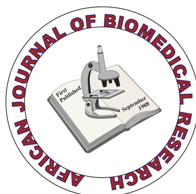

Afr. J. Biomed. Res. Vol. 22 (September, 2019); 295- 301

Research Article

L-arginine Attenuates Oxidative Stress and Regulates the Inflammatory Actions of Tumor Necrosis Factor and Interleukin-10 in a Rat Model of Pre-eclampsia

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ABSTRACT

Maternal endothelial dysfunction, oxidative stress and inflammation are parts of the theories associated with preeclampsia. L-arginine is the natural substrate for NO synthase and responsible for the production of NO, This study investigated the effect of L-arginine on blood pressure, oxidative stress and inflammatory markers in pregnant rats administered N-nitro-arginine methyl ester (L-NAME). Thirty-six nulliparous female Sprague-Dawley rats weighing between 150-170 g were divided into 4 groups as follows: control (normal saline), L-NAME (50mg/kg b.w. intraperitoneal injection from days 13-18 of pregnancy), L-NAME + L-arginine (50mg/kg b.w. and 1g/kg b.w. of L-arginine from days 13-18 of pregnancy) and L-arginine (1g/kg b.w. administered orally from days 13-18 of pregnancy). Fetal outcome and blood pressure were measured on day 19 of pregnancy. The placenta was homogenized for oxidative enzyme assay and serum nitric oxide metabolite (NOx), endothelial nitric oxide synthase (eNOS), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) levels were measured. L-arginine reduced blood pressure increase by L-NAME. It also reduced placenta malondialdehyde level and increased glutathione peroxidase level thereby countering the effects of L-NAME. It increased eNOS and NOx levels and decreased TNF-α level compared with L-NAME. L-arginine supplementation in rat pregnancy prevents increase in blood pressure by improving eNOS and NOx levels thereby reducing oxidative stress and reducing proinflamatorry actions of TNF-α.

Keywords: *Tumor necrosis factor-alpha, Preeclampsia, L-arginine, Oxidative stress, Inflammation*

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Received: March 2019; Accepted: August, 2019

Abstracted by:
Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius

INTRODUCTION

Preeclampsia is a hypertensive disorder of unknown etiology, about 11% of first pregnancies are estimated to be affected with hypertension, in which half of these cases are associated with preeclampsia (Hofmeyr *et al*., 2019). The dysfunction of the maternal vascular endothelium is one of the theories ascribed to causing preeclampsia (Tsukimori *et al*., 2006; Valencia-Ortega *et al*., 2019). The endothelial nitric oxide synthase (eNOS), is an enzyme that helps protect the vasculature against vascular diseases by the production of the vasoprotective molecule nitric oxide (NO) (Forstermann and Munzel 2006). Nitric oxide is usually elevated in pregnancy (Choi *et al*., 2002, Oludare and Iranloye 2016) and decreased NO production has been implicated in the pathogenesis of preeclampsia (Sladek *et al.,* 1997)**.** Pregnant rats administered N-nitro-arginine methyl ester (L-NAME), an irreversible inhibitor of constitutive NO synthase, had a reduction in NO synthesis during late pregnancy (days 14–20) with an increase in blood pressure, and a decrease in the weights of the placenta as well as the litters (Thaete *et al.,* 2005, Oludare *et al.,*2018). L-NAME induced hypertension in rats is used to mimic hypertension in humans (Kopincova *et al.,* 2012, Abdel-Rahman *et al.,* 2017). L-NAME-treated rats have down-regulated eNOS protein expression in blood vessels (Silambarasan *et al.,* 2014, Berkban *et al.,* 2015) and depletion of plasma NO levels, which leads to systemic vasoconstriction, increased vascular resistance and high blood pressure. It is also known that L-NAME-induced hypertension induces oxidative stress by increasing levels of oxidative stress markers such as vascular superoxide ($O_2^{\bullet-}$) and plasma malondialdehyde (MDA) (Nakmareong *et al.,* 2011; Berkban *et al.,* 2015). Oxidative stress is recognized to play a central role in the pathophysiology of many different disorders, including complications of pregnancy which includes preeclampsia (Burton *et al.,* 2011). Oxidative stress occurs

when an imbalance exist between free radical formation and the capability of the cells to clear them (Pizzino *et al.,* 2017). Oxidative stress is manifested at the maternal–fetal interface from early pregnancy onwards. It plays a role in both the normal development of the placenta as well as in the pathophysiology of complications such as miscarriage, preeclampsia, intrauterine growth restriction (IUGR), and premature rupture of the membranes (Burton and Jauniaux 2004; Jauniaux *et al.,* 2006).

In the third trimester, pregnancy is also associated with a systemic inflammatory response, all of which produce ROS (Mannaerts *et al.,* 2018). These obviously show the compounding relationship between oxidative stress and inflammation. Oxidative stress and the systemic inflammatory response are observed to a much greater degree in preeclampsia (Redman and Sargent 2009, Mannaerts *et al.,* 2018). The cause for the oxidative stress is thought to be vascular, because early onset preeclampsia is associated with an incomplete trophoblast invasion resulting in a poor spiral artery remodeling (Meekins *et al.,* 1994, Burton and Jauniaux 2011). A normal pregnancy outcome to term is promoted by the fundamental interaction between the pro- and anti-inflammatory cytokines of which interleukin-10 (IL-10) seems to be the key cytokine. Recent findings show that a healthy pregnancy has reduced proinflamatorry cytokines such as TNF-alpha and IL-6 and an increase in the counter regulatory cytokines such as IL-10 (Denney *et al.,* 2011).

L-Arginine, a semi-essential amino acid, is the natural substrate for NO synthase and responsible for the production of NO, which is involved in a wide variety of regulatory mechanisms of the cardiovascular system (Boger and Ron 2005, Macrae, 2016). Since endothelial dysfunction, oxidative stress and inflammation contributes to the pathogenesis of preeclampsia, this study hypothesizes that L-arginine supplementation will prevent oxidative stress and support a state of inflammatory balance in pregnant rats administered L-NAME.

MATERIALS AND METHODS

Chemicals: L-NAME was obtained from Santa-Cruz Biothechnology, Germany, L-Arginine was obtained from Solgar, USA, and the assay kits were obtained from Enzo-life Science, Switzerland and Elabscience, China.

Experimental animals: Thirty-six (36) nulliparous female Sprague-Dawley rats weighing between 150-170 g were used for this study (n= 9 for each group). The rats were kept under standard conditions of 12 h light and dark cycles. They were acclimatized for 2 weeks, kept at room temperature and were allowed to feed and drink water *ad libitum.* Experimental procedures from this study were in compliance with the international principles for laboratory animals as obtained in the Helsinki's declaration (NIH 1985) guide for care and use of laboratory animals.

Experimental groups: Animals were grouped into four groups of rats as follows: group I (control group); received 0.2 ml normal saline from days 13-18 of pregnancy, group II (L-NAME); received intraperitoneal L-NAME injection at a dose of 50 mg/kg body weight from days 13-18 of pregnancy, Group III (L-NAME + L-Arg); received 50 mg/kg b.w. L-NAME (i.p) and L-Arginine 1 g/kg bw orally from days 13-18 of pregnancy and Group IV (L-Arginine): received oral 1 g/kg bw L-Arginine from days 13-18 of pregnancy.

Determination of estrus cycle and mating: The Marcondes technique was employed to determine estrus cycle phases (Marcondes *et al.,* 2002). Two-three drops of normal saline where inserted into the rats vagina using a dropper. This fluid was retrieved and placed on a glass slide to ascertain the cell types in the vaginal smear under a light microscope (X 40). The rats on proestrus phase were allowed to mate with male rats on the evening of proestrus. The presence of sperm cells in the smears of the rats, the following day, confirmed mating and was assumed as day 1 of pregnancy.

Blood pressure and fetal parameter measurements: On day 19 of pregnancy, the rats were anaesthetized with a solution of 25% (w/v) urethane and 1% (w/v) α- chloralose injected intraperitoneally at a dose of 5 ml/kg body weight. Animal that has totally lost its righting reflex was then dissected. The trachea was exposed and cannulated as well as one carotid artery. Blood pressure measurements were obtained from the cannulated carotid artery. A polyethylene cannula filled with 1% heparinized saline was inserted into the artery, tied in place. The cannula inserted into the artery was connected to a pressure transducer (model SP 844, Physiological Pressure Transducer. AD Instruments) that was attached through MLAC11 Grass adapter cable to a computerized data acquisition system with LabChart-7 pro software [Power Lab-4/24T, model MLT844/P; AD Instruments Pty Ltd., Castle Hill, Australia] to obtain blood pressure values (Oludare *et al.,* 2017). The animal abdominal cavity was then cut open and the fetuses were counted, removed and weighed and the resorped fetus were observed and counted. This was identified visually as a highly growth retarded fetus that has being disintegrated alongside with its placenta and containing a blood filled amniotic sack.

Collection of urine samples and determination of urine protein: Twelve hour urine samples were collected on day 17 or 18 of pregnancy using a locally constructed metabolic cage. The urine collected was preserved with toluene and used for determining urine protein. Total protein in the urine was assayed using Randox Biuret kits (Tietz 1995).

Determination of oxidative stress parameters: MDA (an index of lipid peroxidation), was determined based on its interaction with thiobarbituric acid to form a pink complex with absorption maximum at 535 nm (Mihara and Uchiyama 1978). The activity of the superoxide dismutase (SOD) enzyme in the placenta homogenate was carried out in 0.05 m sodium carbonate buffer pH 10.3 and was initiated by the addition of epinephrine in 0.005 N HCl (Sun and Zigman 1978). Catalase (CAT) activities was determined by measuring the exponential disappearance of H_2O_2 at 240 nm and expressed in units/mg of protein (Aebi, 1984). Determination of glutathione levels (GSH) was based on the reaction of Ellman's reagent (5,5'‑dithiobis (2‑nitrobenzoic

acid) DNTB) with the thiol group of GSH at pH 8.0 to produce 5-thiol-2-nitrobenzoate, which is yellow at 412 nm (Van Doorn *et al.,* 1978). Absorbance was recorded using Shimadzu recording spectrophotometer.

Determination of Nitric oxide metabolites (NOx): Nitrite/nitrate (stable NO metabolites) in the serum samples were measured based on the Griess reaction. A colorimetric method using Enzo-life Science nitrate/nitrite assay kit was used to measures serum nitric oxide metabolite concentration ([NOx]). The reaction buffer, NADH reagent and nitrate reductase enzyme provided were reconstituted as described by the manufacturer, while the standards for nitrate and nitrite where also prepared in various concentrations as described in the protocol booklet.

Determination of eNOS, TNF-α and Interleukin-10 concentration in serum: Blood samples were collected via the carotid artery from the rats during sacrifice for the estimation of the levels of eNOS, TNF-α and Interleukin-10 using Enzyme-linked-immunosorbent serologic assay (ELISA) techniques. The samples were centrifuged for 15 min at 3000 rpm using a bench top centrifuge and the serum was stored at -20°C. The assay was carried out using the protocol booklet of the manufacturers of the ELISA kits (Elabscience China).

Statistical analysis: All the values were expressed as mean ± standard error of mean (SEM). The values were analysed by one-way ANOVA followed by Student's Newman-Keuls post-hoc test using the Graph Pad software. Differences were considered significant when $p < 0.05$.

RESULTS

Fetal parameters: Fetal weight (Day 19) was significantly reduced in L-NAME and L-NAME+L-Arg groups when compared with control ($p < 0.05$) In addition, there was a significant increase in fetal weight of L-Arg administered rats when compared with both L-NAME and L-NAME+L-Arg groups. The percentage of resorption sites in the L-NAME group (65.11%) and that of L-NAME+L-Arg (27.08%) groups were significantly increased compared with control (Table 1).

Urinary protein and blood pressure parameters: Protein levels were increased in L-NAME administered pregnant rats (0.85 ± 0.12) when compared with control (0.33 ± 0.033) an L-arginine group (0.26 ± 0.04) ($p < 0.05$). The systolic blood pressure and diastolic blood pressure in the L-NAME rats were increased significantly when compared with control. L-arginine administration and L-NAME+L-Arg combination significantly reduced the increased systolic and diastolic blood pressures when compared with L-NAME administered pregnant rats. The mean arterial blood pressure (MABP) followed the same pattern as the systolic and diastolic blood pressures (Table 2).

Table 1:
Fetal parameters of rats administered L-NAME and L-arginine

	Control	L-NAME	L-NAME + L-Arg	L-Arg
Total number of fetus	39	15	35	42
Number of resorptions	6	28	13	4
Percentage of resorptions	13.33	65.11*	27.08 #	8.69#
Fetuses weight (Day 19) (g)	4.02 ± 0.08	2.48 ± 0.06 *	2.98 ± 0.04 *#	3.94 ± 0.06#$
Average number of Fetus	7.8 ±0.49	3.0 ± 1.84	7.0 ± 1.82	8.4 ± 1.12

*Signifies significant difference from control, # signifies significant difference from L-NAME and **$** signifies significant difference from L-NAME + L-Arg ($p < 0.05$).

Table 2:
Values of blood pressure of pregnant rats administered L-NAME and L-arginine

	Control	L-NAME	L-NAME + L-Arg	L-Arg
SBP (mmHg)	115.53 ± 14.13	178.78 ± 5.07*	132.60 ± 16.02#	120.54 ± 4.17#
DBP (mmHg)	81.17 ± 14.87	145.52 ± 2.57*	93.25 ± 16.34#	90.32 ± 6.28#
MABP (mmHg)	92.58 ± 14.59	156.61 ± 3.25*	101.26 ± 16.60#	109.84 ± 7.83#

*Signifies significant difference from control, # signifies significant difference from L-NAME and **$** signifies significant difference from L-NAME + L-Arg ($p < 0.05$). SBP = systolic blood pressure, DBP= diastolic blood pressure, MABP = Mean arterial blood pressure.

Table 3:
Effect of L-arginine supplementation on placental oxidative stress parameters in rats administered L-NAME

	Control	L-NAME	L-NAME + L-Arg	L-Arg
MDA (units/mg prot)	0.12 ± 0.007	0.20 ± 0.003*	0.141 ± 0.003#	0.128 ± 0.004#
SOD (units/mg prot)	4.80 ± 0.47	3.10 ± 0.40*	3.96 ± 0.29	4.61 ± 0.75#
GSH (units/mg prot)	1.62 ± 0.07	0.76 ± 0.08*	1.22 ± 0.017*#	1.77 ± 0.009#$
CAT (units/mg prot)	37.32 ± 2.23	23.61 ± 1.64*	29.82 ± 4.81	38.41 ± 4.85#

*Signifies significant difference from control, # signifies significant difference from L-NAME and **$** signifies significant difference from L-NAME + L-Arg ($p < 0.05$). MDA = Malondialdehyde, SOD = Superoxide dismutase, GSH = Glutathione peroxidase, CAT = Catalase

Placenta oxidative stress parameters: L-NAME significantly increased MDA levels while L-arginine supplementation in L-NAME administered rats reversed the trend ($p < 0.05$). The levels of antioxidant enzymes SOD, GSH and CAT were decreased in L-NAME administered rats compared with control ($p < 0.05$). L-arginine supplementation with L-NAME increased GSH levels significantly but did not raise SOD and CAT levels significantly when compared with L-NAME ($p < 0.05$) (Table 3).

NOx, eNOS, TNF-α and Interleukin-10

Nitric oxide metabolite level was significantly reduced in L-NAME administered rats compared to control. L-arginine supplementation increased NOx levels when compared with L-NAME group ($p < 0.05$; Figure 1). This pattern was also found in the endothelial nitric oxide synthase level which was decreased in the L-NAME administered rats when compared with control. L-arginine supplementation with L-NAME also increased eNOS levels when compared with L-NAME group ($p < 0.05$; Figure 2). TNF-α levels was increased in the L-NAME administered rats compared with control while L-arginine supplementation with L-NAME increased TNF-α levels when compared with L-NAME ($p < 0.05$; Figure 3). No difference was found in the levels of interleukin-10 levels in all the groups compared with control ($p < 0.05$; Figure 4).

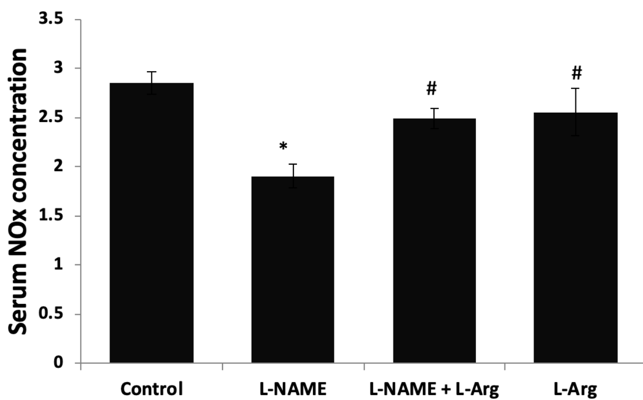

Figure 1:
Effect of L-arginine supplementation of serum nitrate/nitrite level. *signifies significant difference from control, # signifies significant difference from L-NAME

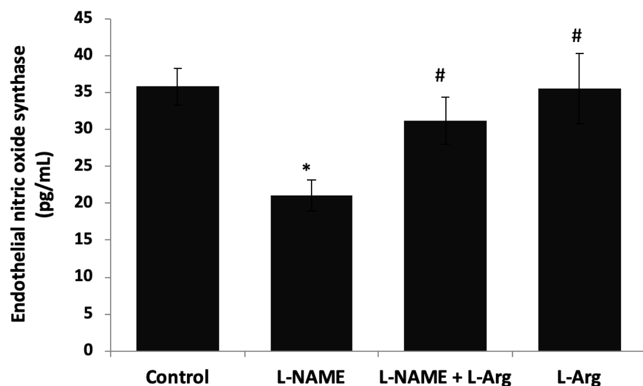

Figure 2:
Effect of L-arginine supplementation of serum endothelial nitric oxide synthase level. *signifies significant difference from control, # signifies significant difference from L-NAME

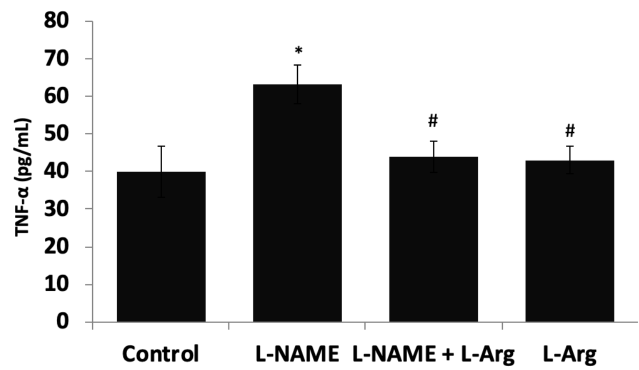

Figure 3:
Effect of L-arginine supplementation on Serum TNF-alpha levels. *signifies significant difference from control, # signifies significant difference from L-NAME

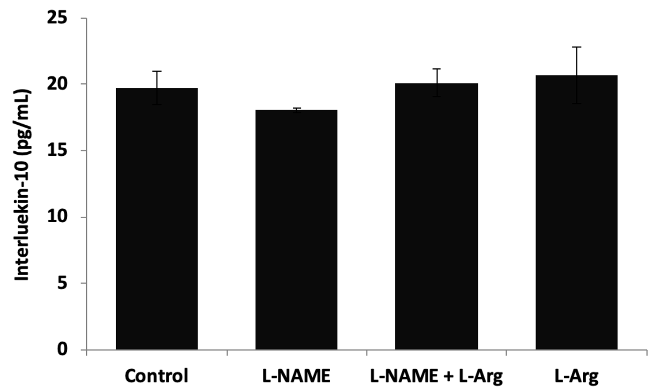

Figure 4:
Effect of L-arginine supplementation on serum interluekin-10 levels. No significant difference in all the groups

DISCUSSION

This study further establishes the fact that L-NAME administration increases blood pressure, reduces the number of viable fetus and decrease the weight of the fetus. There is a drastic increase in blood flow at the feto- and utero-placenta sites at term which correlates with fetal growth and survival of the fetus (Wang and Zheng 2012; Reynolds *et al.,* 2005). The increased fetal resorption and decreased fetal weight in L-NAME administered rats could be due to the actions of L-NAME on NOS which led to vascular constriction and probable decrease in blood flow. L-arginine supplementation with L-NAME reduced the number of resorptions and increased the fetal weight compared to the L-NAME administered rats' fetuses. This is in line with a previous studies that showed that L-arginine reversed blood pressure increase by L-NAME and increased fetal weight (Buhimschi *et al.,* 1995, Oludare *et al.,* 2018).

Increased MDA levels (an index of lipid peroxidation) in the L-NAME administered rats showed an increase in reactive oxygen species in the rats. Oxidative stress is manifested at the maternal-fetal interface for both normal development of the placenta as well as in the pathophysiology of complications such as miscarriage, preeclampsia, intrauterine growth restriction (IUGR), and premature rupture of the membranes (Burton and Jauniaux 2004; Jauniaux *et al.,* 2006).

Evidence exist that the vascular redox state in preeclampsia favors excessive pro-oxidants production and decreased antioxidant protection (Myatt, 2010). Excess ROS and lack of adequate antioxidant levels to protect the surrounding lipids and proteins from damage could cause significant harm to the developing embryo (Jauniaux et al., 2000; Hempstock *et al.,* 2003). The elevated ROS levels further stimulates vascular smooth muscle cell proliferation and increase arterial resistance due to reduced NO availability, leading to the impairment of vascular relaxation (Klima *et al.,* 2013). It has been shown that women suffering from recurrent pregnancy loss possess weakened antioxidant defense systems as well as increased levels of oxidative stress biomarkers (Simsek *et al.,* 1998; Yiyenoglu *et al.,* 2014, Wu *et al.,* 2017); and this could be attributed to the high percentage of resorped fetuses in the L-NAME administered rats in this study. L-arginine supplementation with L-NAME reduced MDA levels and increased the level of the antioxidant enzyme GSH. The increase in antioxidant level coupled with the increase amount of NO produced in this study by L-arginine, reduces the tendency for the formation of peroxinitrite ($ONOO^-$.) a potent oxidant that aids eNOS uncoupling leading to the production of superoxide in place of NO (Forstermann and Munzel 2006, McRae 2016).

Endothelial nitric oxide synthase is the predominant NOS isoform in the vasculature and on red blood cells (Cortese-Krott *et al.,* 2012; Wood *et al.,* 2013). It is responsible for producing most of the NO in the tissue. Recent discoveries have shown that circulating eNOS contributes to the regulation of blood pressure and coronary artery disease (Cortese-Krott *et al.,* 2012; Wood *et al.,* 2013). This study reported decreased eNOS concentration and NOx in the serum of L-NAME administered rats, which were reversed by L-arginine supplementation. The normal function of eNOS requires dimerization of the enzyme, the presence of the substrate L-arginine, and the essential cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH4) (Forstermann and Munzel 2006). Though the body produces L-arginine in small quantities, its supplementation is required in pathological states due to the increased competition for the substrate by arginase an enzyme expressed by endothelial cells as well as eNOS inhibitor asymmetric dimethyl-L-arginine (ADMA) (Berkowitz *et al.,* 2000; Xu *et al.,* 2004; Tsukimori *et al.,* 2008). The result from this study showed that L-arginine is beneficial in a state of gestational hypertension. Other studies have also shown that L-arginine administration has beneficial effects on endothelial cell dysfunction in female rats with renal pathologies, and in the synthesis of NO from the rat mesenteric artery wall, independent of cellular arginine uptake (MacKenzie and Wadsworth 2003, Nesher *et al.,* 2014).

L-NAME increased TNF-α concentration however, L-arginine supplementation with L-NAME reversed the trend. In pregnancy, the immune system ensures the development of normal pregnancy and prevents the development of complications (MacKenzie and Wadsworth 2003, Han *et al.* 2019). TNF-α is a pro-inflammatory cytokine and a therapeutic target in many inflammatory diseases. Its production is increased in obstetrics complications such as recurrent fetal loss, gestational diabetes mellitus, hypertension and fetal growth restrictions (Peracoli *et al.,* 2007, Denney *et al.,* 2011). The current study reports increased TNF-α in L-NAME administered rats as well as increased resorption of fetus and decreased fetal weights. This further buttresses the relationship between L-NAME induced blood pressure, oxidative stress and inflammation as factors contributing to L-NAME being used as a model of preeclampsia in rats. The mechanism by which L-arginine prevents inflammation is not very clear, however, it could be due to its ability to promote NO production by preventing the uncoupling of eNOS such that NO is produced rather than $ONOO^-$ thus reducing oxidative stress and triggers for inflammation. On the other hand, no statistical difference in all groups was found in the concentration of Interluekin-10 a special type of cytokine that plays both stimulatory and immunosuppressive roles (Moreli *et al.,* 2012). Interleukin-10 has a protective effect on the fetal-placental unit because it inhibits the secretions of inflammatory cytokines, such as TNF-α IL-6 and IFN- γ (Thaxton and Sharma 2010; Cheng and Sharma 2015). The dual functions of IL-10 as both a stimulatory and a counter regulatory agent might be responsible for the concentration of IL-10 been unchanged in the groups.

In conclusion, this study reports the contribution of L-arginine supplementation to reduced blood pressure, improved eNOS activity and NOx production in pregnant rats administered L-NAME. Also, L-arginine supplementation reduced oxidative stress and inflammation induced by L-NAME in the pregnant rats. This suggests that L-arginine supplementation in pregnancy might help prevent endothelial dysfunction, oxidative stress and inflammation which are associated with gestational hypertension.

Acknowledgement*: We wish to appreciate the technical assistance of Mr. Sunday and Mr Ota during the research work.*

Conflict of interest disclosure: *All authors disclose no actual or potential conflicts of interest, including any financial, personal, or other relationships with people or organizations*

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