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## The effect of chronic nitric oxide inhibition on vascular reactivity and blood pressure in pregnant rats

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### ABSTRACT

### INTRODUCTION

**Context:** The exact mechanism involved in changes in blood pressure and peripheral vascular resistance during pregnancy is unknown.

**Objective:** To evaluate the importance of endothelium-derived relaxing factor (EDRF) and its main component, nitric oxide, in blood pressure and vascular reactivity in pregnant rats.

**Design:** Clinical trial in experimentation animals.

**Setting:** University laboratory of Pharmacology.

**Sample:** Female Wistar rats with normal blood pressure, weight (152 to 227 grams) and age (90 to 116 days).

**Intervention:** The rats were divided in to four groups: pregnant rats treated with L-NAME (13 rats); pregnant control rats (8 rats); virgin rats treated with L-NAME (10 rats); virgin control rats (12 rats). The vascular preparations and caudal blood pressure were obtained at the end of pregnancy, or after the administration of L-NAME in virgin rats.

**Main Measurements:** The caudal blood pressure and the vascular response to acetylcholine in pre-contracted aortic rings, both with and without endothelium, and the effect of nitric oxide inhibition, N<sup>w</sup>-L-nitro-arginine methyl-ester (L-NAME), in pregnant and virgin rats. The L-NAME was administered in the drinking water over a 10-day period.

**Results:** The blood pressure decreased in pregnancy. Aortic rings of pregnant rats were more sensitive to acetylcholine than those of virgin rats. After L-NAME treatment, the blood pressure increased and relaxation was blocked in both groups. The fetal-placental unit weight of the L-NAME group was lower than that of the control group.

**Conclusion:** Acetylcholine-induced vasorelaxation sensitivity was greater in pregnant rats and that blood pressure increased after L-NAME administration while the acetylcholine-induced vasorelaxation response was blocked.

**Key-words:** Vascular reactivity. Pregnancy. Rats. L-NAME. Nitric oxide.

Various hemodynamic changes characterized by decreased, systemic, vascular resistance and decreased blood pressure are associated with pregnancy.<sup>1-4</sup> The exact physiopathological mechanisms by which these vascular changes take place are not known. Many factors such as decreased blood viscosity, changes in the prostacyclin/thromboxane ratio and a decrease in nitric oxide activity or synthesis are involved.<sup>5, 6</sup>

Nitric oxide is now known to be the principal component in EDRF (endothelium-derived relaxing factor),<sup>7,9</sup> which is released by acetylcholine stimulus. This nitric oxide is produced by the vascular endothelium and it regulates the vascular vasorelaxation tonus and blood pressure.<sup>10,11</sup> Nitric oxide acts by stimulating soluble guanylate cyclase resulting in an increase in the intracellular concentration of cyclic guanosine monophosphate in the smooth muscle cells, which leads to vasorelaxation.<sup>12-14</sup>

The importance of nitric oxide in controlling blood pressure during pregnancy is unknown. Some studies suggest a greater relevance of nitric oxide in pregnant than in non-pregnant subjects,<sup>15,16</sup> while others show the converse.<sup>17</sup>

The present study thus examines blood pressure during pregnancy in the presence and absence of nitric oxide inhibitors, and the vascular reactivity and fetal outcome consequent on this inhibition.

## METHODS

Female Wistar rats with normal blood pressure, weighing between 152 and 227 grams and aged 90 to 116 days were used. The rats were transferred to the laboratory one week before the experiments were started and during this time they were maintained in a quiet environment under a cycle of 12 hours dark to 12 hours light.

The animals were mated, placing a single male rat in each cage with three or four female rats. Vaginal smears were obtained daily; the day on which spermatozooids were found was considered to be the first day of pregnancy. Pregnancy in the rats under our conditions lasts 3 weeks.

The rats were divided in to four groups:

1. Pregnant rats treated with L-NAME (13 rats);
2. Pregnant control rats (8 rats);
3. Virgin rats treated with L-NAME (10 rats);
4. Virgin control rats (12 rats).

The caudal blood pressure was measured in conscious rats previously maintained at 35 to 40° C for 10 min. The rats were covered with cotton tissue during blood pressure measurements.

The caudal blood pressure and body weight of the pregnant rats were obtained at the end of the acclimatization period, and after the first and third weeks. In the virgin rats, the second measurement was obtained after L-NAME administration and after a similar period in the virgin control rats.

N<sup>w</sup>-L-nitro-arginine methyl ester (L-NAME) inhibits nitric oxide synthesis and was administered diluted in the drinking water. During this treatment, the rats were caged individually. The water was changed daily allowing determination of the volume drunk and calculation of the amount of the drug ingested. The concentration of L-NAME in the water was between 35 to 60 mg%, resulting in a daily consumption of L-NAME of approximately 50 to 70 mg/kg/day.

L-NAME treatment lasted 10 days in pregnant and virgin rats. This treatment began on day 10 of pregnancy.

The rats were sacrificed at the end of the

**Table 1 - Caudal blood pressure, weight and age during acclimatization period in the four groups of rats**

	Pregnant L-NAME n = 13	Pregnant control n = 8	Virgin L-NAME n = 10	Virgin control n = 12
Measurements				
Blood Pressure, mmHg	115.86 (11.13)	113.28 (11.87)	108.75 (3.90)	110.41 (13.46)
Weight, grams	196.23 (19.57)	201.50 (16.17)	198.80 (14.65)	206.91 (14.30)
Age, days	101.38 (8.26)	102.12 (6.52)	101.00 (5.08)	101.16 (3.86)

\* p < 0.05; standard deviation given in parenthesis.

**Table 2 - Caudal blood pressure**

Period	Pregnant L-NAME n = 13	Pregnant control n = 8	Virgin L-NAME n = 10	Virgin control n = 12
Acclimatization period	115.86 (11.13)	113.28 (11.87)	108.75 (3.90)	110.41 (13.46)
Week 1 (pregnant)	105.76 (12.61)	107.81 (15.22)	-	-
Week 3 (pregnant) or second measurement (virgin)	137.98 (13.07) <sup>*B§</sup>	98.43 (11.5) <sup>*B</sup>	154.37 (14.80) <sup>*B</sup>	109.89 (6.96)

\*p < 0.05 compared with respective controls; <sup>B</sup> p < 0.05 compared with the acclimatization period; <sup>§</sup> p < 0.05 compared with virgin rats; standard deviation given in parenthesis.

treatment or equivalent control period to perform the vascular reactivity study. The pregnant rats (groups 1 and 2) were sacrificed after approximately 20 days of pregnancy. The virgin rats were sacrificed after treatment with nitric oxide inhibitor in the drinking water.

The rats were anesthetized with 10% chloral hydrate administered subcutaneously. Laparotomy and thoracotomy were performed to obtain the thoracic aorta, which was rapidly immersed in Krebs-Henseleit solution. The aorta was dissected and all conjunctive and adipose tissues were removed. Two rings, 0.4 cm in diameter, were obtained from the aorta and the endothelium was mechanically removed from one of the pair. Each ring was suspended by a fine steel wire; and connected to an isometric tension transducer (Myograph F60); which was connected to a Narco Trace 40 polygraph (Narco-Bio-Systems Inc., Texas, USA). The rings were maintained in 15 ml Krebs-Henseleit solution at 37°C (range 36.5 to 37.5). A tension of 0.5 grams was maintained during an equilibration period of 60 min during which the bathing medium was changed every 20 minutes.

The aortic rings were contracted by adding noradrenalin  $10^{-7}$  M, a concentration that induces 60-70% of the maximum contraction, resulting in a tension from which the magnitude of subsequent vasorelaxation is highest. Progressively increased concentrations of acetylcholine were added to the preparations. The percentage relaxation of the contraction was measured.

Vascular reactivity was studied using dose-response curves and the percentage of relaxation induced by each dose of acetylcholine, and by measurement of the maximum response and sensitivity to acetylcholine.

The maximum response to acetylcholine was obtained from the mean and standard deviation of maximum response for each replicated experiment.

Acetylcholine sensitivity was estimated using EC50, i.e., the acetylcholine dose that produced 50% of the maximum contraction in each experiment. The means and standard deviations were calculated.

The drugs used were: norepinephrine bitartrate, acetylcholine chloride and N<sup>w</sup>-L-nitro-arginine methyl ester (L-NAME) (Sigma Chemical Company); potassium chlorate, calcium chloride and chloral hydrate from Merck; monobasic potassium phosphate (Carlo Erba); magnesium sulfate, glucose and sodium bicarbonate (Ecibra); and EDTA (Fischer Science Company).

Serum creatinine and uric acid were measured using the Jaffé method, modified and adapted for automation (RA-1000 System, Technicon Instruments Corporation, Tarrytown, NY, USA).<sup>18</sup> Uric acid was also measured using a chlorimetric, enzymatic technique adapted for automation.<sup>18</sup> The left kidney was removed and fixed in formalin for histopathological study.

The weight and number of offspring and the placental-fetus unit weight were also obtained.

*Statistical Methods.* The data were analyzed statistically using variance analysis (one or two way ANOVA, MANOVA), Student's T test and the Mann-Whitney and Kruskal-Wallis non-parametric tests (SPSS/PC<sup>®</sup> 3.0+ Microsoft Corp, 1988; and STATISTICA<sup>®</sup> for Windows<sup>™</sup>, release 4.3, Statsoft Inc., 1993).

## RESULTS

*Acclimatization period.* Blood pressure, weight and age were similar among all groups during the acclimatization period (Table 1).

*Blood pressure during pregnancy.* The blood pressure in the pregnant rats decreased during pregnancy. The caudal blood pressures were 114.44 mmHg (SD 11.82), 106.89 mmHg (SD 14.40) and 98.43 mmHg (SD 11.96) in the first, second and third weeks of pregnancy, respectively (Table 2).

*L-NAME effects on blood pressure.* Drinking water containing L-NAME was administered over a 10-day period to pregnant and virgin rats. The quantities of L-NAME ingested were similar between groups. The pregnant group consumed 60.62 mg L-NAME/kg/day (SD 12.81) and the virgin group 61.05 mg/kg/day (SD 10.32) (Table 2).

The mean caudal blood pressures during

the third week were significantly altered as follows (Table 2):

- a) the L-NAME pregnant group (137.98 mmHg, SD 13.07) was greater than the pregnant control group (98.43 mmHg, SD 11.58);
- b) the L-NAME virgin group (154.37 mmHg, SD 14.80) was greater than the virgin control group (109.89 mmHg, SD 6.96);
- c) the L-NAME virgin group was greater than the L-NAME pregnant group;
- d) the caudal blood pressures of the L-NAME-treated groups were greater than those of the control groups;
- e) the caudal blood pressures of the pregnant groups were lower than those of the virgin groups, independent of treatment.

The effects of L-NAME treatment on the blood pressures of pregnant and virgin rats were compared, using the differences in blood pressure between the acclimatization period and final period. In the pregnant group, blood pressure decreased by 9.38 mmHg and, in the L-NAME-treated group, blood pressure increased by 32.22 mmHg, an absolute difference of 41.60 mmHg. In the virgin group blood pressure decreased by 0.52 mmHg and, in the L-NAME-treated group, it increased by 45.62 mmHg, an absolute difference of 46.14 mmHg. These differences were not statistically significant (Table 2).

Analysis of the maximum relaxation obtained with cumulative doses of acetylcholine revealed the following (Fig. 1 and Table 3):

- a) Relaxation in aortic rings with endothelium from pregnant L-NAME-treated rats was blocked;
- b) Relaxation in aortic rings with endothelium from virgin L-NAME-treated rats was blocked;
- c) Relaxation in aortic rings without endothelium from pregnant rats was blocked;
- d) Relaxation in aortic rings without endothelium from virgin rats was blocked;
- e) Relaxation in aortic rings with endothelium from virgin L-NAME-treated rats was completely blocked; some relaxation occurred in the pregnant rats.

The sensitivity was evaluated using EC50. Our data revealed that the pregnant control

groups were more sensitive than the virgin control groups. In the pregnant group, the EC50 was  $1.4 \times 10^{-8}$  M, SD 1.12 and in the virgin group it was  $8.12 \times 10^{-8}$  M, SD 10.73 ( $p < 0.05$ ).

*Renal histopathology.* Renal histopathology was similar among the four groups.

*Plasma uric acid and creatinine.* Plasma creatinine and uric acid concentrations were similar among the four groups.

*Fetal outcome.* Analysis of the fetal outcome revealed the following:

- a) fetal weights in the L-NAME-treated, pregnant group (33.81 g, SD 14.76) were lower than in the control group (61.03 g, SD 33.76), although this was not statistically significant ( $p = 0.0638$ );
- b) the number of fetuses in the L-NAME-treated, pregnant group (9.46 fetuses, SD 3.45) was the same as in the control group (9.87 fetuses, SD 3.21) ( $p = 0.7501$ );
- c) the fetal-placental unit weights of the L-NAME-treated, pregnant group (3.89 g, SD 1.65) were lower than those of the pregnant control group (5.87 g, SD 1.92) ( $p = 0.0302$ ) (Table 5).

## DISCUSSION

Decreased blood pressure is characteristic of human and animal pregnancies. We confirmed this in Wistar rats when they became pregnant; blood pressure was also lower at the end of pregnancy. This fact has also been demonstrated by Molnar & Hertelendy,<sup>19</sup> Chu & Beilin,<sup>20</sup> Ahokas et al,<sup>16</sup> Allen et al.,<sup>21</sup> Aoi et al,<sup>21</sup> Baylis & Davinson<sup>22</sup> and Baylis & Engels.<sup>23</sup> Other animal models for hypertension, like the SHR, DOCA-salt and Goldblatt rats, also show decreased blood pressure during pregnancy.<sup>21,24-28</sup>

Decreased blood pressure is probably due to a decrease in peripheral vascular resistance and of vascular reactivity to vasoactive substances.

Pregnancy in rats and humans show some similarities like plasma volume expansion, increased glomerular filtration rates, decreased blood pressure and decreased vascular reactivity

to vasoactive substances.<sup>29,32</sup> Rats thus appear to be good animal models for the study of hemodynamic alterations during pregnancy.

Blood pressure control is determined by many factors. Endothelium-derived relaxing factor (EDRF), now known as nitric oxide, is one factor that can modulate blood pressure. Thus, analysis of the effect of nitric oxide inhibitors in rats may reveal the importance of nitric oxide in blood pressure control. In both pregnant and non-pregnant rats, administration of L-NAME in the drinking water increased blood pressure, revealing the importance of nitric oxide in controlling blood pressure, independently of whether the animal is pregnant or not.

The mean blood pressure was higher in the virgin L-NAME-treated group than in the pregnant L-NAME treated-group. This suggests that although inhibition of an important vasodilatation factor did occur, other pressure reducing mechanisms avoided serious hypertension during pregnancy, possibly using the same mechanism that decreased blood pressure in the pregnant control rats. If nitric oxide were the only mechanism controlling blood pressure, then the mean blood pressures of pregnant and virgin rats would be similar.

The correlations between the quantity of L-NAME ingested and blood pressure were not always linear. When small quantities of the drug

**Table 3 - Mean percentage relaxation induced by increasing doses of acetylcholine in aortic rings with endothelium**

Groups	Acetylcholine					
	10 <sup>-8</sup> M	3x 10 <sup>-8</sup> M	10 <sup>-7</sup> M	3x 10 <sup>-7</sup> M	10 <sup>-6</sup> M	3x 10 <sup>-6</sup> M
Pregnant L-NAME	1.37 (3.98) <sup>*B</sup>	3.25 (4.79) <sup>*B</sup>	5.51 (6.73) <sup>*BS</sup>	8.46 (10.08) <sup>*B</sup>	11.58 (13.33) <sup>*B</sup>	13.83 (15.63) <sup>*B</sup>
Pregnant control	35.68 (21.12) <sup>S</sup>	58.45 (25.87) <sup>S</sup>	66.42 (22.47) <sup>S</sup>	73.05 (20.82) <sup>S</sup>	76.05 (18.52) <sup>S</sup>	76.05 (18.52) <sup>S</sup>
Virgin L-NAME	-0.69 (1.36)	-4.49 (7.65) <sup>*</sup>	-3.01 (6.44) <sup>*</sup>	-2.93 (7.21) <sup>*</sup>	-1.75 (8.73) <sup>*</sup>	-1.51 (10.01) <sup>*</sup>
Virgin control	20.25 (16.35)	39.50 (24.29) <sup>S</sup>	61.16 (26.43) <sup>S</sup>	77.45 (19.10) <sup>S</sup>	87.96 (11.43) <sup>S</sup>	90.29 (9.05) <sup>S</sup>

\*p < 0.05 compared with concerning controls; <sup>B</sup> p < 0.05 compared with virgin rats; <sup>S</sup> p < 0.05 compared with aorta without endothelium; the values are the mean and standard deviations.

**Table 4 - Mean percentage relaxation induced by increasing doses of acetylcholine in aortic rings without endothelium**

Groups	Acetylcholine					
	10 <sup>-8</sup> M	3x 10 <sup>-8</sup> M	10 <sup>-7</sup> M	3x 10 <sup>-7</sup> M	10 <sup>-6</sup> M	3x 10 <sup>-6</sup> M
Pregnant L-NAME	0.07 (2.97)	-0.42 (4.31)	-0.60 (5.31)	-0.10 (7.54)	0.48 (9.62)	0.28 (10.84)
Pregnant control	-4.09 (2.82)	-5.54 (3.20)	-6.11 (3.63)	-6.90 (4.94)	-6.76 (5.92)	-7.06 (6.82)
Virgin L-NAME	-0.88 (1.27)	-2.54 (2.53)	-4.34 (3.42)	-5.99 (4.68)	-6.45 (6.91)	-6.62 (7.11)
Virgin control	-1.77 (3.65)	-1.91 (6.93)	-2.75 (9.33)	-2.87 (13.00)	-4.31 (14.86)	-2.90 (16.06)

\*p < 0.05 compared with the respective control; <sup>B</sup> p < 0.05 compared with virgin rats; the values are the mean and standard deviations.

**Table 5 - Fetal outcome**

Fetal outcome	Pregnant L-NAME-treated n = 13	Pregnant control n = 8
Total weight, grams	33.81 (14.76)	61.03 (33.76)
Number of fetuses	9.46 (3.45)	9.87 (3.21)
Weight of fetal-placental unit, grams	3.89 (1.65) <sup>*</sup>	5.87 (1.92)

\* p < 0.05; standard deviation given in parenthesis.

were ingested, a linear correlation was observed. However, with large amounts, blood pressure reached a limit and stabilized (data not shown). This limit was lower in the pregnant rats than in the virgin group (data not shown). This fact reinforces the idea that other hypotensive mechanisms are present which avoid hypertension in the pregnant rats.

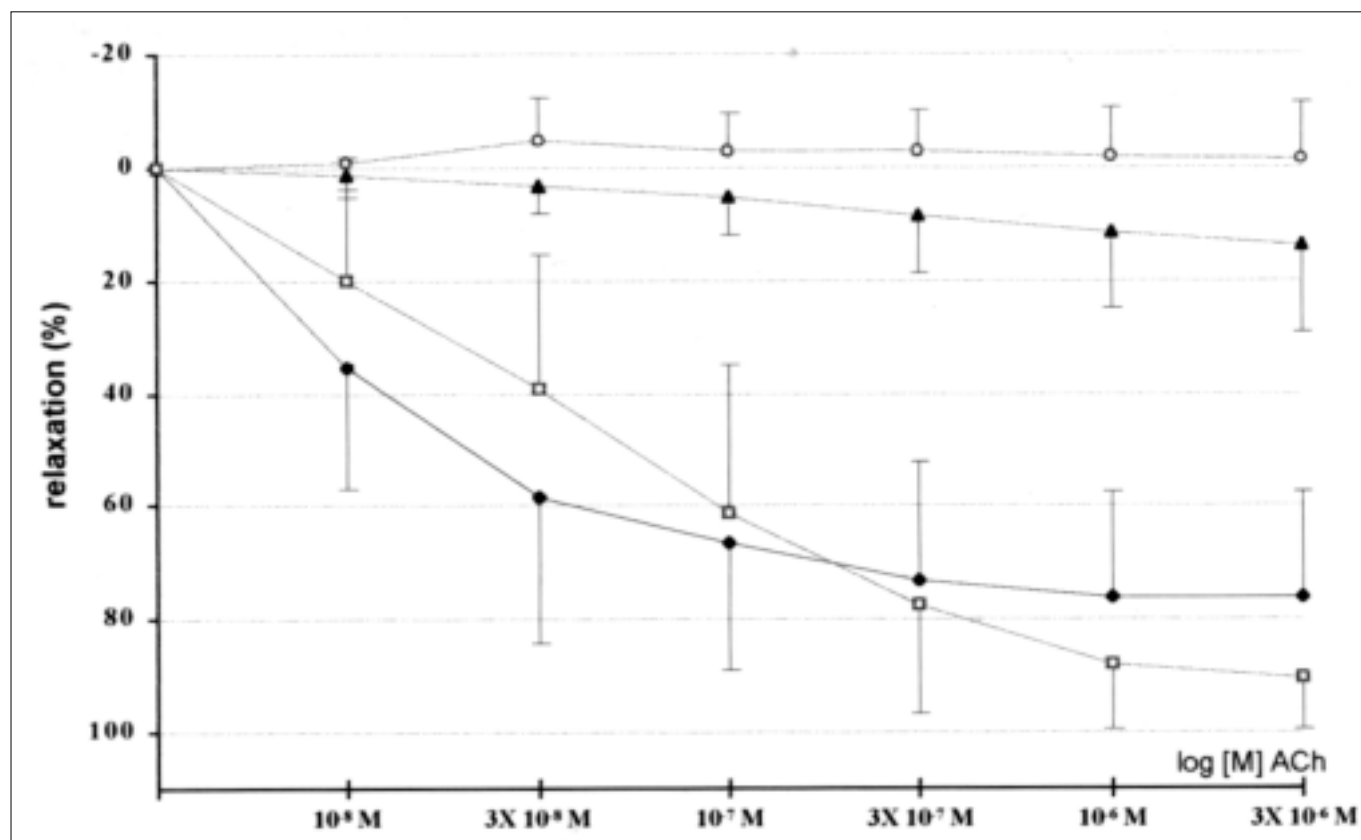
The blood pressure increased with the administration of a nitric oxide inhibitor and was higher in the virgin group than in the pregnant group. However, the mean blood pressures in the two groups were not statistically significant. These data suggest that nitric oxide does not play a more important role during pregnancy than during non-pregnancy.

We believe that during pregnancy many hypotensive mechanisms are activated and that when one of these is deactivated, there is a compensatory increase in the other mechanisms, which maintains adequate, normal, blood

pressure during gestation. Many hypertensive mechanisms are present in humans, providing protection against hemorrhagic shock, and various mechanisms may furnish protection against hypertension during normal pregnancy. Thus, inhibition of the mechanism may not result in great variations in blood pressure.

Allen et al.<sup>17</sup> demonstrated greater increase in blood pressure in virgin rats than in pregnant rats when both were treated with N<sup>G</sup>-monomethyl-L-arginine (LNMMA). Other investigators have found contrary data.<sup>15,16,19</sup>

We observed that the sensitivity of aortic rings from pregnant control rats to acetylcholine was greater than that of the virgin rats. This provides confirmation of the major effect of EDRF in aortic rings from pregnant rats, which may be due to an increase in nitric oxide release or effect, due to the release of another EDRF, due to increased sensitivity and quantity of muscarine receptors for acetylcholine, or due to increased



**Figure 1** - Percentage relaxation as a function of increasing doses of acetylcholine in aorta rings with endothelium. The values were expressed as mean  $\pm$  standard deviations ( $\circ$  - L-NAME virgin group;  $\blacktriangle$  - L-NAME pregnant group;  $\square$  - Control virgin group;  $\blacklozenge$  - Control pregnant group).

sensitivity of smooth muscle to cyclic AMP. Weiner et al.<sup>33</sup> obtained similar results.

St-Louis & Sciotte<sup>27</sup> obtained discordant data, although these authors did not observe greater sensitivity or an increase in the maximum response in pregnant rats, compared to virgin rats.

In the groups of rats treated with L-NAME, the relaxation effect was blocked. This suggests that nitric oxide is the principal factor responsible for the relaxation of aortic rings by acetylcholine and subsequent control of blood pressure.

In the virgin rats, the L-NAME treatment completely blocked relaxation. In the pregnant rats, residual relaxation was still present. This data suggests reduced efficiency of L-NAME in inhibiting the release of EDRF during pregnancy, which may result from the lower absorption or higher dilution of L-NAME, or from the presence of another EDRF, like EDHF (endothelium-derived hyperpolarizing factor).<sup>34</sup>

A comparison between pregnant and virgin rats is difficult, as the precise mechanism for L-NAME action, and its pharmacokinetics and pharmacodynamics during pregnancy, are unknown. Thus, caution is necessary when comparing virgin and pregnant rats.

Treatment with L-NAME had a detrimental effect on fetal outcome. Our data showed a decrease in fetal-placental unit weight. Yallampalli & Garfield<sup>35</sup> and Baylis & Engels<sup>23</sup> have also shown a poor fetal outcome. This may result from the direct action of the drug or from increased blood pressure.

The true function of nitric oxide during pregnancy is controversial. Our data suggest that EDRF is increased during pregnancy, probably by another EDRF, the action of which could not be blocked by L-NAME. This would explain the residual relaxation seen in pregnant rats, the similar differences in relaxation after L-NAME in the two groups, the lower increase in blood pressure in the pregnant rats, and the similar differences in blood pressure after L-NAME treatment in the two groups. However, the possibility of differential absorption and hemodilution of the drug cannot be completely excluded.

## CONCLUSION

Acetylcholine-induced vasorelaxation sensitivity is greater in pregnant rats than in virgin rats and that, after L-NAME administration, blood pressure increases, acetylcholine-induced vasorelaxation response is blocked and fetal outcome is detrimentally affected.

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## RESUMO

**Contexto:** Os mecanismos envolvidos na diminuição da pressão arterial e da resistência vascular periférica da gravidez não são totalmente esclarecidos, assim como a real importância do fator relaxante derivado do endotélio (Endothelium-derived relaxing factors - EDRF), um importante modulador do tônus vascular. **Objetivo:** O trabalho avaliou a importância do EDRF e seu principal componente, óxido nítrico, na prenhez das ratas. **Tipo de estudo:** Experimentação clínica em animal. **Local:** Laboratório Universitário de Farmacologia. **Variáveis estudadas:** Foram avaliados, em ratas Wistar normotensas, virgens ou prenhes, a influência da inibição crônica da síntese de óxido nítrico, utilizando o N<sup>w</sup>-nitro-L-arginina (L-NAME) diluído na água ingerida pelos animais durante 10 dias e comparadas com os respectivos grupos controles. Foram analisados as repercussões da prenhez e do L-NAME sobre a pressão arterial, reatividade vascular da aorta a acetilcolina *in vitro* e resultados perinatais. **Resultados:** A pressão arterial caudal diminuiu no final da prenhez. A sensibilidade da aorta das ratas prenhes à acetilcolina foi maior que nas virgens. O tratamento com L-NAME determinou aumento da pressão arterial caudal em ambos os grupos de animais e determinou bloqueio no relaxamento induzido pela acetilcolina, de modo semelhante a retirada mecânica do endotélio da aorta. O grupo de ratas prenhes que ingeriram L-NAME apresentou peso da ninhada e do peso de cada feto menor que o grupo controle. **Conclusões:** Demonstramos maior sensibilidade para acetilcolina na gestação, contribuindo com a diminuição da pressão arterial e um bloqueio no relaxamento induzido pela acetilcolina após administração de L-NAME.

**Palavras-chave:** Reatividade vascular. Prenhez. Ratas. L-NAME. Óxido nítrico.