.

The blood pressure examination on gestational day 13 showed normotensive conditions in all the groups. The negative control group did not experience an increase in blood pressure at both examination times. The average systolic blood pressure measurements for the negative control group on days 13 and 19 of pregnancy were 107.25±8.77 mmHg and 113.5±6.14 mmHg, respectively. The positive control group exhibited an increase, with average systolic blood pressure measurements on days 13 and 19 of pregnancy being 108.00±14.35 mmHg and 149.5±15.15 mmHg, respectively. Treatment Group 1 had a systolic blood pressure of 108.5±29.70 mmHg on gestational day 13. Subsequently, they were injected with L-NAME and administered 30 mg/kgBW/day curcumin via gavage until gestational day 19. The blood pressure examination on gestational day 19 showed an increase to 150.5±29.70 mmHg. Treatment Group 2 had a systolic blood pressure of 106.25±24.93 mmHg on gestational day 13. Following L-NAME injection and gavage of 50

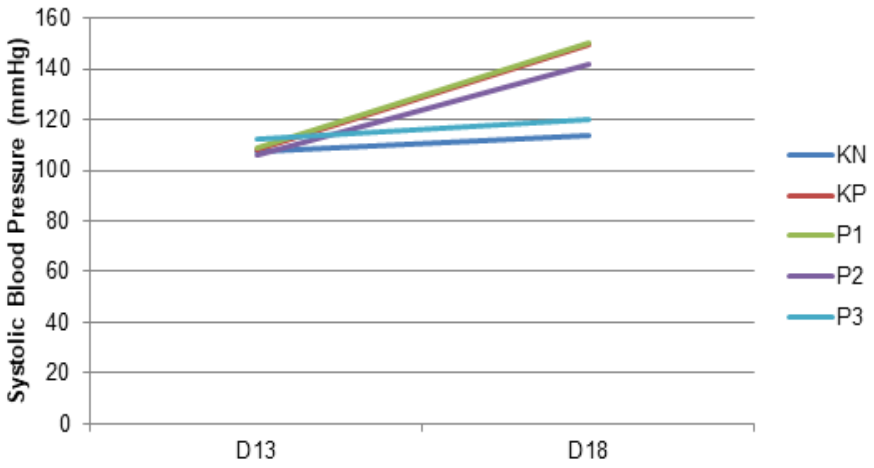

Figure 1 Characteristics of Systolic Blood Pressure in Preeclamptic Rat Models before and after Intraperitoneal L-NAME Injection and Curcumin Gavage

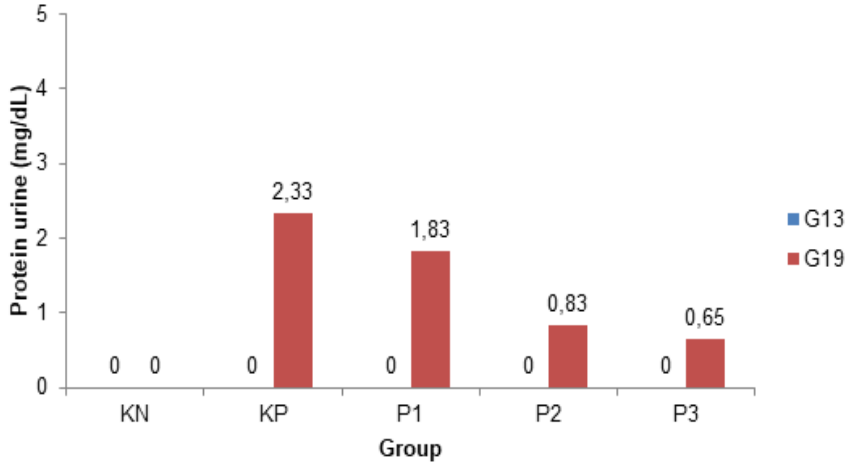

Figure 2 Characteristics of Protein Urine in Preeclamptic Rat Models before and after Intraperitoneal L-NAME Injection and Curcumin Gavage

KN = negative control, KP = positive control, P1 = treatment group 1, P2 = treatment group 2, P3= treatment group

mg/kgBW/day curcumin on gestational day 19, the blood pressure increased to 141.5±24.93 mmHg. Treatment Group 3 had a systolic blood pressure of 112±5.66 mmHg on gestational day 13. Subsequently, they were injected with L-NAME and administered 100 mg/kgBW/day curcumin via gavage. The blood pressure examination on gestational day 19 showed an increase to 119±5.66 mmHg.

Urinary protein levels were assessed on Days 13 (DH-13) and 19 (D-19). No urinary protein was detected on gestational day 13 in all the groups. On Day 19, the positive control group had the highest increase in mean urinary protein (from 0 to 2.33 g/L), while Treatment Group 1 (P1) increased by 1.83 g/L, Group 2 (P2) by 0.83 g/L, and Group 3 (P3) by 0.65 g/L. The blood pressure and urine analyses on Day 13 revealed neither increase in mean systolic blood pressure nor urinary protein in the negative control group

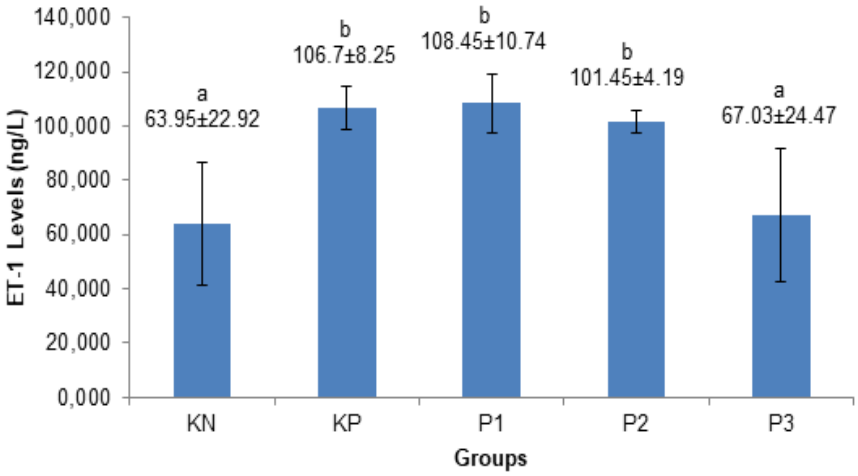

Figure 3 The ET-1 Level in Each Group

KN = negative control; KP = positive control; P1 = treatment group 1; P2 = treatment group 2; P3 = treatment group 3. Analysis was performed using One-Way ANOVA followed by Post Hoc LSD. (a,b), differing notations indicate significant differences ($p<0.05$).

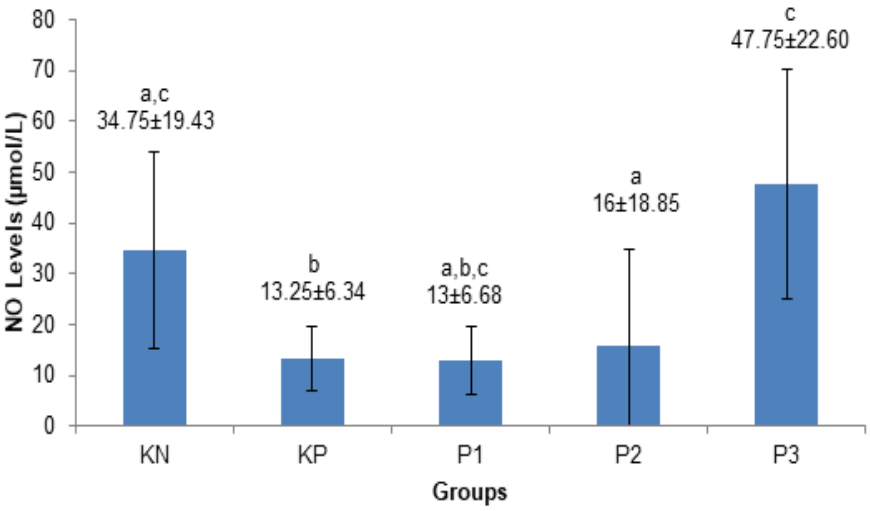

Figure 4 The NO Level in Each Group
KN = negative control; KP = positive control; P1 = treatment group 1; P2 = treatment group 2; P3 = treatment group 3. Analysis was performed using One-Way ANOVA followed by Post Hoc LSD. (a,b), differing notations indicate significant differences ($p<0.05$)

(KN). The positive control group (KP) exhibited increased systolic blood pressure and urinary protein. Treatment Groups 1, 2, and 3 (P1, P2, and P3) showed no hypertension on Day 13. However, on Day 19, P1 had the highest mean blood pressure, while P3 had the lowest.

Based on Figure 3, differences in the ET-1 levels between groups were evident. The results of the statistical analysis indicated that the administration of L-NAME to induce preeclampsia significantly increased ET-1 levels in the positive control group compared to the negative control group. Meanwhile, in treatment group P3 (with a dose of 100 mg/kgBW), the serum ET-1 level was significantly the lowest, approaching the level of the negative control group.

Based on Figure 4, the NO levels in the negative control group were significantly higher than the ET-1 levels in the positive control group. There was a difference in NO levels between the positive control group and the treatment groups. The results of the satistical analysis showed that treatment group 2 (50 mg/kgBW) and treatment group 3 (100 mg/kgBW) had significantly higher NO levels compared to the negative control group.

Discussion

This study utilized pregnant rats as a preeclamptic model. Preeclampsia was induced on the pregnant rats on gestational days 13-19 by administering NG-nitro-L-arginine methyl ester (L-NAME) at a dose of 125 mg/kgBW/day intraperitoneally.[19] s.[20]

The resulting on the 19th day, blood samples were taken from the rat hearts and killed while they were pregnant. Preeclamptic rat model exhibited symptoms such as an increase in blood pressure and urine protein levels in the positive control group, while these symptoms were not observed in the negative control group treated with PBS. In this study, the elevation of blood pressure (>140/90 mmHg) occurred on day 19 or T3 of pregnancy after L-NAME induction. This is consistent with the findings of Rahardjo *et al.,* where the positive control group also experienced an increase in blood pressure that persisted until day 19 of pregnancy. Zhu *et al.* conducted research by administering L-NAME at a dose of 125 mg/kgBW/day from gestational day 13 to day 19 and found an increase in systolic blood pressure in rats.[14] This condition indicates the success of creating a preeclamptic rat model. Other studies show that rats given L-NAME also exhibit symptoms such as increased protein in urine, elevated blood pressure, endothelial damage, and an increase in sFlt-1 and PLGF levels. [15,21]

Increased protein levels in urine are another symptom of preeclampsia. In preeclampsia, the presence of protein in urine occurs due

to endothelial dysfunction in blood vessels, leading to increased blood vessel permeability. This increased permeability allows proteins to pass through the glomeruli.[22] The results of this study showed changes in urine protein levels in the positive control group, indicating impaired kidney function in pregnant rats, resembling the clinical presentation of preeclampsia.

This study showed that curcumin effectively reduced ET-1 levels, especially in the P3 treatment group given a dose of 100mg/kgBW. ET-1 is a peptide with 21 amino acids produced by endothelial cells of blood vessels and functions as a potent vasoconstrictor.[7] Other studies indicate that ET-1 measurements in normal and preeclamptic pregnancies show a two to threefold increase in ET-1 in the circulation of patients with preeclampsia compared to normal pregnancies. The activation of protein kinase C influences the release of ET-1 and is affected by various factors such as LDL, ADH, angiotensin-II, interleukin, TGFβ, TNFα, leptin, adrenaline, and arginine.[23]

The curcumin dose considered most effective in reducing ET-1 levels is 100 mg/kgBW. Treatment group 3 approached the mean value of the negative control. Furthermore, there was a significant difference in the mean ET-1 levels between treatment groups 1 and 3 (p=0.001), indicating the ability of curcumin to reduce ET-1 levels at doses of 50 mg/kgBW and 100 mg/kgBW, with higher curcumin doses correlating to lower ET-1 levels in the serum of experimental animals. A study by Shu et al., using two doses of curcumin (50 mg/kgBW/day and 100 mg/kgBW/day), demonstrated that both doses could lower blood pressure and improve endothelial dysfunction. Specifically, the 100 mg/kgBW dose could prevent changes in the aortic wall and reduce ACE activity and oxidative stress.[19]

In this study, the administration of curcumin at doses of 50 mg/kgBW and 100 mg/kgBW significantly increased NO levels compared to the positive control. In preeclampsia, there is an imbalance between antioxidant and pro-oxidant mechanisms, attributed to the low remodeling of spiral arteries. This leads to repeated ischemia-reperfusion injuries in the myometrium. Consistent with this hypothesis, in vitro studies show an increase in reactive oxygen species (ROS) levels in human placental tissue after ischemia-reperfusion injury. The most common ROS include superoxide, hydrogen peroxide, and hydroxyl radicals. In this condition, there is also an increase in reactive nitrogen species (RNS), consisting of nitric oxide and peroxynitrite (Aouache et al., 2018). The elevation of ROS and RNS can reduce the levels of vasodilators, including Nitric Oxide (NO).[24]

Nitric oxide (NO) is a soluble gas mediator that functions in vascular homeostasis and vascular tone modulation. Reactive oxygen species (ROS) can disrupt vascular tone homeostasis by reducing NO production. Endothelial NO synthase (eNOS), expressed in vascular endothelium, regulates vascular tone through NO synthesis and decreases due to excessive ROS production. Inhibition of endothelial NO synthesis leads to dysregulation in vascular tone modulation, as well as platelet and leukocyte adhesion due to ROS.[24]

Curcumin possesses antioxidant activity, allowing it to reduce reactive oxygen species (ROS). Curcumin reduces ROS production by enzymes through the inhibition of lipoxygenase/cyclooxygenase and xanthine dehydrogenase/oxidase enzymes. Additionally, curcumin enhances the activity of superoxide dismutase (SOD) and peroxidase (POD), known as the primary enzymes combating ROS. Furthermore, curcumin is known to elevate the antioxidative enzymes, including catalase and glutathione.[25]

The findings of this study are supported by several previous research efforts. Some studies indicate that curcumin can increase NO levels. Research by Santos-Parker[25] demonstrates that curcumin acts as a vasodilator by elevating NO levels and reducing peripheral resistance in the brachial artery in both adults and the elderly.[25] Other research found that curcumin reduces iNOS protein and NO levels, through various mechanisms, including tyrosine phosphorylation inhibition and the ERK 1/2 pathway.[25] A study by Longobardi[16] affirms that curcumin exhibits antiapoptotic, anti-inflammatory, and antioxidant properties, particularly in countering the toxic effects induced by ochratoxin in rats.[16]

This study indicated that curcumin could reduce ET-1 levels and increase NO, suggesting its potential use in preventing preeclampsia. However, there are several limitations to this research. Firstly, the study did not assess the toxicity and teratogenic effects of curcumin, highlighting the need for further investigation in this area. Secondly, the combined effects of curcumin with other antihypertensive drugs remain unknown, necessitating further research.

In conclusion, the findings of this study indicate that the administration of curcumin at a dose of 100 mg/kg BW/day effectively reduces ET-1 levels in the serum of preeclamptic Wistar rats. Additionally, this dose of curcumin results

in a significant increase in NO levels in the serum of these rats. Moving forward, further research is crucial to confirm these conclusions. It would be beneficial to conduct more frequent blood pressure and urine protein measurements to better understand the stages of curcumin's effects on preeclampsia. More complex animal models and larger-scale human studies should be explored to provide more applicable and comprehensive insights for the prevention of eclampsia during pregnancy. Furthermore, detailed analyses regarding the potential toxicity and teratogenic effects of curcumin in preeclamptic rats are essential. Lastly, investigating the effects of combining curcumin with other antihypertensive drugs will provide valuable information for future research in this field.

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