 

# *Evaluation of Low-dose Endotoxin Administration during Pregnancy as a Model of Preeclampsia*

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***Background:*** **Recent evidence implicates nitric oxide ($^{\cdot}NO$) in the pathogenesis of preeclampsia. The authors tested the hypothesis that administration of low-dose endotoxin to pregnant rats mimics the signs of preeclampsia in humans and that $^{\cdot}NO$ and $^{\cdot}NO$-derived species play a role in that animal model.**

***Methods:*** **Endotoxin was infused at doses of 1, 2 and 10 μg/kg over 1 h to rats on day 14 of pregnancy. Mean arterial pressure, urinary protein, urinary and plasma nitrite plus nitrate ($NO_2^- + NO_3^-$) concentrations, and platelet count were measured before and after the endotoxin infusion. In another group of pregnant rats, the nitric oxide synthase inhibitor L-nitroarginine methyl ester (L-NAME) was administered in drinking water at a dose of $3\ mg \cdot kg^{-1} \cdot d^{-1}$ starting on day 7 of pregnancy. Endotoxin was then infused at 10 μg/kg on day 14 of pregnancy. Kidneys and uteroplacental units were examined histologically and analyzed immunohistochemically for 3-nitrotyrosine.**

***Results:*** **Endotoxin administration at doses of 2 and 10 μg/kg caused proteinuria and thrombocytopenia in pregnant rats, but did not result in hypertension. Urinary $NO_2^- + NO_3^-$ concentration, reflective of tissue $^{\cdot}NO$ production rates, was significantly elevated in pregnant rats that received endotoxin at 10 μg/kg. Ingestion of L-NAME caused hypertension. Tissues from pregnant rats treated with L-NAME, endotoxin at 10 μg/kg, and a combination of L-NAME and endotoxin had increased 3-nitrotyrosine immunoreactivity.**

***Conclusion:*** **Nitric oxide either directly or through secondary species plays a significant role in the biochemical and physiologic changes that occur in a rodent model of endotoxin-induced injury. (Key words: Animal model; 3-nitrotyrosine; nitric oxide.)**

PREECLAMPSIA is one of the most significant health problems in human pregnancy, affecting 6–8% of all pregnancies and accounting for 20–25% of total perinatal mortality. Preeclampsia also presents anesthetic challenges resulting from tissue edema, difficult airway management, intravascular volume depletion, hypertension, and thrombocytopenia.[1]

The pathophysiology of preeclampsia remains unclear. A major limitation has been the inability to develop an animal model that successfully mimics all of the clinical features of preeclampsia. One model used the infusion of endotoxin 1 μg/kg to rats on day 14 of pregnancy.[2] Endotoxin infusion at these doses produced hypertension, proteinuria, and thrombocytopenia in pregnant rats, but the mechanism was not elucidated. It has been suggested that the hypertensive response resulting from this endotoxin infusion is mediated by endothelin.[2] Some clinical studies have shown good correlation between endothelin levels and the incidence of preeclampsia,[3] whereas other studies have failed to demonstrate a correlation.[4] Low-dose endotoxin has also been reported to induce a generalized Shwartzman reaction when infused in pregnant rats.[5] This reaction shares some of the features of preeclampsia, including thrombocytopenia and disseminated intravascular coagulation, especially in the glomerular microvasculature of the kidneys, where cortical necrosis may eventually occur.[6] On the other hand, high-dose endotoxin infusion has been demonstrated to cause systemic hypotension through increased synthesis of nitric oxide ($^{\cdot}NO$) in pregnant rats.[7] The opposing blood pressure responses to endotoxin infusion appear to be dose-dependent.

The toxicity of $^{\cdot}NO$ is thought to result, at least in part, from the reaction of $^{\cdot}NO$ with superoxide ($O_2^-$) to produce the potent oxidizing and nitrating species peroxynitrite ($ONOO^-$).[8] Evidence for the reactions of $ONOO^-$ *in vivo* is based predominantly on the detection of 3-nitrotyrosine in injured tissues, although other reaction pathways involving $^{\cdot}NO$ production in the presence of oxidative stress could result in tyrosine nitration as well.[9] Immunoreactive 3-nitrotyrosine has been demonstrated in placental villous tissue from normotensive human pregnancies complicated by preeclampsia; however, a causal link between 3-nitrotyrosine formation and the tissue dysfunction associated with preeclampsia remains elusive.[10]

In the present study, we tested the hypothesis that administration of low-dose endotoxin to pregnant rats mimics preeclampsia in humans and that $^{\cdot}NO$ and $^{\cdot}NO$-derived species play a contributory role in reproducing the clinical features of preeclampsia in the proposed animal model. The criteria chosen as indicative of preeclampsia-like phenomena in pregnant rats included hypertension, proteinuria, and low platelet count. Although thrombocytopenia is not a consistent feature of preeclampsia, it is a diagnostic criterion for severe disease and is a feature of the variant HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets). Insight into the mechanism of preeclampsia could lead to improved therapy and improved prognosis.

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## Materials and Methods

### *Surgical Preparation*

Animal use approval was obtained from the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Female Sprague-Dawley rats (n = 40) 14 weeks of age, 250–300 g, were kept in a constant temperature (75°F) and light-controlled room (lights on 6 AM to 6 PM) with free access to food and water. Using sterile technique, radiotelemetric blood pressure implants (TA11PA-C40, Data Sciences International, Saint Paul, MN) were inserted into the abdominal aorta through a midline laparotomy incision during ketamine–xylazine anesthesia (9 mg and 1.5 mg/kg, respectively).[11] After 10 days of recovery from surgery, rats were mated with fertile males. A positive vaginal smear for sperm defined day 1 of pregnancy (duration of gestation is 21 days). On day 14 of pregnancy, rats were anesthetized, and the femoral artery and vein were cannulated. Blood samples (200 μl each) were collected from the femoral artery cannulae for platelet count and urinary and plasma nitrite plus nitrate ($NO_2^- + NO_3^-$) measurement, and then endotoxin was infused through the femoral vein cannulae over 1 h. The cannulae were then removed, and the animals were allowed to recover.

### *Preparation of Endotoxin and L-Nitroarginine Methyl Ester*

Endotoxin (*Escherichia coli*, 0.55: B5, Whittaker MA Bioproducts, Walkerville, MD) was prepared in 2 ml of pyrogen-free normal saline to be infused *via* multisyringe pump (KD Scientific, Boston, MA) over 1 h (constant infusion) in the femoral vein of the corresponding group of rats. L-Nitroarginine methyl ester (L-NAME; Sigma, Saint Louis, MO) was administered orally by dissolving 3 $mg \cdot kg^{-} \cdot d^{-1}$ in drinking water. The concentration was adjusted to daily water intake.

### *Radiotelemetric Blood Pressure Measurement*

Mean arterial pressure (MAP) was continuously monitored and recorded every 20 min. A 24-h moving average was calculated by Advanced Research Technology data acquisition system software (Data Sciences International) and exported into Microsoft Excel software (Microsoft Corporation, Redmond, WA) for further statistical analysis.

### *Experimental Design*

Experiments were organized into two series: series 1 was designed to study the dose-dependent effects of endotoxin single infusion (single constant infusion over 1 h on day 14 of pregnancy) on MAP, urinary and plasma protein content, and platelet count. Plasma and urinary $NO_2^- + NO_3^-$ were determined to demonstrate increased $\cdot$NO metabolites in urine and plasma. On the last day of experiment (day 21), the localization and extent of 3-nitrotyrosine immunoreactive protein in kidneys and uteroplacental units of pregnant rats who had received endotoxin (n = 21) was examined.

On day 14 of pregnancy, 200-μl blood samples were drawn from the femoral artery of the anesthetized rats, and then endotoxin was infused immediately over 1 h at doses of 1 μg/kg (n = 6), 2 μg/kg (n = 7), and 10 μg/kg (n = 8). A sham control group of pregnant rats (n = 7) received 2 ml of pyrogen-free saline infusion over 1 h. Both cannulae were then removed, and all animals were allowed to recover from anesthesia.

Two blood samples were drawn from the rats, one before the infusion of endotoxin from the femoral artery (on day 14 of pregnancy), and one drawn on day 21 of pregnancy by exsanguinating and killing the animals during anesthesia. A portion of the whole blood sample (25 μl) was used for platelet count, whereas the remainder was centrifuged to obtain the plasma for quantitation of $NO_2^- + NO_3^-$. Urine samples were collected over 24 h using metabolic cages on days 6, 12, 15, and 19 of pregnancy. The urine samples were used for measurement of protein and $NO_2^- + NO_3^-$ in urine. Blood pressure was continuously measured and automatically saved every 20 min using the radiotelemetric method throughout the experiment period (days 1–21 of pregnancy). The mean of the 24-h blood pressure was calculated using the data acquisition software. The mean blood pressure for 7 days before and 14 days after the infusion of endotoxin was calculated.

Series 2 was designed to further elucidate the role of $\cdot$NO in endotoxin-induced injury. The NOS inhibitor L-NAME was introduced in drinking water of pregnant rats at a dose of 3 $mg \cdot kg^{-1} \cdot d^{-1}$ starting from day 7 of pregnancy (n = 8). The group of pregnant rats receiving endotoxin infusion at a dose of 10 μg/kg on day 14 of pregnancy (n = 4) was designated as the PLE group. As a positive control group (PL group), some pregnant rats (n = 4) received L-NAME only in drinking water and a saline infusion on the date that corresponded to day 14 of pregnancy. The sham negative control group (PC group, n = 4) consisted of pregnant rats that had saline infusion on day 14 of pregnancy. Blood samples were drawn one time from the femoral artery before the infusion of endotoxin (on day 14 of pregnancy) and a second time (on day 21 of pregnancy by exsanguinating the rats) for platelet count and quantitation of $NO_2^- + NO_3^-$ in plasma. Urine samples were collected over 24 h twice on days 6 and 12 before the endotoxin infusion and twice on days 15 and 19 after the single endotoxin infusion. The urine sample on day 6 would be the only sample before the start of L-NAME in drinking water. Urine samples were analyzed for protein and $NO_2^- + NO_3^-$ content. Blood pressure was measured as described above.

On day 21 (last day) of pregnancy, the rats were killed by exsanguination during ketamine–xylazine anesthesia, and the kidneys and uteroplacental units were collected

for histologic examination and immunohistochemical detection of protein tyrosine nitration.

*Platelet Count Determination*

Samples (25 μl whole blood) were diluted 1:100 in ammonium oxalate (11.45 g/l $H_2O$), thimerosal (0.1 g/l $H_2O$) and Sorensen's buffer, pH 6.8. The diluted samples were mounted on a microcell-counter (Sysmex F 800, Toa Medical Electronics, Kobe, Japan) and counted.

*Determination of Urinary Albumin Excretion*

Urine samples were stored at −70°C until analysis. Protein concentration was determined by a modification of the bicinchoninic acid method (Pierce Chemical, Rockford, IL).[12]

*Urinary and Plasma $NO_2^-$ + $NO_3^-$ Assay*

Total $NO_2^-$ + $NO_3^-$ was measured in plasma and urine by a modification of the Griess reaction,[13] using *E. coli* to convert $NO_3^-$ to $NO_2^-$ before measurement at 520 nm. Calibration curves were prepared using 0–100 μM $NO_2^-$ standards.

*Histology and Immunohistochemistry*

Tissues were fixed with freshly prepared 4% paraformaldehyde in 0.1 M potassium phosphate, pH 7.4, for 16–18 h. Tissues were then transferred to 70% ethanol. Samples were further dehydrated through ascending ethanol concentrations, embedded in paraffin, and sectioned. Immunohistochemical staining was performed using the avidin-biotin peroxidase labeling (DAKO Corporation, Carpinteria, CA) *via* a biotinylated secondary antibody (Jackson Immuno Research, Westgrove, PA).[14] To determine the specificity of immunostaining, controls were performed for each experimental group. To determine specificity of the primary antisera, antibody binding the primary antibody was preadsorbed with 5 mM free 3-nitrotyrosine. Positive controls were also generated by directly nitrating tyrosine in tissue sections with 1 mM peroxynitrite in 50 mM phosphate buffer, pH 7.4, for 5–10 s. Another group of slides was stained with hematoxylin and eosin for histology. A pathologist blinded to the protocol evaluated the slides by selecting a representative sample that reflected the treatment group. All animals were observed for any sign of illness such as behavioral changes, loss of appetite, or death. All fetuses were weighed, counted, and inspected for gross anatomic abnormalities.

*Statistical Analysis*

Data are presented as mean ± SD. All study parameters were analyzed using the SigmaStat software by Jandel Scientific (San Rafael, CA). A paired *t* test was used to compare the data before and after treatment within the same group. One-way analysis of variance was used to compare data between multiple groups. Student-Newman-Keuls and Dunn's method was used for pairwise multiple comparison procedures and applied as appropriate. *P* less than 0.05 was considered statistically significant.

## Results

All rats that were exposed to endotoxin infusion recovered fully and did not develop any sign of illness such as lethargy, behavioral changes, or loss of appetite. None of the treated animals died as a result of the treatment.

Infusion of endotoxin at 1 μg/kg (n = 6), 2 μg/kg (n = 7), and 10 μg/kg (n = 8) did not significantly alter MAP (fig. 1A). Similarly, infusion of saline to the sham control group did not significantly change MAP (fig. 1A). Infusion of endotoxin at 2 and 10 μg/kg, but not 1 μg/kg, caused significant proteinuria ($P < 0.05$; fig. 1B). Infusion of endotoxin at 2 and 10 μg/kg, but not 1 μg/kg, caused a significant decrease in platelet count ($P < 0.05$; fig. 1C) (normal count is 865 ± 50).[2] However, when L-NAME was administered, MAP was increased in both the PL (n = 4) and PLE (n = 4) groups ($P < 0.05$; fig. 2). When endotoxin was administered on day 14 (PLE group), the MAP decreased to pre–L-NAME or control group levels (fig. 2).

Endotoxin infusion at 10 μg/kg significantly increased urinary (fig. 3A) and plasma (fig. 3B) $NO_2^-$ + $NO_3^-$ ($P < 0.05$). There was a slight increase in the plasma $NO_2^-$ + $NO_3^-$ in the control and 1- and 2-μg/kg endotoxin groups, but this increase did not reach statistical significance (fig. 3B). Oral administration of L-NAME significantly decreased urinary $NO_2^-$ + $NO_3^-$ in both the PL and PLE groups ($P < 0.05$; fig. 4A), but after endotoxin infusion in the PLE group, that effect was reversed, and urinary $NO_2^-$ + $NO_3^-$ increased significantly (fig. 4A). Ingestion of L-NAME resulted in significant proteinuria ($P < 0.05$) in pregnant rats (fig. 4B). Infusion of endotoxin on day 14 of pregnancy resulted in significant augmentation of the proteinuria caused by L-NAME administration (fig. 4B; $P < 0.05$). There was no change in urinary $NO_2^-$ + $NO_3^-$ in the PC group (fig. 4A).

Oral administration of L-NAME had no effect on platelet count (slight decrease that did not reach statistical significance; fig. 5A). Endotoxin infusion in presence of L-NAME treatment caused a significant decrease in platelet count (fig. 5A), similar to the decrease in platelet count that occurred after 10 μg/kg endotoxin infusion without prior L-NAME treatment. Plasma $NO_2^-$ + $NO_3^-$ increased significantly in the PLE group after endotoxin infusion (fig. 5B). There was no change in the plasma $NO_2^-$ + $NO_3^-$ in the PC group (fig. 5B).

Endotoxin (fig. 6) caused a dose-dependent thickening of the media of placental vessel walls (figs. 6B and 6C) compared with the control group (fig. 6A). Endotoxin caused a dose-dependent glomerular endotheliosis with

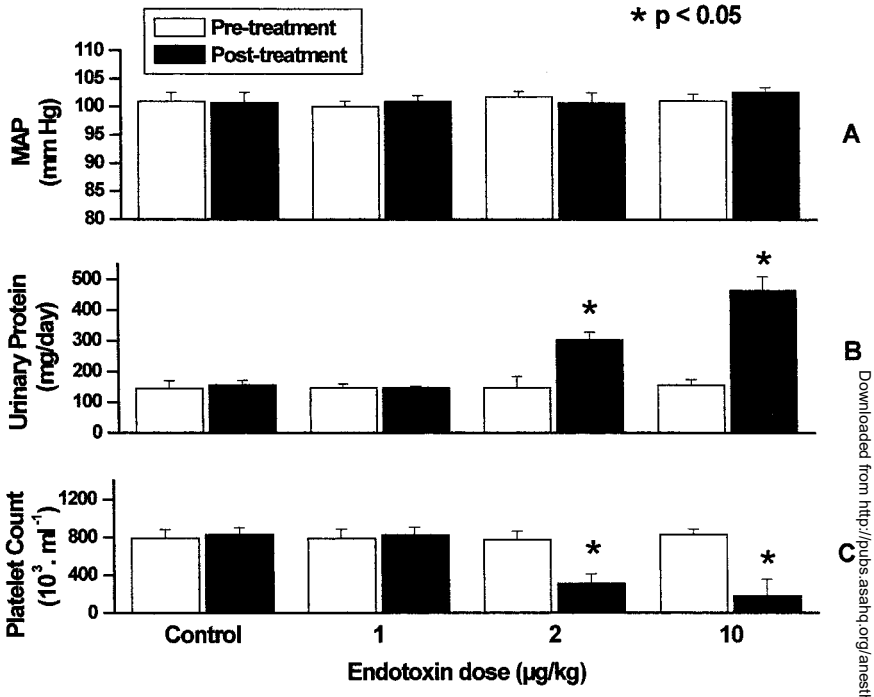


**Fig. 1. (*A*) The blood pressure effect of intravenous infusion of endotoxin at 1 (n = 6), 2 (n = 7), and 10 (n = 8) μg/kg on day 14 of pregnancy compared with sham control animals (n = 7). The mean arterial pressure (MAP) from days 1 to 14 of pregnancy (pretreatment, white bars) was compared with the mean value from days 14 to 21 of pregnancy (posttreatment, black bars). No significant change of MAP was observed after endotoxin infusion. (*B*) The effect of intravenous infusion of endotoxin on urinary protein content. Endotoxin at 2 and 10 μg/kg significantly increased the urinary protein excretion. (*C*) The effect of intravenous infusion of endotoxin on platelet count. Endotoxin at 2 and 10 μg/kg significantly decreased the platelet count. *$P < 0.05$.**

accumulation of inflammatory mononuclear and polynuclear cells and proliferation of the glomerular tufts (figs. 6E and 6F) compared with control (fig. 6D). Administration of L-NAME caused thickening of media of the kidney arteriolar walls (fig. 6G). Immunohistochemistry of the same tissues (fig. 7) showed 3-nitrotyrosine immunoreactivity in the placental and kidney vessel walls of the 10-μg/kg endotoxin group (figs. 7C and 7D) compared with controls (figs. 7A and 7B). Immunohistochemistry showed 3-nitrotyrosine immunoreactivity of the placental and kidney vessel walls in both the PL (figs. 7E and 7F) and PLE (figs. 7G and 7H) groups. There was accumulation of polymorphonuclear leukocytes (PML) with intense 3-nitrotyrosine immunoreactivity in the kidneys of both PL and PLE groups (figs. 7E and 7G). The 3-nitrotyrosine immunostaining was more intense in the kidneys of pregnant rats treated with endotoxin plus L-NAME (fig. 7G).

Endotoxin had no effect on the litter size or fetal weight. However, L-NAME ingestion resulted in significant decrease in the fetal weight (control 6.1 ± 0.1 g *vs.* 5.4 ± 0.2 g in the PL and PLE groups, respectively; $P < 0.05$), whereas the litter size remained unchanged. No fetal anomalies were observed in any group.

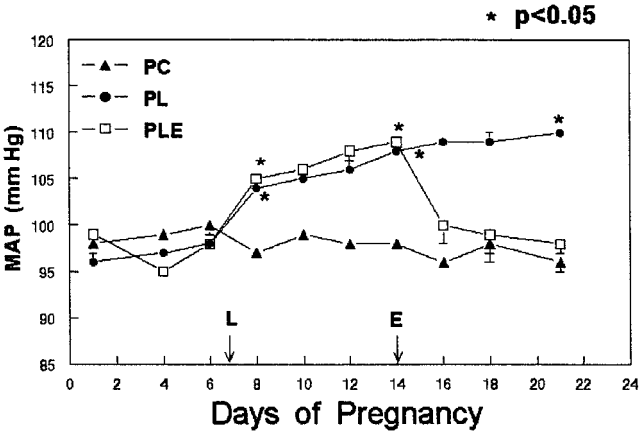


**Fig. 2. The effect of oral administration of L-NAME (L) on day 7 of pregnancy and 10-μg/kg intravenous infusion of endotoxin (E) on day 14 of pregnancy on mean arterial pressure (MAP). The results of MAP were averaged every 2 days. No change in MAP was observed in the pregnant control group (PC, triangles). Administration of L-NAME to the positive control group (PL, circles) resulted in hypertension, whereas further infusion of E to the PLE group of animals (PLE, squares) resulted in further decrease of MAP to pre L-NAME administration levels. *$P < 0.05$.**

## Discussion

Endotoxin infusion at 2 and 10 μg/kg on day 14 of pregnancy caused proteinuria and thrombocytopenia in a dose-dependent fashion. Other investigators have demonstrated lack of effect of endotoxin on virgin cyclic rats at similar doses.[2,15] At 10 μg/kg endotoxin, the $NO_2^-$ + $NO_3^-$ content of both urine and plasma increased significantly, suggesting increased synthesis of ˙NO. However, neither endotoxin dose resulted in hypertension, contrary to the findings of other investigators.[2] Therefore, in our hands, administration of endotoxin to pregnant rats did not mimic the clinical disease of preeclampsia. Administration of the NOS inhibitor L-NAME to pregnant rats resulted in hypertension and proteinuria. There is some clinical evidence that the levels of the endogenous NOS inhibitors, such as asymmetric dimethyl-arginine, increase in preeclampsia.[16] Other investigators have also shown that L-NAME causes

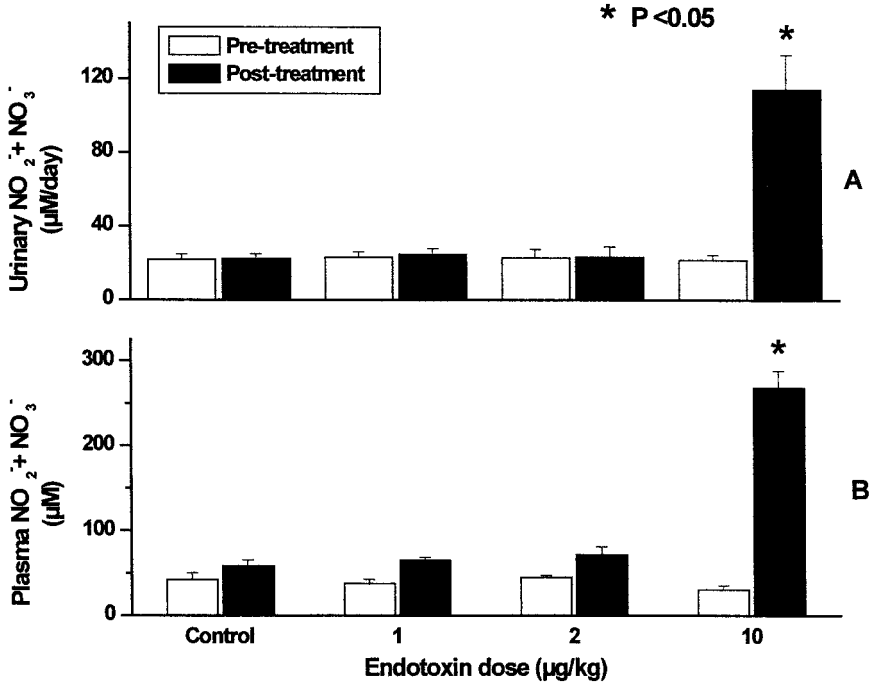


**Fig. 3. The effect of intravenous infusion of endotoxin on urinary and plasma nitrite plus nitrate ($NO_2^-$ + $NO_3^-$). (*A*) Endotoxin at 10 μg/kg resulted in significant increase in urinary $NO_2^-$ + $NO_3^-$. (*B*) Similarly, it resulted in significant increase in plasma $NO_2^-$ + $NO_3^-$. **P* < 0.05.**

hypertension only in pregnant female rats and not in virgin controls.[17] Administration of endotoxin to L-NAME–treated pregnant animals resulted in augmentation of the proteinuria but attenuated the hypertension produced by L-NAME. 3-Nitrotyrosine immunoreactivity was identified in the renal vasculature and uteroplacental units of pregnant rats treated with endotoxin at the 10-μg dose, L-NAME, or L-NAME plus endotoxin. 3-Nitrotyrosine immunoreactivity was more intense in the uteroplacental units of the pregnant rats treated with L-NAME plus endotoxin.

Endotoxemia has been linked to a variety of human disease, including sepsis, inflammatory bowel disease, liver failure, and pancreatitis.[18] Endotoxin has also been used to generate animal models for septic shock,[19] pulmonary inflammatory and immune diseases,[20] acute anterior uveitis,[21] and preeclampsia.[2] In our experimental model, there was thickening of the media of the blood vessels of the uteroplacental units and kidneys of the pregnant rats treated with endotoxin at 2 and 10 μg/kg and the PL group, respectively, changes that are commonly observed in preeclampsia. Administration of L-NAME to

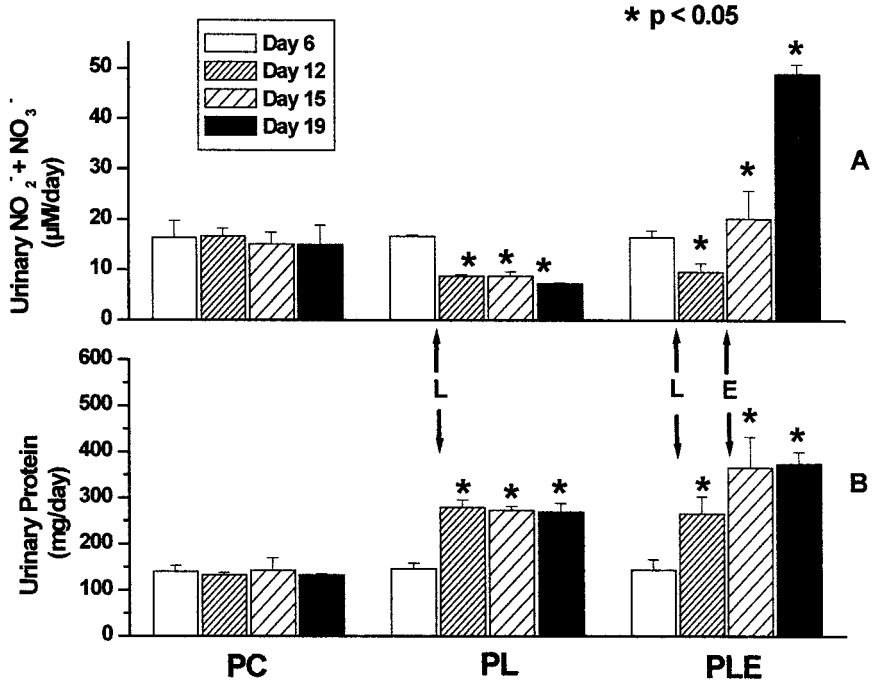


**Fig. 4. (*A*) The effect of oral administration of L-NAME (L) and 10 μg/kg intravenous infusion of endotoxin (E) on urinary levels of nitrite plus nitrate ($NO_2^-$ + $NO_3^-$). Urine samples were obtained before L-NAME on day 6, after L-NAME but before endotoxin on day 12, and after endotoxin on days 15 and 19 of pregnancy. L-NAME resulted in decrease in urinary $NO_2^-$ + $NO_3^-$ levels in the pregnant positive control group (PL) and the group of pregnant rats that were administered oral L-NAME starting on day 7 and then a single 10-μg/kg endotoxin infusion on day 14 of pregnancy (PLE). Endotoxin infusion in the PLE group resulted in reversal of that effect and significant increase in the urinary $NO_2^-$ + $NO_3^-$ levels. (*B*) The effect of oral administration of L-NAME and 10-μg/kg intravenous infusion of endotoxin on urinary protein levels. L-NAME resulted in significant proteinuria, which was augmented by endotoxin infusion. **P* < 0.05. PC = pregnant negative control group.**

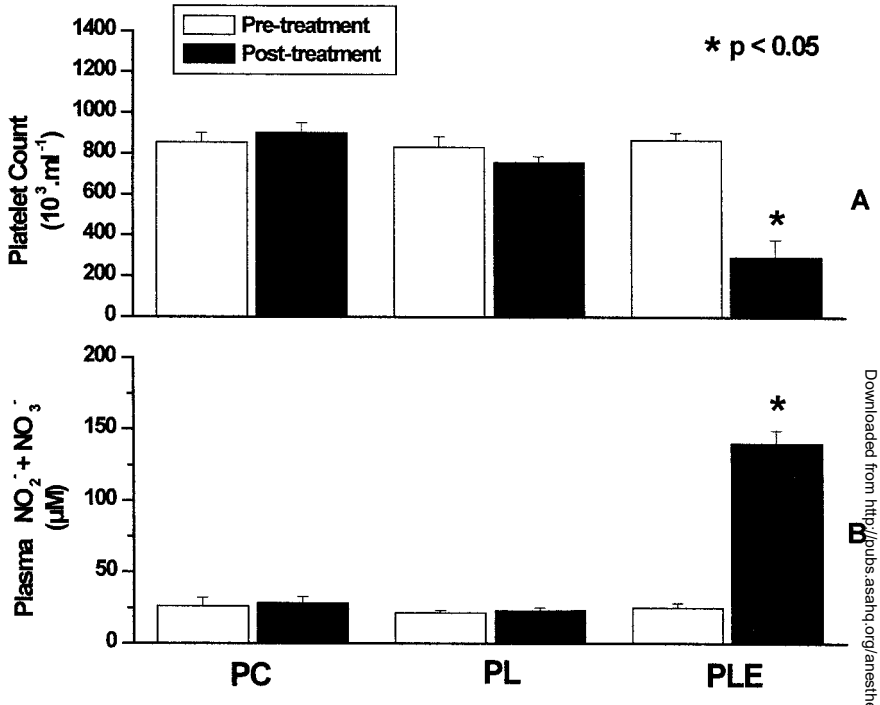


**Fig. 5. (*A*) The effect of L-NAME and endotoxin on platelet count. Pretreatment (white bars) refers to treatment with endotoxin on day 14 of pregnancy, whereas posttreatment (black bars) refers to the sample after treatment with endotoxin on the last day of pregnancy (day 21). Endotoxin resulted in significant decrease in platelet count. (*B*) The effect of L-NAME and endotoxin on plasma nitrite plus nitrate ($NO_2^- + NO_3^-$) levels. Endotoxin resulted in significant increase in plasma $NO_2^- + NO_3^-$ levels. *$P < 0.05$. PC = pregnant negative control group; PL = pregnant positive control group; PLE = group of pregnant rats that were administered oral L-NAME starting on day 7 and then a single 10-μg/kg endotoxin infusion on day 14 of pregnancy.**

pregnant rats resulted in increased MAP, possibly through partial inhibition of NOS. Endotoxin infusion normalized the L-NAME–induced increase in MAP, probably a result of increased · NO biosynthesis, mediated through cyclic guanosine monophosphate.[22] This interpretation is inferred from the increase of $NO_2^- + NO_3^-$ levels in both the plasma and urine after 10 μg/kg of endotoxin infusion on day 14 of pregnancy. It has also been reported that endotoxin at ultralow dose (1 μg/kg) can cause systemic hypertension through release of endothelin,[2] rather than · NO. This is an unlikely explanation for the observed change of MAP after endotoxin infusion because the · NO metabolites, $NO_2^- + NO_3^-$, increased after endotoxin infusion, with an accompanying decrease in MAP, suggestive of · NO- rather than endothelin-mediated change. Other investigators have demonstrated a transient increase of endothelin levels after endotoxin infusion.[23] Recently published evidence from studies of cultured human placental endotheliocytes suggests that there is some cross-link between the · NO and endothelin regulatory mechanisms.[24] The endotoxin effects also seem to be dose-dependent, resulting in paradoxical systemic hypotension[7] and pulmonary hypertension[25] at high doses (≥ 10 μg/kg), mainly *via* enhanced · NO synthesis.

The apparent discrepancy between the endotoxin effect on MAP in this study and previous studies may be attributed to differences in the strain of rat or methodology used for measurement of blood pressure. In the present study, Sprague-Dawley rats were used, whereas previous investigations used Wistar rats. Strain differences may increase the susceptibility of Wistar strain to endotoxin-mediated hypertension. In the present study, MAP was measured *via* an invasive radiotelemetric method rather the indirect tail-cuff method used by other investigators. Radiotelemetry provides a number of benefits over conventional methods for monitoring blood pressure, including the ability to collect data continuously without intervention; the elimination of the stress caused by handling, restraint, and tethering; and the elimination of the need for anesthesia during measurement.[26] A systolic blood pressure of up to 37 mmHg higher was reported when blood pressure was measured with the tail-cuff method compared with simultaneous aortic cannulation.[27] On the other hand, other investigators have reported good correlation between the tail-cuff method and the direct invasive monitoring.[28] Infusion of endotoxin at doses higher than 10 μg/kg results in hypotension and a sepsis-like picture in rats.[19] Endotoxin infusion at the three doses that were used in this experiment did not result in hypotension. It is unclear why other investigators have detected hypotension with infusion of endotoxin at 6.5 μg/kg in light of the fact that they could not detect any endotoxin levels after infusing it at that dose.[2] Although we did not measure the temperature of the animals in the present experiments, they did not develop any sign suggestive of sepsis or severe illness such as lethargy, loss of appetite, or behavioral changes.

Preeclampsia is characterized by the presence of inflammatory stimuli that enhance tissue $O_2^-$ production, which may then react with · NO and contribute to the pathophysiologic changes observed in preeclampsia, namely, the formation of cytotoxic reactive species and impaired · NO-dependent vascular function.[29] Immunohistochemical analysis of the blood vessels of the utero-

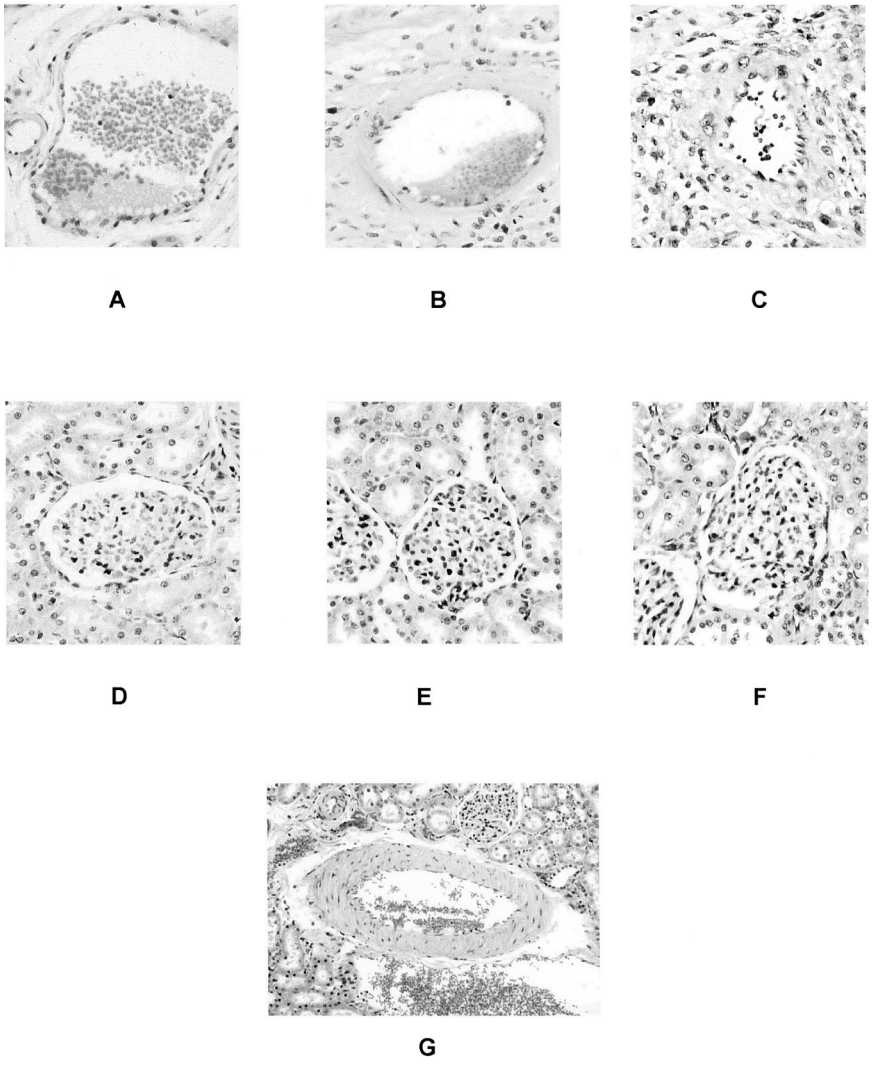


**Fig. 6. (*A*) Histologic hematoxylin and eosin examination of the placental vessel of the control group of animals. There was a dose-dependent thickening of the placental vessel wall at 2 μg/kg (*B*) and 10 μg/kg (*C*) endotoxin regimen. (*D*) Histologic hematoxylin and eosin examination of the kidney of the control animals. There was a dose-dependent glomerular endotheliosis with accumulation of inflammatory mononuclear and polynuclear cells and proliferation of the glomerular tufts (*E* and *F*) (original magnification, 400×). (*G*) Histologic hematoxylin and eosin examination of the kidney vessels of the group of animals that received L-NAME shows thickening of the media of the kidney vessels (original magnification, 200×).**

placental tissues showed 3-nitrotyrosine immunoreactivity in the 10-μg/kg endotoxin, PL, and PLE groups, resembling the changes observed in the placental villous tissue[10] and vasculature of preeclamptic women.[30] However, in this model we cannot firmly associate any of the findings with human preeclampsia because of failure to generate the entirety of the disease.

The 3-nitrotyrosine immunoreactivity in response to L-NAME treatment suggests increased $^{\cdot}$NO production in the presence of other oxidative mediators, conflicting with the common agreement that L-NAME decreases $^{\cdot}$NO production, therefore decreasing the substrate for 3-nitrotyrosine formation. Part of these results may reflect the low dose of L-NAME causing only partial inhibition of $^{\cdot}$NO synthesis while still equimolar with $O_2^-$ to form 3-nitrotyrosine. An alternative mechanism is that L-NAME administration causes a paradoxical increase in tissue $^{\cdot}$NO release determined electrochemically and by $NO_2^- + NO_3^-$ assay.[31] One suggested mechanism by which L-NAME could increase the production of $^{\cdot}$NO is that it induces *NOS II* gene expression, a NOS isoform less susceptible to inhibition by L-NAME.[31] In pregnancy, *NOS II* appears to play a role that is normally assumed by *NOS III*, namely, smooth muscle relaxation.[31] Another mechanism by which L-NAME could increase the production of $^{\cdot}$NO is that it decreases $^{\cdot}$NO production in the larger, conducting vessels (*e.g.*, the uterine arteries) leading to compensatory $^{\cdot}$NO-mediated vasodilatation in the smaller vessels within the tissues[32] and possibly reacting with $O_2^-$, leading eventually to the formation of 3-nitrotyrosine. Apoptosis and 3-nitrotyrosine immunoreactivity were identified in the uteroplacental units of the L-NAME– and endotoxin-treated pregnant rats.[31]

Although tyrosine nitration is commonly used as a marker of $ONOO^-$ formation *in vivo*, recent studies have challenged this paradigm and explored alternate mechanisms for nitration of tyrosine as through myeloperoxidase[9] and other pathways that are beyond the scope of this study. Immunoreactivity to 3-nitrotyrosine was identified in PMLs, infiltrating the kidneys of the pregnant rats treated with L-NAME plus endotoxin. Myeloperoxidase is present in PML of rodents, which suggests that the myeloperoxidase-mediated pathway of nitration of tyrosine could also be an operative mechanism

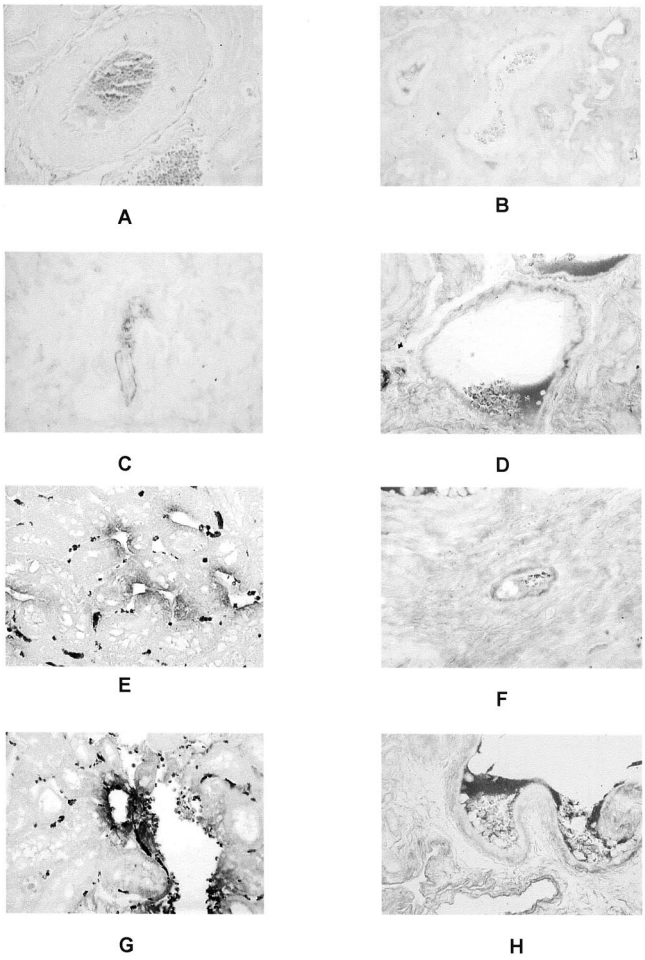


**Fig. 7. (*A* and *B*) Absence of 3-nitrotyrosine immunostaining of the kidneys and placentas, respectively, of pregnant control animals. (*C* and *D*) 3-Nitrotyrosine immunoreactivity in the kidneys and placentas, respectively, of the pregnant rats that were administered a single 10-μg/kg endotoxin infusion. (*E* and *F*) 3-Nitrotyrosine immunoreactivity in the kidneys and placentas, respectively, of the pregnant rats that received oral L-NAME. (*G* and *H*) More intense 3-nitrotyrosine immunoreactivity of the kidneys and placentas, respectively, of the pregnant rats that received L-NAME plus endotoxin. There was accumulation of polymorphonuclear leukocytes with intense 3-nitrotyrosine immunoreactivity in the kidneys of the pregnant rats that were administered oral L-NAME and the pregnant rats that were administered L-NAME plus endotoxin (original magnification, 400×).**

of nitration of tyrosine in the present experiments. Infiltration of the kidneys with PML is not a common feature of the human disease, which may be a limitation to this model.

Significant proteinuria was observed in pregnant rats treated with L-NAME and endotoxin at 2 and 10 μg/kg and was augmented after infusion of endotoxin into L-NAME–treated animals. Pregnancy profoundly activates the neutrophils in the glomeruli of pregnant rats treated with even low doses of endotoxin, leading to the accumulation of inflammatory cells that are potential producers of $O_2^-$ in the glomeruli.[15] Endotoxin infusion also enhances the chances of developing glomerular thrombosis and endotheliosis in pregnant rats.[33] Glomerular endotheliosis was detected histologically and 3-nitrotyrosine was localized immunohistochemically in the kidney vessels of the pregnant rats treated with endotoxin at 10 μg/kg. The kidneys of preeclamptic women usually show swelling of the glomerular cells with fibrinogen deposition, a picture that closely resembles the glomerular endotheliosis observed in these experiments. The observation of 3-nitrotyrosine immunoreactivity in the kidneys suggests that endotoxin may produce glomerular injury in pregnant rats by either oxidation or nitration-induced inactivation of essential proteins. In general, oxidation and nitration can disrupt the structure and catalytic function of proteins, and in the case of tyrosine nitration, it can impair tyrosine kinase–mediated signal transduction.[8]

Glomerular damage, together with marked renal vasoconstriction and elevation of glomerular blood pressure, could explain the L-NAME–induced proteinuria in pregnant rats.[34] Other investigators have demonstrated that pregnancy causes rats to be more susceptible to NOS inhibition than nonpregnant rat controls.[35] Both the glomerular filtration rate and effective renal plasma flow declined more in the pregnant rats than controls when L-NAME was infused.[35] The combination of endotoxin and L-NAME in the current experiment resulted in augmentation of the proteinuria in pregnant rats, possibly through a combination of decreased glomerular filtration rate, glomerular endotheliosis, and damage and production of cytotoxic reactive species. This possibility is consistent with the presence of 3-nitrotyrosine immunoreactivity in the kidneys of pregnant rats treated with endotoxin, L-NAME, and the combination of L-NAME plus endotoxin. The 3-nitrotyrosine immunostaining was more intense in the kidneys of pregnant rats treated with endotoxin plus L-NAME, suggesting more severe urinary injury and, subsequently, more proteinuria in the pregnant rats treated with both endotoxin and L-NAME.

In addition to proteinuria, thrombocytopenia is a problematic manifestation of preeclampsia. Thrombocytopenia occurs in 15–30% of women with preeclampsia.[1] Platelet count decreased significantly in pregnant rats treated with endotoxin at 2 and 10 μg/kg, whereas it remained unchanged in the PL group after L-NAME ingestion. Once endotoxin was infused in the PLE group, the platelet count decreased significantly. Endotoxin has been demonstrated to cause a generalized Shwartzman reaction in pregnant rats and is characterized by thrombocytopenia and disseminated intravascular coagulation.[5]

Preeclampsia can be categorized as severe if there is evidence of intrauterine growth restriction.[1] Preeclamptic women have decreased uteroplacental perfusion, which is believed to be the reason for development of intrauterine growth restriction in their fetuses.[1] In these

experiments, the weight of pups of rats treated with L-NAME was less than control, although the number of the pups was not different, and no fetal anomalies could be detected in any of the pups. NOS inhibition with L-NAME has been reported to cause intrauterine growth restriction and hind-limb degeneration by other investigators.[36] The lack of hind-limb degeneration in the pups from our experiments could be a result of the lower dose of L-NAME used in our studies. The presence of low-birth-weight pups in our experiments mimics the intrauterine growth restriction seen in human preeclampsia.

Human studies have shown decreased,[37] increased,[38] or unchanged[39] levels of $NO_2^- + NO_3^-$ in women with preeclampsia compared with normal pregnancies. The majority of these clinical studies did not control the diet of the patients before measurement of $^{\cdot}NO$ metabolites, ignoring the fact that $^{\cdot}NO$ metabolite concentration is critically dependent on dietary factors.[40] In two recent reports using rigorous dietary control, one reported a reduction in 24-h urinary $NO_2^- + NO_3^-$ in a group of preeclamptic women compared with normal women in the third trimester,[41] whereas the other showed significantly higher levels of $NO_2^- + NO_3^-$ in the serum of preeclamptic women.[42] This leads us to believe that there is no consensus on the rate of $^{\cdot}NO$ production in preeclampsia. This potential problem of dietary control is eliminated in animal studies because the rats can be fed a standardized diet low in $NO_2^- + NO_3^-$.

We conclude that endotoxin infusion in this animal model succeeded in reproducing two of the clinical features of the human disease of preeclampsia, proteinuria and thrombocytopenia, but failed to reproduce a cardinal feature, hypertension. In this endotoxin-induced injury model, $^{\cdot}NO$ may either directly or through nitration of tyrosine mediate the hematologic and physiologic changes, although a firm causal relationship cannot be confirmed from the present studies. The intermediary steps between the generation of $^{\cdot}NO$ and the formation of 3-nitrotyrosine require further investigation.

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