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Protective Functions of L-Arginine on Implantation in Pregnant Rats Administered L-NAME

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ABSTRACT: Nitric oxide (NO) plays a role in estrogen-mediated uterine receptivity. We hypothesize that L-arginine a NO substrate will increase NO production and boost pregnancy outcomes in rats with inhibited NO synthase activity. Thirty-two pregnant rats were randomly divided into four groups, control (10 mL/kg of water), L-NAME (50 mg/kg), L-NAME + L-Arg (50 mg/kg and 500 mg/kg) and L-Arg only (500 mg/kg). L-Arg and L-NAME were administered orally and subcutaneously respectively from day 3-11 of pregnancy. Blood samples were collected on day 8 for the assay of nitric oxide, TNF-α and interleukin-10 concentrations. The rats were sacrificed on day 12 of pregnancy. L-NAME-administered rats had decreased maternal body weight and number and weight of implantation sites when compared with the control ($p< 0.05$). Co-administration of L-NAME with L-Arg significantly increased the body weights and fetal weights when compared with L-NAME. L-Arginine co-administration with L-NAME significantly boosted NO and interleukin-10 concentrations and significantly reduced TNF-α concentration when compared with L-NAME. Malondialdehyde (MDA) level was significantly reduced while activities of superoxide dismutase (SOD) and catalase (CAT) increased in the L-NAME + L-Arg group compared with L-NAME only group. The study reveals that L-arginine improves fetal development through its antioxidant and anti-inflammatory actions.

Keywords: L-Arginine, L-NAME, Implantation, Pregnancy, Antioxidant

Introduction

Embryo implantation is a critical phase in reproduction that determines if pregnancy will be achieved successfully or fail. It is the stage of pregnancy at which the developing embryo adheres to the wall of the uterus (Dekel *et al.,* 2014). Implantation requires a distinct collaboration between the embryo and the uterus which must be accomplished within a limited period called the window of implantation (Guzeloglu-Kayisli *et al.,* 2009; Cakmak and Taylor, 2011). Any breach in this communication during this time usually leads to an implantation failure. In humans, this window is represented as day 3 to day 9 after ovulation while rats which have a very short gestational period, have their implantation window corresponding to 24 hours before day 4 and day 5 of pregnancy (Strowitzki *et al.,* 2006, Sposito and Santos, 2011). During this window, the uterus experiences some functional and structural modifications which are induced by progesterone and estrogen that aids the process of implantation.
The changes in the uterine environment include the conversion of the stromal cells of the uterus into larger rounded decidual cells (Dunn et al 2003), also, there is the growth and development of several secretory glandules and the

appearance of microvilli on the luminal epithelium (Paria *et al.,* 2002). The functional modifications in the uterus also include changes in the expression of different growth factors, cytokines, and adhesion molecules. This change is also accompanied by increased vascularization and intrusion of the immune cells from the blood to the endometrial tissue (van Mourik *et al.,* 2009, Dekel *et al.,* 2014). Prostaglandins, NO, sex steroids, some anti-inflammatory drugs, cytokines, and notch signalling receptors are groups of molecules designated collectively as blastocyst implantation essential factors (BIEF). These factors are indispensable for the successful implantation of a mature and healthy blastocyst (Martin *et al.,* 2007; Cuman *et al.,* 2014).

Embryo implantation appears like an inflammatory reaction, because of the infiltration of leukocytes, oedema, and vasodilation (Dekel *et al.,* 2014). It has been relatively known that increased concentrations of helper Th1 cells are associated with embryo rejection while elevated helper Th2 cells level is associated with pregnancy (Nakagawa *et al.,* 2015; Bashir *et al.,* 2018). Tumour necrosis Factor alpha (TNF-α) a cytokine produced by the helper Th1 cells is believed to suppress trophoblastic growth and promote inflammation and thrombotic responses in maternal uterine blood vessels which unfavorably affects implantation. Cytokines produced by the helper Th2 cells such as IL-4, IL-6 and IL-10 inhibit Th1 cell-induced tissue factor by monocytes (Nakagawa *et al.,* 2015; Bashir *et al.,* 2018)

Nitric oxide contributes to the modulation of periovulatory ovarian blood flow (Mitsube *et al.,* 2002). It is also suspected to be involved in follicular growth and ovulation (Mahran *et al.,* 2016). During pregnancy, NO contributes to the estrogen-mediated uterine receptivity to support the process of implantation (Biswas *et al.,* 1998; Battaglia *et al.,* 2003). Biswas's research group reported that when 2.5mg of L-NGNitro arginine methyl ester (L-NAME) was injected into the uterine horn on day 3 of pregnancy, it led to implantation failure when observed on day 8 of pregnancy in rats. The authors reported embryo growth retardation because of decreased preimplantation permeability changes in the uterus brought about by L-NAME injection. Although NO synthesis is required in regulating endometrial activity, its role in embryonic implantation has not been fully explored. The current study explored the role of nitric oxide on blastocyst implantation and development in rats. The study explored the possible roles of L-arginine administration during the peri-implantation and post-implantation states in rats with diminished nitric oxide production induced by L-NAME.

Materials and methods

Experimental animals and groups: Thirty-two nulliparous Sprague-Dawley rats weighing between 100-110 g were used in this study. The rats were kept under similar conditions of 12-hour light and dark cycles. They were acclimatized for two weeks, kept at room temperature, and allowed free access to food and water. The experimental protocols were carried out in compliance with the international principles for the care and use of experimental animals for use in research (National Research Council, 2011). The rats were randomly divided into four groups of rats as described below:

Group I (control group) received distilled water (10 mL/kg orally).
Group II (L-NAME): received intraperitoneal injection of L-NAME (50 mg/kg subcutaneously).
Group III (L-NAME + L-Arg): received L-NAME (50 mg/kg) and L-arginine (500 mg/kg orally).
Group IV (L-Arginine): received L-Arginine (500 mg/kg body weight orally).
L-NAME and L-Arginine were administered from day 3 of pregnancy to day 11 of pregnancy.

Determination of oestrous cycle, mating, sacrifice and fetal parameter measurements: Prior to mating, the female rat's oestrous cycle was examined using the wet method techniques of Marcondes *et al.,* (2001). The phases of the cycle were examined under the light microscope (X 40). Sexually matured male rats were allowed to mate with female rats on the evening of their proestrus phase. Mating was confirmed by the presence of sperm cells in the vaginal smears the following day and was assumed as day 1 of pregnancy. On day 12 of pregnancy, the animals were sacrificed by cervical dislocation and the rats were cut open to access the number of implants and their weights.

Measurement of the concentrations of nitric oxide, TNF-α, and IL-10: Blood samples were collected from the rats and centrifuged for 15 min at 3000 rpm using a bench-top centrifuge and the serum was extracted and kept at -20 degree Celsius. A colourimetric assay kit from Enzo-life science was used to determine nitric oxide level while ELISA kits from Elabscience were used to measure the concentrations of TNF-α, and IL-10. The assays were carried out as described in the protocol booklet of the manufacturers.

Measurements of MDA, SOD and CAT levels: The uterus of the rats was collected and homogenized in phosphate buffer and stored at -20 degrees Celsius before the assay of malondialdehyde (MDA), superoxide dismutase (SOD)

and catalase (CAT) activities. The method of Mihara and Uchiyama, (1978) was used to determine the MDA levels. This method is based on the interaction of thiobarbituric acid with the samples to form a pink complex at an absorption maximum of 535 nm. The activities of the antioxidant enzyme superoxide dismutase were determined in the uterus homogenate using the protocol described by Sun and Zigma, (1978). The reaction 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. The activity of catalase was determined as described by Aebi, (1978). This was based on the exponential disappearance of hydrogen peroxide at 240 nm and expressed in units/mg of protein. The absorbances were recorded using PG instruments T70 UV/VIS (Lutterworth, UK)

Statistical analysis: The data derived from the measurements were analyzed using one-way ANOVA followed by Student's Newman-Keuls post-hoc test using Graph pad prism 6 software. The values are expressed as mean ± standard error of mean (SEM) and differences were considered significant when $p < 0.05$.

Results

Influence of L-arginine on implantation and organ weights: Table 1 shows the animal weights, organ weights and implant weight and number. The table shows that there was a significant decrease in the body weight of rats administered L-NAME when compared to the control ($p<0.05$). No change was observed in the liver weight, but the uterus weight was significantly decreased in the L-NAME-administered rats compared with the control. The number of implants and the implant weights were decreased in the L-NAME group as well as the L-NAME and L-arginine group when compared with the control. However, the average number of implants increased in the L-NAME + L-arginine group compared with the L-NAME group.

Table 1: Weight of implantation sites, organ and animal weights of rats administered L-NAME and L-Arginine

	Control	L-NAME	L-NAME + L-Arg	L-Arg
Animal weight (g)	153.16 ± 6.79	139.5 ± 6.92*	142.66 ± 7.64	142.16 ± 9.08
Liver weight (g)	5.81 ± 0.64	4.32 ± 0.33	6.02 ± 0.67	5.41 ± 0.43
Uterus weight (g)	0.8 ± 0.08	0.58 ± 0.03 *	0.63 ± 0.03	0.64 ± 0.03
Implantation sites (g)	0.34 ± 0.008	0.21 ± 0.009 *	0.24 ± 0.006 *	0.31 ± 0.005 * # $
The average number of implantation sites (Absolute number of implantation sites)	8.0 ± 0.31 (40)	3.6 ± 0.51 (18) *	5.2 ± 0.58 (26) * #	7.4 ± 0.51 (37) # $

*=significant difference from control, #= significant difference from L-NAME and $ signifies significant difference from L-NAME + L-arginine

Influence of L-arginine on the concentrations of TNF-α, Interleukin 10 and nitric oxide metabolites: Concentrations of TNF-α, revealed in Figure 1, show that TNF-α was significantly increased in the L-NAME group compared with the control ($P<0.05$). L-arginine supplementation significantly reduced the increased concentration of TNF-α in the L-NAME + L-arginine group as well as the L-arginine group when compared with L-NAME. Figure 2 shows the concentration of interleukin 10 measured in the serum of the pregnant rats. The figure shows that L-NAME decreased interleukin 10 concentrations when compared with the control. Furthermore, the figure showed that L-arginine supplementations increased Interleukin 10 concentrations when compared with L-NAME. The result on nitric oxide metabolites is revealed in figure 3. The figure shows that NO concentrations were reduced by L-NAME significantly when compared to the control group while L-arginine supplementation with or without L-NAME administration increased NO metabolite levels when compared with L-NAME ($p<0.05$).

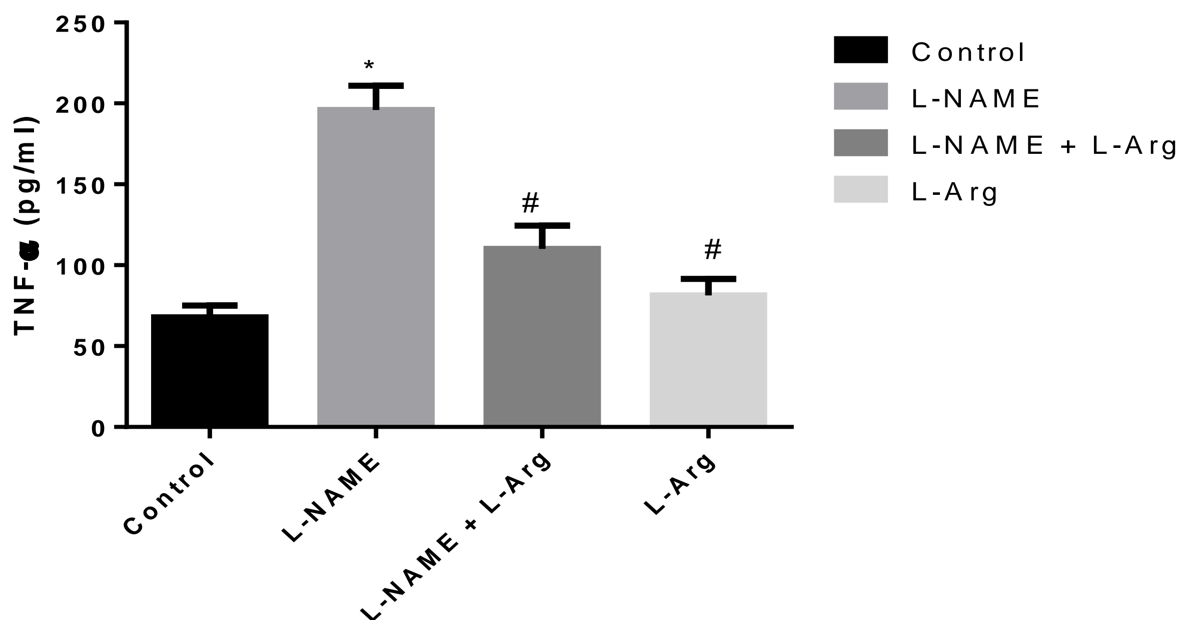

Figure 1: Concentrations of TNF-α on day 8 of pregnancy in rats administered L-NAME and L-Arginine
*=significant difference from control, #= significant difference from L-NAME

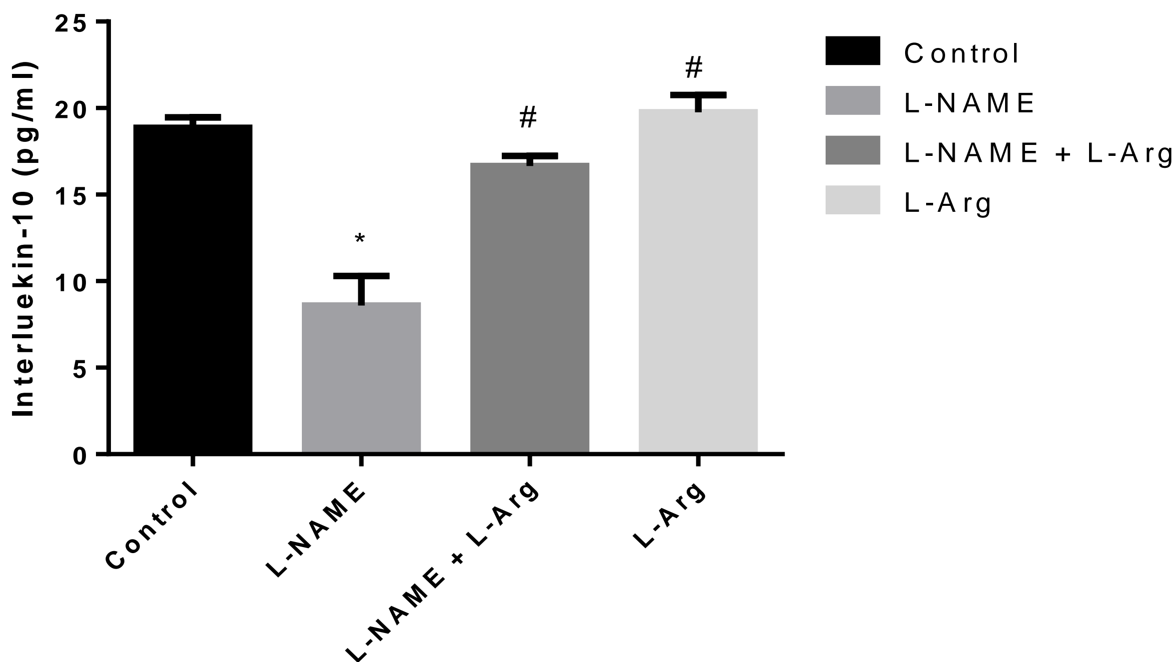

Figure 2: Concentrations of Interleukin-10 on day 8 of pregnancy in rats administered L-NAME and L-arginine
*=significant difference from control, #= significant difference from L-NAME

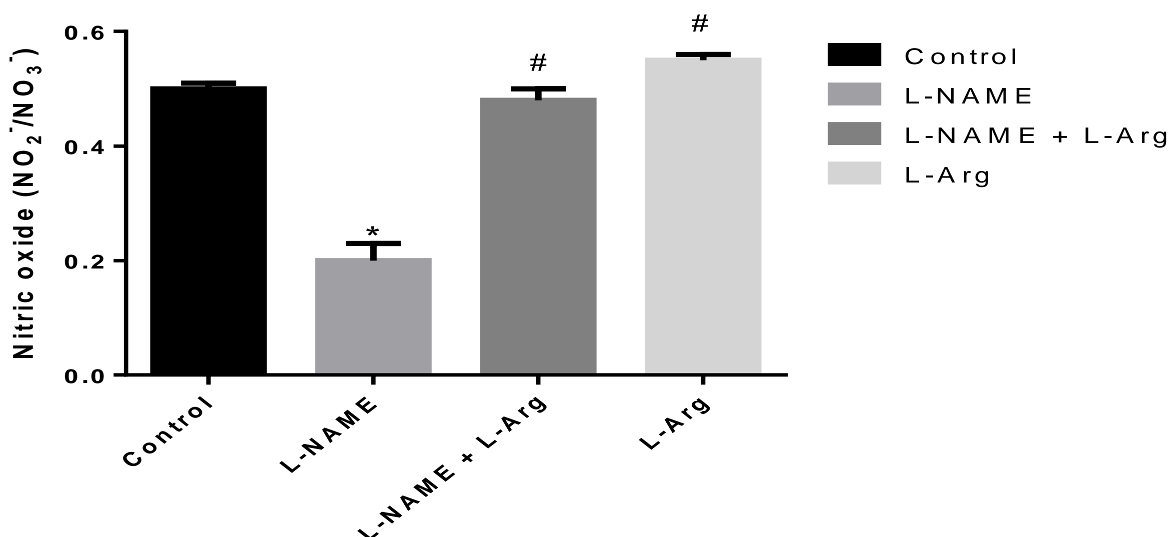

Figure 3: The concentrations of nitric oxide metabolites level on day 8 of pregnancy in rats administered L-NAME and L-arginine.
*=significant difference from control, #= significant difference from L-NAME

Influence of L-arginine on oxidative stress status of the uterine environment of pregnant rats administered L-NAME: Table 2 shows that the L-NAME-administered rats' MDA levels were significantly increased compared with the control ($p<0.05$). This increase by L-NAME was significantly reduced in the pregnant rats supplemented with L-arginine with or without L-NAME administrations. The table also revealed the activities of intracellular antioxidants SOD and CAT. The results showed that L-NAME significantly decreased the activities of SOD and CAT compared with the control values ($p<0.05$). In the L-NAME + L-Arg group, SOD was significantly decreased when compared with the control, but the values of SOD were significantly increased when compared with L-NAME. Catalase activity was significantly increased in the L-arginine-supplemented rats when compared with L-NAME ($p<0.05$).

Table 2: Antioxidant status of the uterine environment in pregnant rats administered L-NAME and L-arginine on Day 13 of pregnancy

	Control	**L-NAME**	**L-NAME + L-Arg**	**L-Arg**
Malondialdehyde (µmol/ml)	0.16 ± 0.01	0.24 ± 0.02*	0.17 ± 0.01 #	0.16 ± 0.02#
Superoxide Dismutase (units/mg protein)	5.61 ± 0.47	4.03 ± 0.40*	4.86 ± 0.29*#	5.44 ± 0.75#
Catalase (units/mg protein)	50.58 ± 8.01	35.67 ± 8.23*	48.67 ± 3.62 #	48.75 ± 5.10 #

*=significant difference from control, #= significant difference from L-NAME

Discussion

The purpose of this study was to ascertain if L-arginine a functional amino acid and a substrate for NO production, could play an important role in the uterine environment of rats with inhibited NO synthesis. Experimentally, L-NAME has been used to induce hypertension and preeclampsia in laboratory animals. We accessed the role of L-arginine during embryonic implantation on the activities of pro-inflammatory and anti-inflammatory cytokines as well as on nitric oxide production and oxidative status in the uterine environment.

Findings from this study showed that L-NAME reduced the number and weight of implantation. This is consistent with various studies that used L-NAME to simulate preeclampsia which reported decreased fetal weight and number of implants (Zhu *et al.,* 2017, Oludare *et al.,* 2018, de Alwis *et al.,* 2022). Although the mechanism around the early onset of preeclampsia is not very clear, there are indications that a perturbed NO production at the uterine environment during the window of implantation could bring about an imbalance in the production of cytokines

which could affect the implantation step. NO is crucial during implantation as the rate of neovascularization increases in the uterine environment during the implantation stage. Vascular dysfunction resulting from poor placentation is thought to be a primary cause of preeclampsia (Lam *et al.,* 2005). Decreased blood flow brought about by L-NAME inhibition of NO synthesis could affect the process of implantation (Matsubara *et al.,* 2016). The result from this study showed that L-NAME decreased NO synthesis while L-Arg supplementation improved NO production. This could explain why the number and weights of implantation sites in the L-arginine-supplemented rats were increased.

Helper T cells produced at different stages of pregnancy determine the activity at those stages (Wang *et al.,* 2020). The Th1 cells that produce more proinflammatory cytokines are dominant during the peri-implantation period which is supposed to play a beneficial role in the fetus. However, shortly after implantation, the endometrial environment is shifted from the early inflammatory Th1 immunity to the anti-inflammatory Th2 immune responses (Wang *et al.,* 2020). This dominant TH2 immunity then overrules the TH1 immunity at the placental implantation site and protects the fetus by ensuring that the fetus and the placental develop normally. Should the inflammatory responses of Th1 cells continue without check, it could result in the inhibition of trophoblast invasion and in endothelial cell dysfunction via activated allograft immunity that can lead to spontaneous abortion and preeclampsia (Saito and Sakai, 2003).

Our study reported that an increase in the level of TNF-α a proinflammatory cytokine on day 8 of pregnancy in the L-NAME pregnant rats with a decrease in the concentration of interleukin-10 an anti-inflammatory cytokine. Th1/Th2 imbalance appears to be important in mediating the pathogenesis of preeclampsia (Saito *et al.,* 1999). Thus, since the L-NAME model exhibits this characteristic, this study further buttresses the importance of nitric oxide at the window of implantation on the type of cytokines produced. Supplementation with L-Arg showed a reverse in the concertation of these cytokines. Thus, confirming our results on the ability of L-arginine supplementation to increase NO synthesis. A recent study by Shafiq *et al.,* (2021) reports that increased levels of interleukin 1β and Interleukin 17A (both of which are proinflammatory cytokines) during pregnancy in women are associated with adverse birth outcomes and infant growth deficits.

Pregnancy is well-known to increase oxidative stress because of the normal systemic inflammatory response, which produces elevated concentrations of reactive oxygen species (ROS). Oxidative stress contributes to embryonic resorption, recurrent pregnancy loss, intrauterine growth restriction, preeclampsia development and fetal death (Gupta *et al.,* 2007, Sharma *et al.,* 2013, Chiarello *et al.,* 2020). The increased MDA concentration and reduced SOD and CAT activities in the L-NAME administered group confirms oxidative imbalance between the prooxidants and the antioxidants. This could be brought about by the oxidation of nitric oxide to form peroxynitrite and reactive oxygen radicals which further exacerbates the inflammatory environment and NO deficiency created by L-NAME action. The study also reported that the pregnant rats supplemented with L-arginine reversed this trend in the L-NAME group. MDA levels were reduced, and antioxidant enzymes SOD and CAT activities were increased. About 50–60% of cases involving RPL have been observed to implicate the presence of OS (Gupta *et al.,* 2007, Gupta *et al.,* 2009).

The study reveals the antioxidant and anti-inflammatory actions of L-arginine. The study also showed that L-arginine improved fetal development and increased the production of nitric oxide a key signalling molecule that plays a crucial role during pregnancy. Therefore, it is suggested that L-arginine supplementation prior to implantation could protect from the perturbed environment created by NOS inhibition and endothelial dysfunction. This has promising implications for women susceptible to hypertensive disorders in pregnancy as L-Arginine supplementation is potentially therapeutic in improving mother and fetus health.

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