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Dose-dependent Effects of Lipopolysaccharide in a Fetal Lamb Model of Endotoxaemia

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Abstract Perinatal and neonatal infections are associated with a high mortality particularly in the presence of multiple organ failure. A better understanding in its underlying pathogenesis would optimise the design of treatment for this devastating condition. The end products of nitric oxide, nitrates and nitrites, have been found to be elevated in neonatal sepsis. This study reports that inducible nitric oxide synthase expression, the major source of nitric oxide production in a fetal lamb model of endotoxaemia, is up-regulated in liver and spleen tissues in a dose-dependent manner following systemic administration of lipopolysaccharide.

Key words Nitric oxide; Lipopolysaccharide; Endotoxaemia

Introduction

Neonatal infection continues to be a major cause of mortality and morbidity, particularly in developing countries.[1] Although the annual neonatal mortality associated with sepsis had fallen by 25% over a 16-year study period, those born prematurely remained at high risk as almost half of the sepsis-related deaths occurred in these vulnerable infants.[2] Recent human and animal studies have also found an adverse association between maternal chorioamnionitis and neuro-developmental outcome.[3,4] Nitric oxide (NO) derived from the inducible isoform of nitric oxide synthase (iNOS) has been implicated in the pathogenesis of septic shock.[5] In neonatal sepsis, the end metabolites of NO, nitrites and nitrates, were reported to be elevated in plasma.[6] Similar findings were found in critically ill children with sepsis in a case control study.[7] In patients who survived, plasma nitrite and nitrate levels at presentation of sepsis predicted the development of sequential pulmonary, hepatic and renal organ failure.[8] As the liver and kidney are involved in the metabolism of nitrates and nitrites, failure of these organs could result in an increase in plasma levels without an increase in nitric oxide production.[9] Steinhorn and Cerra had shown a dose-dependent, pro-inflammatory response in cultured hepatocytes from newborn rats stimulated with lipopolysaccharide (LPS) similar to that of adult rats.[10] This response had also been demonstrated in fetal hepatocytes exposed to LPS with up-regulation of iNOS mRNA.[11] In vivo experiments have shown that LPS could induce injury to the liver and spleen.[12] However systemic administration of LPS to pregnant rats induced iNOS mRNA in maternal liver but not in the fetus.[13] iNOS protein had been localized in the red pulp of spleen tissues in endo-toxaemic adult rats.[14] Whether fetal spleen tissues could mount a similar response is not known. In this study, we hypothesized fetal lambs exposed to LPS would result in up-regulation of iNOS expression in liver and spleen tissues in a dose-dependent manner.

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Materials and Methods

Subjects

All surgical and experimental procedures were performed in accordance with the guidelines established by the National Health and Medical Research Council of

Australia, and were approved by the Monash Medical Centre Committee on Ethics in Animal Experimentation.

Five pregnant Merino-Border Leicester cross ewes (93-100 days gestation, term=147 days) were anaesthetised (Propofol 5 mg/kg) and then intubated and ventilated (1.5% halothane, 50% oxygen, balance nitrous oxide). Each fetus was instrumented using sterile surgical techniques. A mid-line incision was made in the ewe's abdomen and the fetus delivered through the hysterotomy. The fetal internal jugular veins and common carotid arteries were catheterized and used for drug administration and haemodynamic monitoring respectively. After returning the fetus to the uterus, the uterine incision was closed and made watertight. The ewe's abdominal incision was closed in layers and the catheters were tunneled subcutaneously to the ewe's right flank where they exited via a small incision. Antibiotics (Ilium Penstrep, Troy Laboratories Pty, Inc.) were administered daily to the ewe throughout the period of study.

After 48 hours of recovery LPS (L3129, Sigma) at 2 mg/kg, 20 μg/kg or 2 μg/kg was infused into the fetus over 30 minutes. Blood pressure, cardiac output and heart rate were monitored continuously from one hour before LPS infusion for 24 hours. Two control fetal lambs underwent the same surgery but had saline infused instead of LPS. At the end of experimentation or time of death, liver and spleen tissues were harvested and immediately frozen in liquid nitrogen until processing.

Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Total RNA were extracted by Chomczynski's modified method of RNA extraction according to manufacturer's instructions (Promega).[15] Briefly, tissues were homogenized in denaturing solution (26 mM sodium citrate, pH 4.0, 0.5% N-lauryl sarcosine, 0.125 M β-mercaptoethanol, 4 M guanidine thiocyanate). Sodium acetate (2 M, pH 4.0) was added and mixed thoroughly. Phenol:Chloroform: Isoamyl alcohol (125:24:1, pH 4.7) was then added an, mixed vigorously and kept on ice for 15 minutes. RNA was extracted from the top aqueous phase after centrifugation at 10,000 g for 20 minutes at 4°C. An equal volume of isopropanol was added to the aqueous extract and the RNA precipitated at -20°C overnight. The RNA was pelleted by centrifugation at 10,000 g for 10 minutes at 4°C and then washed with 1 ml of ice cold 75% ethanol. After breaking up the pellet and centrifuging for another at 10,000 g for 10 minutes at 4°C, the RNA was air dried and dissolved in nuclease-free water. All procedures were performed in a RNase-free environment. Integrity of extracted RNA was ascertained by measuring the A260/A280 relative absorbance with a spectrophotometer (Biophotometer, Eppendorf) and visualized by running the RNA in a 1% denaturing agarose gel (2.2 M formaldehyde). Two micrograms of total RNA was used for subsequent RT-PCR.

A two-step RT-PCR was employed. All reactions were performed using the Perkin Elmer GeneAmp PCR system 2400. For the reverse-transcription, reactions were set up using 2 μl of 10x RT buffer, 2 μl (5 mM) dNTP mix, 1 μl of RNase inhibitor (10 units/μl, Qiagen), 4 units of Omniscript reverse-transcriptase (Qiagen), reverse iNOS primer (1 μM), 2 μg of total RNA and RNase-free water to make up to 20 μl per reaction. All reactions were maintained at 37°C for 60 minutes. Reaction mix for the PCR step consisted of 5 μl of cDNA from the RT reactions, 5 μl of 10x PCR buffer, 1 μl of dNTP mix (10 mM), 0.5 μM each of reverse (5'-3' ATGAGCTGGGCGTTCCAGAC) and forward (5'-3' CTCATCTTCGCCACCAAGCA) iNOS primers, 1.25 units of HotStarTaq DNA polymerase (Qiagen) and nuclease-free water to make up to 50 μl per reaction. The iNOS primers used were specifically designed for the ovine iNOS sequence and the 239 base pair PCR product was shown to have 98% homology to the corresponding bovine sequence (data not shown). The conditions of PCR were set at 15 minutes at 95°C for activation of DNA polymerase, 36 cycles of 1 minute denaturation at 94°C, 30 seconds of annealing at 60°C and 1 minute of extension at 72°C. Reactions were complete by a 10-minutes final extension step. Preliminary experiments were performed to establish the optimal conditions for PCR and cycle numbers within the linear phase of reaction (data not shown). Positive and negative controls were run in parallel in each experiment. Five microlitres of PCR products were fractionated by agarose gel electrophoresis and stained with ethidium bromide.

Immunohistochemistry

Liver and spleen tissues were fixed in 4% formaldehyde in phosphate-buffer saline (PBS) for 4 hours at 4°C and washed in PBS for 10 minutes three times. Tissues were cryo-protected by immersing in 10% sucrose-PBS for 30 minutes before 12 mm sections were mounted on SuperFrost Plus slides (Menzel-Glaser, Germany). Sections were stained with primary antisera specific for iNOS at 1:100 dilution for 18 hours (rabbit polyclonal against murine iNOS; Upstate Biotechnology, USA) at room temperature. Slides were counterstained with donkey anti-mouse Texas Red antibody (Jackson Laboratory) at 1:150 dilution for 1 hour at room temperature.

Results

Animal Outcomes

At a high LPS dose of 2 mg/kg, the fetus died within 1 hour of administration. At 20 μg/kg, the fetus survived for 5.5 hours. The fetus that received 2 μg/kg of LPS and the 2 control animals survived to the end of experimentation. The mean fetal blood pressure fell by an average of 41.4% (48.3±2.9 mmHg to 26.3±14.0 mmHg). The haemodynamic responses of the LPS treated animals are summarized in Figure 1.

Reverse-transcription Polymerase Chain Reaction

Despite the early demise, iNOS up-regulation was observed in both liver and spleen tissues of the fetus that received 2 mg/kg of LPS. This expression was relatively higher in the spleen than the liver. iNOS expression was absent in the liver but present in the spleen in the fetus that received low dose (2 μg/kg) LPS. For the fetus with 20 μg/kg of LPS, similar iNOS up-regulation was seen in the liver and spleen. Tissues from the control animals did not have iNOS expression (Figure 2).

Immunohistochemistry

Intense positive iNOS staining, particularly in areas adjacent to the venous centralis, was found in liver tissues of fetuses treated with 2 mg/kg or 20 μg/kg of LPS (Figure 3A). No iNOS staining was seen in the liver of the fetus that received 2 μg/kg of LPS. Spleen tissues of all LPS treated animals showed isolated positive staining (Figure 3C). Tissues from control animals were negative for iNOS (Figures 3B & 3D).

Discussion

The most advanced and serious stage of ascending intrauterine infection is fetal infection. The overall mortality rate of neonates with perinatal neonatal sepsis ranges between 25% and 90%.[16-19] Infected infants born before 33 weeks of gestation were twice as likely to die than non-infected.[20] Chorioamnionitis had been proposed as a precedent for fetal infection.[21] Carroll and Nicolaides reported that fetal bacteraemia is found in 33% fetuses with positive amniotic fluid culture compared with 4% in those

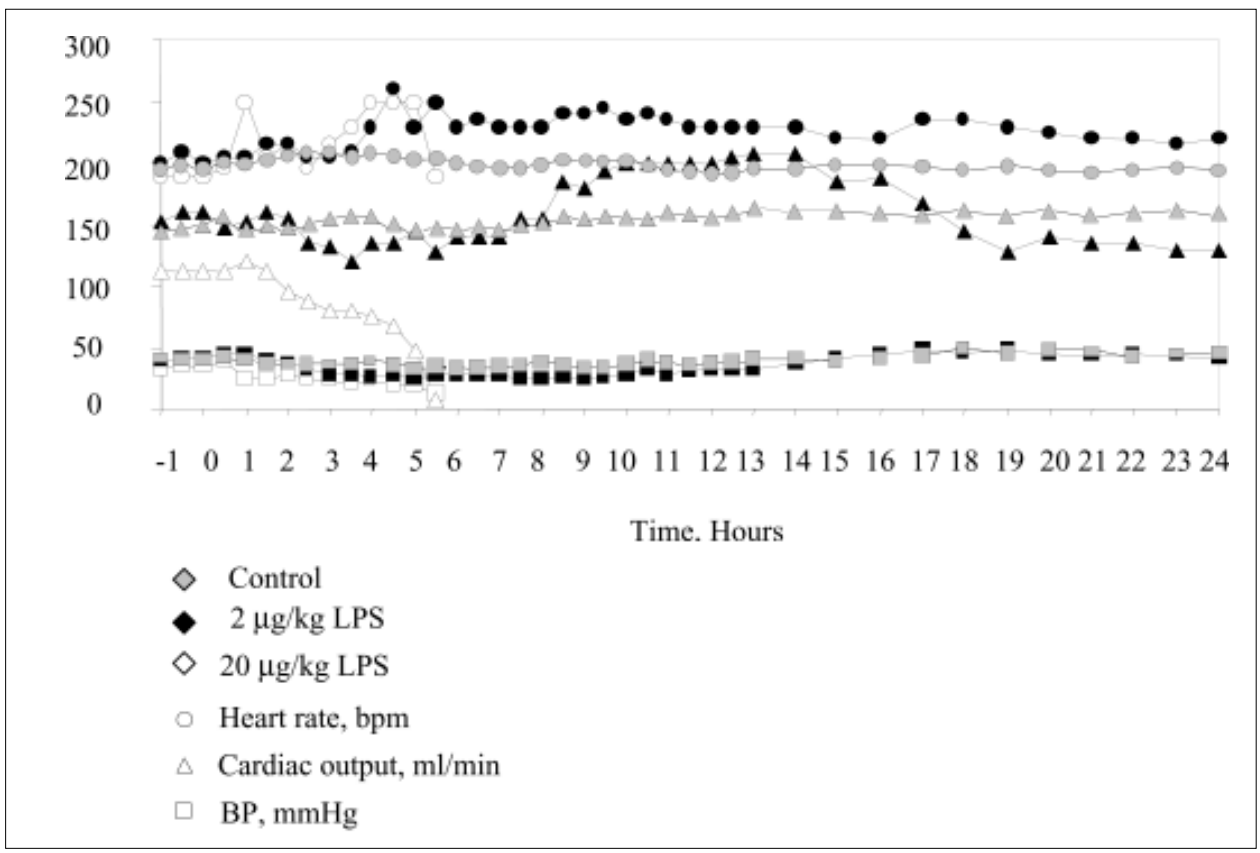
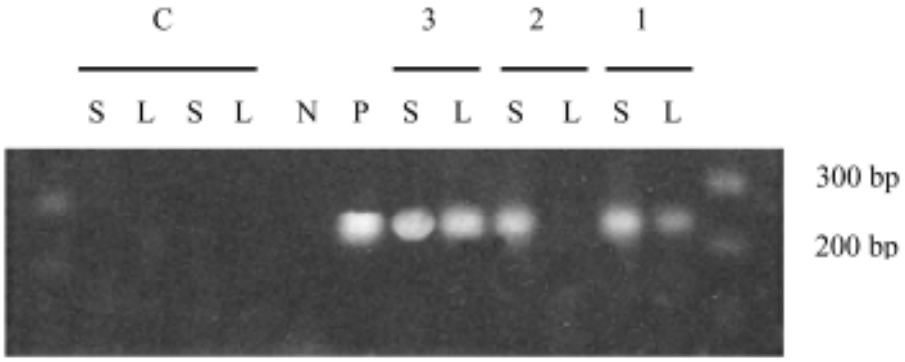
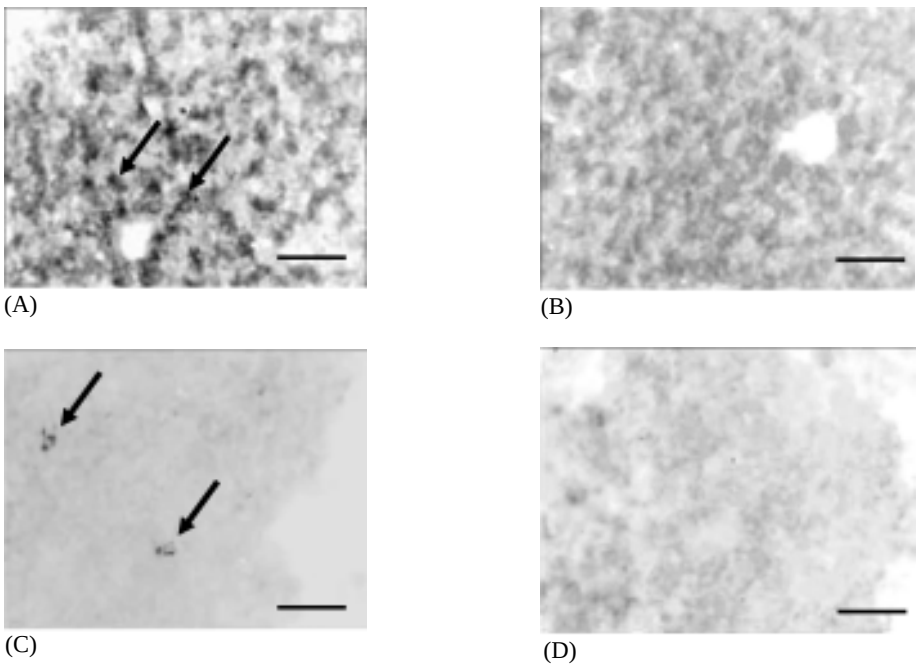


Figure 1 Haemodynamic responses after LPS at time 0 hour.



1 = 2 mg/kg LPS; 2 = 2 µg/kg LPS; 3 = 20 µg/kg LPS; C = Control; L = Liver;
S = Spleen; P = Positive control; N = Negative control

Figure 2 iNOS RT-PCR products.



Positive iNOS staining indicated by arrows.
Scale bars = 20 µm.
Magnification = x200.

Figure 3(A-D) iNOS immunohistochemistry in liver (3A) and spleen (3C) of LPS treated animals and liver (3B) and spleen (3D) of control animals.

with negative amniotic fluid culture.[22] More recent studies have found an association between chorioamnionitis and poor neuro-developmental outcome.[3,4] Raised pro-inflammatory cytokines are postulated to stimulate the underlying pathogenesis in white matter damage.[23] Reports on animal models of endotoxaemia have shown that fetal and neonatal animals are more resistant to the effects of LPS than adults.[24,25] Our study shows that mid gestation fetal lambs respond to LPS in a dose-dependent manner. This is mirrored by the up-regulation of iNOS mRNA expression in fetal liver and spleen tissues.

Bech-Jansen et al studied the acute circulatory responses of near term fetal lambs to systemically administered LPS ranging from 300 μg/kg to 1.1 mg/kg. Within the one-hour study period, they found no significant changes in fetal cardiac output, umbilical blood flow and total systemic resistance compared with controls.[24] In our study, 2 mg/kg LPS resulted in fetal death within one hour of drug infusion. With 20 μg/kg of LPS, blood pressure and cardiac output decreased between 1 and 2 hours post-infusion, then plateaued before dropping again at two and a half hours before death. In the fetus treated with 2 mg/kg LPS, there was an initial rise in blood pressure 1 hour after infusion and returning to baseline level 5 hours afterwards. Cardiac output decreased during the first 6 hours after drug infusion, rebounded to a higher level than baseline between 8-17 hours before dropping to near baseline level. This biphasic response to LPS could be due to the early release of vasoactive substances including catecholamines, histamine, kinins, prostaglandins and nitric oxide follow by oxygen-derived free radicals during reperfusion as the animal recovers. The animals we used in our study were more immature than those reported in Bech-Jansen's study and this could have accounted for the increase in mortality. The haemodynamic changes might have been observed if the duration of their study was prolonged.

Nitric oxide production from iNOS has been linked with cardiac dysfunction and renal impairment in sepsis.[26-28] In addition, raised plasma nitrate and nitrite levels in children with multiple organ failure associated with sepsis were found to have a higher mortality.[8] The role of iNOS in sepsis induced injury to the liver and spleen is not known. Our study clearly shows that iNOS mRNA expression is up-regulated by LPS in these tissues.

In vitro studies on the time course of iNOS mRNA expression in rat glomerular incubates after LPS administration suggests iNOS activity peaking at 4-6 hours and absent by 16 hours.[29] Similarly, iNOS immunostaining of tissues from endotoxaemic rats were present at 3-5 hours but absent at 24 hours.[14] Our study shows that with high dose of LPS (2 mg/kg), iNOS is present in liver and spleen tissues within 1 hour. Using 20 μg/kg LPS, this expression is maintained despite the demise of the fetus at 5.5 hours. Differential iNOS expression is observed in liver and spleen tissues of the fetus that received 2 μg/kg LPS at 24 hours. This may reflect the difference in iNOS sensitivity of the organs to LPS. Sade et al proposed the absence of iNOS in glomerular tissues at 16 hours could be due to non-viable specimens.[29] We found iNOS mRNA to be present in tissues from recently killed animals. Furthermore, iNOS mRNA was absent in viable tissues 24 hours after LPS administration. It is more likely that the liver could have recovered from the insult and stimulation of iNOS expression from LPS had subsided by 24 hours.

iNOS immunohistochemistry in this study reflects the changes observed in iNOS mRNA expression. We have not sought to identify the cell types that expressed iNOS. However anatomical distribution of iNOS protein in the liver with immunohistochemistry is similar to that reported by Cook et al.[14] Whether early iNOS expression in these cells precedes cell death remains to be determined.

Clinical trials on NOS inhibition has been hampered by the lack of safe NOS inhibitors. This problem was highlighted when a phase III trial of the non-selective NOS inhibitor N(G)-methyl-L-arginine hydrochloride (546C88) was terminated when interim analysis reported a significant increase in mortality in the treatment group despite promising initial results.[30,31] Recently selective iNOS inhibition with agmatine aldehyde has demonstrated an increase in survival of endotoxaemic rats.[32] Its clinical safety and usefulness remains to be seen.

Conclusions

This study investigating the effects of LPS in mid gestational fetal lambs confirms the haemodynamic responses and iNOS expression in the liver and spleen are dose-dependent. Different dosage of LPS would be necessary for future investigations on acute and chronic outcomes in models of endotoxaemia. Further studies using specific iNOS inhibitors would help to define the role of iNOS in multiple organ failure associated with sepsis.

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