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Spontaneous pregnancy loss mediated by abnormal maternal inflammation in rats is linked to deficient uteroplacental perfusion

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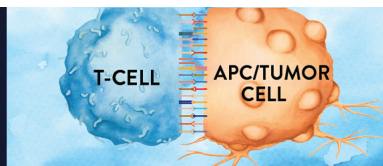
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Spontaneous Pregnancy Loss Mediated by Abnormal Maternal Inflammation in Rats Is Linked to Deficient Uteroplacental Perfusion

Stephen J. Renaud,¹ Tiziana Cotechini, Jill S. Quirt, Shannyn K. Macdonald-Goodfellow, Maha Othman, and Charles H. Graham

Abnormal maternal inflammation during pregnancy is associated with spontaneous pregnancy loss and intrauterine fetal growth restriction. However, the mechanisms responsible for these pregnancy outcomes are not well understood. In this study, we used a rat model to demonstrate that pregnancy loss resulting from aberrant maternal inflammation is closely linked to deficient placental perfusion. Administration of LPS to pregnant Wistar rats on gestational day 14.5, to induce maternal inflammation, caused fetal loss in a dose-dependent manner 3–4 h later, and surviving fetuses were significantly growth restricted. Pregnancy loss was associated with coagulopathy, structural abnormalities in the uteroplacental vasculature, decreased placental blood flow, and placental and fetal hypoxia within 3 h of LPS administration. This impairment in uteroplacental hemodynamics in LPS-treated rats was linked to increased uterine artery resistance and reduced spiral arteriole flow velocity. Pregnancy loss induced by LPS was prevented by maternal administration of the immunoregulatory cytokine IL-10 or by blocking TNF- α activity after treatment with etanercept (Enbrel). These results indicate that alterations in placental perfusion are responsible for fetal morbidities associated with aberrant maternal inflammation and support a rationale for investigating a potential use of immunomodulatory agents in the prevention of spontaneous pregnancy loss. *The Journal of Immunology*, 2011, 186: 1799–1808.

It is estimated that 20–30% of women who become pregnant experience one or more spontaneous pregnancy losses—that is, the loss of a pregnancy without outside intervention before 20 wk of gestation (1). Although some cases of fetal loss can be attributed to chromosomal, endocrinological, or anatomical abnormalities, the precise cause of fetal loss in most instances is unknown (2). Based on the fact that successful pregnancy requires intricate interactions between the maternal immune system and the semiallogeneic conceptus, it has been hypothesized that alterations in maternal immune function contribute to a substantial number of spontaneous pregnancy losses (3–8). However, the precise role of an aberrantly activated maternal immune system in the pathophysiology of spontaneous pregnancy loss is largely unknown.

Various studies have revealed differential expression of various immunological factors in women suffering pregnancy loss versus women whose pregnancy continued to term. For example, in-

creased expression of proinflammatory molecules by uterine and peripheral blood leukocytes is frequently reported in women suffering recurrent pregnancy losses. Among the increased proinflammatory molecules identified in these cases, TNF- α has been of particular interest, at least partly because of its pleiotropic actions on various cell types and because of its central role in the initiation and propagation of inflammatory responses. Interestingly, a causal role for TNF- α in fetal demise has been shown in various well-studied rodent models of pregnancy loss, including those involving maternal exposure to bacterial LPS (9), environmental stress (10), and the CBA/J \times DBA/2 genetic mouse model of spontaneous pregnancy loss (11), as well as in other pathological states such as a rat model of diabetes (12) and a mouse model of anti-phospholipid Ab syndrome (13). Therefore, TNF- α may be involved in the mediation of fetal death as a consequence of a maternal response to a variety of insults. The precise mechanisms by which TNF- α mediates fetal death are unclear but may involve luteal hormone insufficiency, facilitation of trophoblast apoptosis leading to placental disruption, or alternatively, could be the result of uteroplacental vascular insufficiency leading to functional deficits in uteroplacental or umbilical blood flow (5, 14, 15).

Reduced uteroplacental perfusion is a likely contributing factor in pregnancy loss and pregnancy-related morbidities. Irregular uterine artery Doppler waveforms characterized by an increased pulsatility index indicative of high arterial impedance are frequently observed in pregnancies with eventual adverse outcomes such as pre-eclampsia, intrauterine growth restriction, and spontaneous pregnancy loss (16–18). Functional deficits in uteroplacental perfusion may relate to insufficient conversion of the musculoelastic spiral arterioles by invasive trophoblast cells (19). Alternatively, inherited and acquired thrombophilias have emerged as leading causes of pregnancy loss and pregnancy-related morbidities (20). Sporadic clot formation could act to interrupt uteroplacental blood flow, inevitably leading to placental insufficiency and intrauterine fetal demise.

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Abbreviations used in this article: DIC, disseminated intravascular coagulation; EDV, end-diastolic velocity; GD, gestational day; LY30, percentage clot lysis within 30 min; MA, maximum amplitude; PSV, peak systolic velocity; R, time until initial blood clot formation; RI, resistance index; TEG, thromboelastography.

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The purpose of this study was to determine whether there is a causal association between aberrant inflammation and altered placental hemodynamics in relation to pregnancy loss. For this, we used a well-established rodent model of inflammation-mediated pregnancy loss, in which rats were injected with LPS to induce a maternal inflammatory response. A variety of studies have identified various pro-inflammatory molecules and immune cells as essential components of the detrimental cascade that leads to fetal death after maternal LPS administration (7, 21–25). However, surprisingly little is known about the precise mechanisms that initiate fetal death. Our findings indicate that intrauterine death occurs within 4 h of LPS administration and that it is mediated by TNF- α . Moreover, fetal death was preceded by severe alterations in uteroplacental hemodynamics.

Materials and Methods

Animals

Studies involving rats were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee. Virgin female Wistar rats (Charles River Laboratories, Montreal, Quebec, Canada) aged 3–4 mo and weighing ~300 g were kept in controlled conditions of light (lights on from 7 AM to 7 PM) and humidity. Rats were fed ad libitum and had free access to tap water. Daily vaginal smears were taken, and rats with regular 4-d estrus cycles were selected for experiments. Pregnancy was achieved by housing female rats on the night of proestrus with a fertile male at a 2:1 ratio. The morning when spermatozoa were detected in the vaginal lavage was designated as gestational day (GD) 0.5.

Experimental protocol

In the first series of experiments, pregnant rats received saline (0.1 ml/100 g) or LPS (50 or 100 μ g/kg in saline; *Escherichia coli* serotype 0111:B4; Sigma-Aldrich, Oakville, Ontario, Canada) i.p. on GD 14.5, and dams were sacrificed 72 h later to assess for fetal viability and weight. Administration of LPS at a dose of 100 μ g/kg was used for all subsequent experiments because it resulted in >95% fetal loss. Preliminary experiments revealed that resorptions could be distinguished from viable fetuses 24 h after administration of LPS. Therefore, for studies determining morphological evaluation of fetal viability, dams were sacrificed 24–72 h after LPS, saline, IL-10 (R&D Systems, Minneapolis, MN), or etanercept (Enbrel; Amgen, Thousand Oaks, CA and Wyeth Pharmaceuticals, Collegeville, PA) administration. For all other experiments, rats were sacrificed 1–24 h after injections.

For the series of experiments in which ultrasound biomicroscopy was conducted, an impartial technician who was blinded to the experimental treatment recorded all measurements. Fetal viability was determined by the presence of a heartbeat 2, 6, 12, or 18 h after maternal administration of LPS. Based on these results, other experiments were designed to analyze placental vascular flow either continuously from a single fetus per rat beginning preinjection and continuing every 20 min until the time of fetal death (real time), or beginning 2 h after injection in 3–5 implantation sites per rat. Each pregnant rat had ~15 implantation sites.

For experiments involving IL-10, 12.5 μ g carrier-free recombinant rat IL-10 dissolved in saline was injected i.p. 20 min after LPS or saline administration. Control rats received saline only. Fetal viability after maternal administration of saline \pm IL-10 or LPS \pm IL-10 was determined by scoring the number of viable and resorbed fetuses at least 24 h after injections. Experiments were also conducted in which pregnant rats were treated with the TNF- α inhibitor etanercept (10 mg/kg) 6 h before LPS administration. The timing and dose of etanercept administration were chosen based on a previous study that showed high circulating levels of this molecule 6 h after a 10-mg/kg injection in mice (26). With the exception of rats treated with saline + IL-10 and etanercept + saline, at least four dams were used per group, each having a minimum of 12 implantation sites. For the groups of rats treated with saline + IL-10 and etanercept + saline, three dams were used.

Tissue preparation

Rats were euthanized using a single i.p. injection of sodium pentobarbital (Ceva Santé Animale, Libourne, France) at a dose of 85 mg/kg. To assess serum TNF- α levels, blood was collected by cardiac puncture before euthanasia and allowed to clot for at least 1 h before centrifugation (10 min, 4000 \times g). For experiments assessing blood hemostasis, blood was col-

lected by cardiac puncture with sodium citrate-containing syringes before thromboelastography (TEG). For analysis of placental histology, uterine horns were carefully ligated (to avoid clot formation around implantation sites caused by dissection), removed, and dissected to isolate the fetus, placenta, and decidua basalis/mesometrial triangle. Amniotic fluid was collected using a 26 5/8-gauge needle, centrifuged (8 min, 750 \times g), and stored at -80°C until further analysis. The wet weights of viable fetuses were recorded. For histological analysis, implantation sites (placenta + uterus) were removed and fixed by submerging in 4% paraformaldehyde for at least 24 h. After fixation, tissues were transferred to 70% ethanol at room temperature, dehydrated, and embedded in paraffin according to standard procedures. Implantation sites were carefully cut in two equal pieces before paraffin embedding and strategically placed in the blocks to ensure that histological observation was consistently conducted within the midportion of the implantation site. All tissues were cut at 8- μ m thickness and stained using H&E, or in conjunction with immunohistochemistry.

Ultrasound

Ultrasound biomicroscopy and Doppler waveform recordings were used to compare blood flow patterns in the uterine artery and in the uteroplacental and umbilicoplacental circulations using a method adapted from Mu and Adamson (27). In brief, rats were lightly anesthetized with ~1.5% isoflurane in oxygen by face mask during ultrasound examinations. Maternal heart and breathing rates were monitored throughout the assessments and maintained at a steady-state by adjusting the flow of isoflurane. All hair was removed from the abdomen by shaving followed by a chemical hair remover. Prewarmed gel was used as an ultrasound-coupling medium. Rats were imaged transcutaneously with the use of an ultrasound biomicroscope and a 30- or 40-MHz transducer operating at two frames per second (model Vevo 770; VisualSonics). In Doppler mode, the high-pass filter was set at 6 Hz, and the pulsed repetition frequency between 4 and 48 kHz, to detect low and high blood flow velocities, respectively. A 2.5- to 4.5-mm wall filter was used, and the angle between the Doppler beam and the vessel assessed. For Doppler waveform recordings of the uterine artery, measurements were recorded from the artery where it crossed the mesometrium adjacent to the conceptus being analyzed. Waveforms were recorded for later offline analysis. Peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured from five to seven consecutive cardiac cycles not affected by motion caused by maternal breathing, and the results were averaged. The resistance index (RI = [PSV - EDV]/PSV) was calculated when EDV > 0 to quantify the pulsatility of arterial blood velocity waveforms. Because spiral arteriole flow rate was relatively constant (PSV \approx EDV), blood flow velocity was recorded. At least three arterioles were selected in random areas of the mesometrial triangle to ensure an accurate representation of spiral arteriole flow rate in each conceptus. To determine percentage of placental perfusion, placental volume was calculated by selection of contour areas for three-dimensional reconstruction and volume calculation. Contours were refined electronically based on pixilation density, for improved accuracy. Percent perfusion was calculated using Doppler density within a selected volume. For the exception of real-time analysis, measurements were recorded in at least three conceptuses from at least four dams per group. For real-time ultrasound analysis, a single implantation site from each of three dams was assessed. Systolic and diastolic flow parameters were assessed in the uterine artery and spiral arterioles at 20-min intervals for a total of 200 min. Because of the time constraints of the real-time experimental protocol, percent placental perfusion was not analyzed at each time interval. Measurements at each time point were subsequently normalized to the preinjection parameters by dividing the average measurement for each time point by the mean values assessed before injection in each conceptus. This normalization was used to facilitate comparison of data obtained from different dams. The duration of anesthesia was limited to 3 h. There was no noticeable effect of the duration of isoflurane on maternal or fetal circulatory parameters.

Vascular corrosion casting

A modification of the method of Adamson et al. (28) was used to prepare corrosion casts of the maternal uteroplacental vasculature. In brief, rats were anesthetized with sodium pentobarbital (0.4–0.6 ml) injected i.p. and allowed to reach surgical plane. Uterine horns and the abdominal aorta were exposed. A 23-gauge butterfly catheter was inserted in the aorta just above the bifurcation of the common iliac, and the distal portion of the uterine artery and vein were cut to allow the outflow of perfusate. Rats were perfused with 30 ml warmed PBS + heparin at a rate of 6 ml/min to remove blood, followed by perfusion with 30 ml ice-cold PBS/heparin. Components of the casting compound (Batson's #17; Polysciences, Warrington, PA) (28) were mixed on ice and injected at a rate of 4 ml/min. After polymerization, the uterine horns were removed and placed in 20%

KOH to digest the tissue. Casts were examined under a stereomicroscope or sputter-coated with gold for scanning electron microscopy.

Thromboelastography

TEG was performed on citrated blood collected from at least three pregnant rats treated with LPS or saline to determine the type and extent of maternal clotting parameters. TEG is a global hemostatic assay that provides advanced monitoring of hemostasis and rapid detection of disorders linked to coagulopathies. Unlike conventional blood/plasma-based tests of coagulation, which do not account for the impact of platelets and thrombin on hemostasis, nor assess the strength/stability of the clot, TEG determines the kinetics of clotting, starting from the initial formation of fibrin threads and the formation of a stable clot to its eventual lysis. The speed and strength of clot formation is measured and depends on the activity of the coagulation system, platelet function, fibrinolysis, and other factors.

For these studies, blood was collected from pregnant rats by cardiac puncture 1 or 4 h after LPS injection. Saline-treated rats were used as controls. In brief, 0.34 ml citrated whole blood recalcified with 20 μ l 0.2 M CaCl₂ was placed into a cuvette that rotates gently to imitate venous flow and activate coagulation. Subsequently, a sensor shaft was inserted into the sample to initiate clot formation between the sensor and cuvette, and kinetics of clot formation were analyzed using a Computerized Thromboelastograph Coagulation Analyzer 5000 (Haemoscope Corporation, Skokie, IL). All TEG analyses were performed by the same operator, and care was taken to maintain consistency of sample handling. Tracings were recorded for at least 90 min. With each TEG analysis, the following parameters were recorded: time to clot initiation (R), clot propagation (α angle), maximum amplitude (MA [measure of clot stability/strength]), and the fibrinolytic parameter, percentage of clot lysis within 30 min (LY30).

Determination of hypoxia by pimonidazole immunohistochemistry

To assess placental and fetal hypoxia after maternal LPS exposure, immunohistochemical staining for pimonidazole was performed. Pimonidazole is a compound that selectively binds thiol groups in proteins of cells exposed to hypoxia ($pO_2 < 10$ mm Hg). Protein adducts of reductively activated pimonidazole are stable and can be detected immunohistochemically in paraffin sections.

Rats (GD 14.5) were injected i.p. with pimonidazole hydrochloride (Hypoxyprobe-1, 60 mg/kg; Natural Pharmacia, Burlington, MA) 90 min before sacrifice. Uterine horns were ligated and excised, and fetuses and placentas were paraffin-embedded after fixation in 4% paraformaldehyde. Sections were dewaxed in Hemo-D and rehydrated in graded ethanol washes. Antigenic sites were retrieved by immersing slides in 0.05% trypsin/0.1% CaCl₂ (pH 7.8) for 40 min at 40°C. Pimonidazole detection was performed using mouse monoclonal anti-pimonidazole Ab (1:50 Hypoxyprobe-1), followed by addition of biotin-conjugated anti-mouse IgG Ab (1:200; BA-2000; Vector Laboratories, Burlingame, CA). Staining was developed using avidin-conjugated peroxidase (Vectastain Elite; Vector Laboratories) followed by diaminobenzidine hydrochloride (Sigma-Aldrich). Sections were lightly counterstained with hematoxylin, dehydrated, and mounted using Permount (Fisher Scientific, Fairlawn, NJ).

Determination of TNF- α levels by enzyme immunoassay

Maternal serum or fetal amniotic fluid was collected from at least three dams at various times from rats treated with LPS or LPS + IL-10. Saline-treated rats were used as control animals. Levels of TNF- α were subsequently quantified by ELISA according to the manufacturer's instructions (Alpco Diagnostics, Salem, NH) (29, 30).

Statistical analysis

Values are expressed as mean \pm SEM. Statistical significance was analyzed by *t* test when comparing two groups, and ANOVA with Bonferroni correction when comparing three or more groups. Statistical significance for normalized ultrasound data was determined by one-sample *t* test. All statistical tests were two-sided, and differences were considered significant at $p < 0.05$.

Results

Administration of LPS results in altered placental perfusion leading to fetal demise

To examine the effect of LPS on fetal viability, we administered LPS (50 or 100 μ g/kg) or saline i.p. to pregnant rats on GD 14.5,

and rats were sacrificed 72 h later. As shown in Fig. 1A, LPS administration significantly decreased fetal survival in a dose-dependent manner. Compared with fetuses from saline-treated control rats, viable fetuses from LPS-treated rats were significantly growth restricted as indicated by an 18% decrease in wet weight (average fetal weight after maternal administration of 50 μ g/kg LPS: 1.3033 g versus 1.5822 g after saline treatment; $p < 0.05$). At the greatest dose of LPS (100 μ g/kg), there was >95% fetal loss. Therefore, this dose was used for subsequent experiments.

Although LPS-induced fetal loss is a well-studied phenomenon, research to date has not elucidated the precise timing of the detrimental outcomes induced by LPS. Therefore, we characterized the cascade of events taking place between maternal LPS administration and fetal death. Morphological evidence of resorption (as indicated by an amorphous necrotic mass) was evident by 24 h of LPS administration. However, because maternal circulating TNF- α levels were substantially increased 2 h after LPS administration (Fig. 1B), we postulated that LPS-induced fetal demise occurs at a much earlier time point than previously thought. Fetal viability was subsequently assessed by ultrasonography to determine the presence or absence of a fetal heartbeat at 6, 12, and

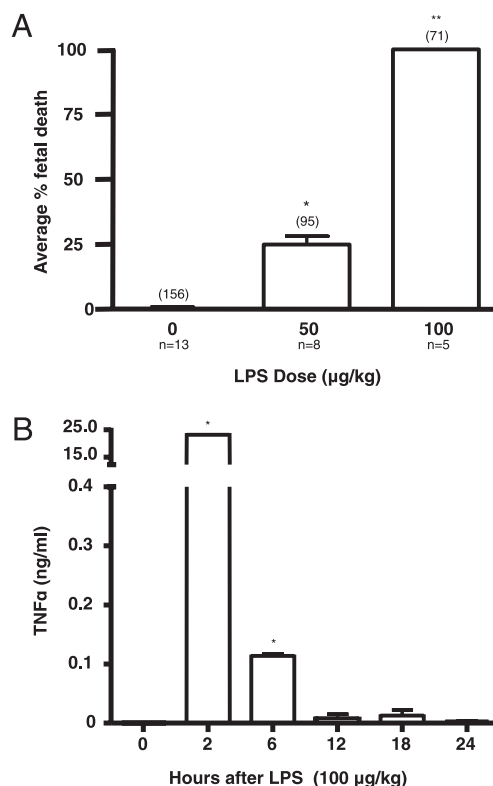


FIGURE 1. Effect of LPS on fetal viability and maternal TNF- α levels. *A*, Pregnant rats were administered 0, 50, or 100 μ g/kg LPS on GD 14.5, and fetal viability was assessed 72 h later. LPS caused fetal death in a dose-dependent manner. Single asterisk denotes significantly different values ($p < 0.05$) from control (0 μ g/kg), whereas double asterisk denotes significantly different values ($p < 0.05$) from 50 μ g/kg LPS. Numbers in brackets indicate the total number of implantation sites for each treatment group, whereas the numbers below the axis labels indicate the number of pregnant dams in each treatment group. *B*, Maternal systemic TNF- α levels after saline administration ($t = 0$) or 2, 6, 12, 18, or 24 h after LPS administration (100 μ g/kg). Asterisk denotes significantly different values ($p < 0.05$) from 0 h. Statistically significant differences were determined by ANOVA followed by Bonferroni correction. TNF- α levels at each time point were measured in at least three pregnant rats.

Table I. Effect of maternal LPS administration on placental blood flow as determined by Doppler ultrasound

Treatment	No. of Placentas Analyzed	Percent Placental Perfusion	Spiral Arteriole Flow Velocity (cm/s)	Uterine Artery Resistance Index
Saline	11	2.37 ± 0.33	22.79 ± 4.07	0.67 ± 0.05
LPS (100 µg/kg)	11	1.21 ± 0.27 ^a	10.36 ± 3.58 ^a	0.79 ± 0.02 ^a

Recordings were taken from implantation sites of rats treated 2.5 h prior with saline or LPS (100 µg/kg). Three to five implantation sites from at least three separate dams were used per group.

^aSignificantly different from values obtained from saline-treated rats ($p < 0.05$).

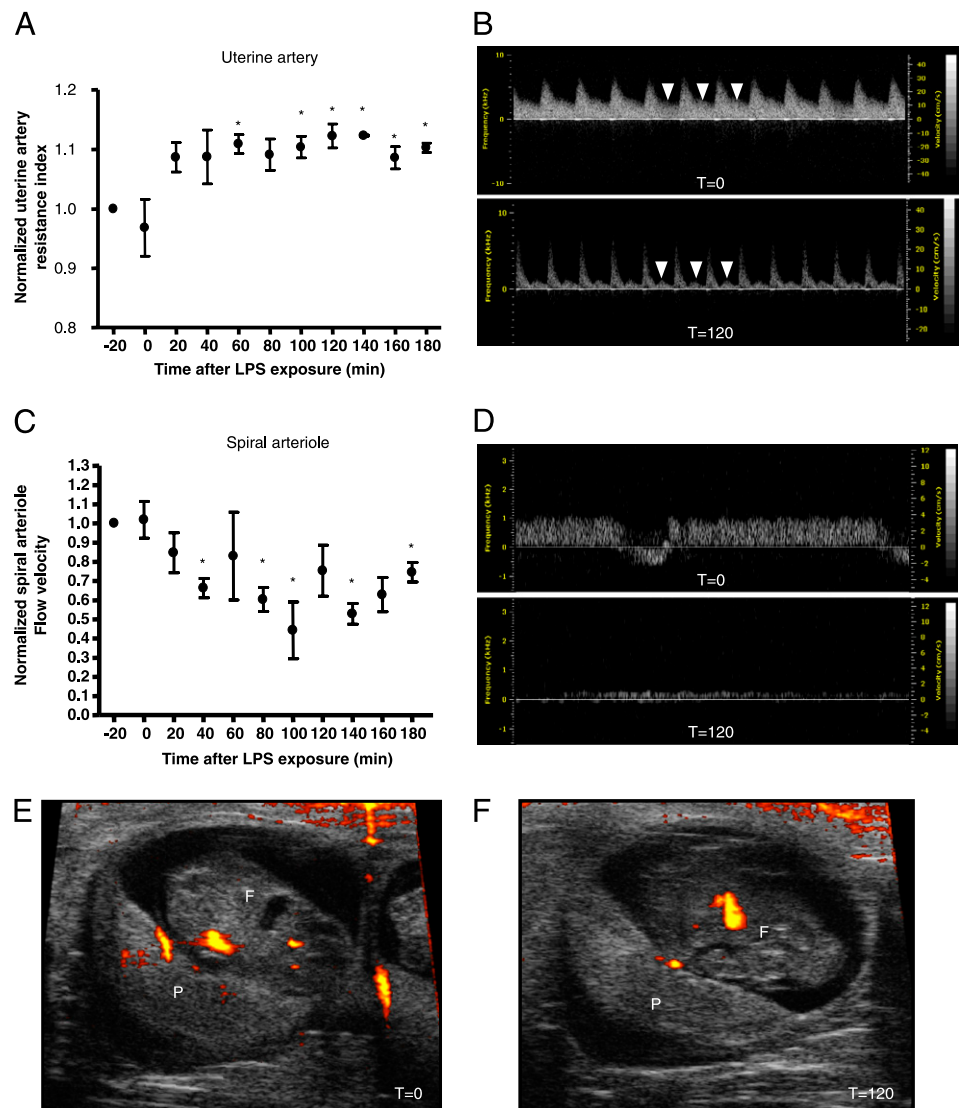
18 h after LPS administration. Surprisingly, fetal heartbeat was absent at all of these time points, indicating that maternal LPS had a deleterious effect on fetal viability within 6 h of administration. Further studies revealed that fetal heart rate consistently decreased and became arrhythmic, ultimately leading to arrest within 3–4 h of LPS administration. Injection of saline into control pregnant rats did not result in fetal cardiac arrest at any of the time points examined.

To determine whether LPS-induced fetal death was associated with alterations in uteroplacental hemodynamics, Doppler recordings were performed to quantify the percentage cross-sectional area of blood flow within the placenta and to determine the spiral arteriole flow velocities and uterine artery resistance indices 2.5 h after saline or LPS administration. Compared with placentas of dams treated with saline, placentas of dams treated with LPS had

a 50% reduction in the total volume that was found to contain detectable blood flow (Table I; $p < 0.05$). Even more striking was the number of placentas that showed <1% total area with detectable blood flow after maternal LPS administration relative to saline injection (7 of 11 placentas from LPS-treated dams versus 2 of 11 placentas from saline-treated dams). Likewise, Doppler waveform recordings of spiral arterioles after LPS administration showed a 55% decrease in mean flow velocity compared with saline-injected rats (Table I; $p < 0.05$). These reductions in spiral arteriole flow velocity correlated with an 18% increase in uterine artery resistance compared with saline-treated rats (Table I; $p < 0.05$) 2.5–3 h after maternal LPS administration and immediately preceding fetal death.

Our next set of experiments involved real-time analysis of changes in the uterine and placental vasculature leading to fetal

FIGURE 2. Real-time assessment of uterine artery and spiral arteriole flow patterns after maternal LPS administration. **A**, Mean normalized uterine artery resistance indices determined by Doppler waveform recordings beginning 20 min before LPS (100 µg/kg) injection and continuing every 20 min for a total of 200 min. **B**, Representative recordings at 0 and 120 min are shown. Arrowheads indicate diastolic flow. **C**, Average normalized spiral arteriole flow velocities determined by Doppler waveform analysis beginning 20 min before LPS (100 µg/kg) injection and continuing every 20 min for a total of 200 min. **D**, Representative recordings at 0 and 120 min are shown. **E** and **F**, Representative power-Doppler analysis of the same implantation site at the time of LPS injection (**E**; 0 min) and 120 min later (**F**) are shown. Values significantly different from $t = -20$ min in **A** and **C** are indicated by an asterisk ($*p < 0.05$), and significant differences were determined by one-sample t test. Recordings were obtained from a single randomly selected individual implantation site in each of three pregnant rats. F, fetus; P, placenta.



demise. For this, parameters from a single placenta from each of three dams were assessed starting 20 min before LPS injection and continuing every 20 min after injection for a total of 200 min. Doppler recordings revealed a marked increase in uterine artery RI (indicative of arterial impedance) within 60 min of LPS exposure, and this increase was maintained for the duration of the recordings (Fig. 2A; $n = 3$; $p < 0.05$). The increased RI was primarily due to reduced flow velocity during diastole, which was characterized by the presence of a prominent diastolic notch (Fig. 2B, arrowheads). Increased uterine artery resistance was accompanied by reduced flow velocity within the spiral arterioles starting at 40 min after LPS administration (Fig. 2C; $n = 3$; $p < 0.05$). This reduction in flow velocity persisted for the duration of the recordings. Representative recordings of flow velocities in the spiral arterioles at 0 and 120 min after LPS administration are shown in Fig. 2D. Spiral vessels constitute the major blood supply to the placenta; therefore, decreased flow velocity in these arteries may account for the reduced detection of placental blood flow as indicated earlier. Similar to our previous observations, compared with $t = 0$ (Fig. 2E), percentage placental perfusion was greatly diminished within 120 min of LPS administration (Fig. 2F).

Rats experience development of a disseminated intravascular coagulopathy-like condition after LPS administration

We performed TEG in pregnant rats at GD 14.5 after injection of LPS. Blood samples were withdrawn from 12 rats and tested for

TEG at 1 ($n = 6$) and 4 h ($n = 3$) after LPS injection, and the results were compared with those obtained from saline-injected control rats ($n = 3$). Representative tracings are shown in Fig. 3A, and the various clotting parameters are graphically represented in Fig. 3B (R), 3C (speed of clot formation; α angle), 3D (MA [clot stability/strength]), and 3E (LY30). At 1 h after LPS injection, three rats showed a disseminated intravascular coagulation-like (DIC-like) condition stage I (hypercoagulability with secondary fibrinolysis). In comparison with saline-injected rats, there was significant shortening of R time (2.2 compared with 10.2 min in saline control), increase in α angle, increase in overall clot index (global tendency to clot), and increase in LY30 ($p = 0.0001, 0.002, 0.03$, and 0.05 , respectively). The three other rats showed DIC stage II (hyperfibrinolysis). Compared with saline control, there was significant shortening of R time, reduction in clot index, and increase in LY30, but no significant changes in α angle or MA ($p = 0.02, 0.03, 0.03, 0.3$, and 0.6 , respectively). After 4 h of LPS injection, rats showed enhanced hyperfibrinolysis and hypocoagulability indicating DIC stage III. In these rats, there were no significant changes in R time or α angle, but there was a significant reduction in MA, clot index, and LY30 ($p = 0.9, 0.8, 0.03, 0.03$, and 0.001 , respectively). The LY30 was 13% in stage I, 26% in stage II, and 39% in stage III compared with 1.7% in saline control. These results indicate that rats experience development of various stages of DIC after LPS, and that TEG can detect these hemostatic changes within 1 h after LPS administration.

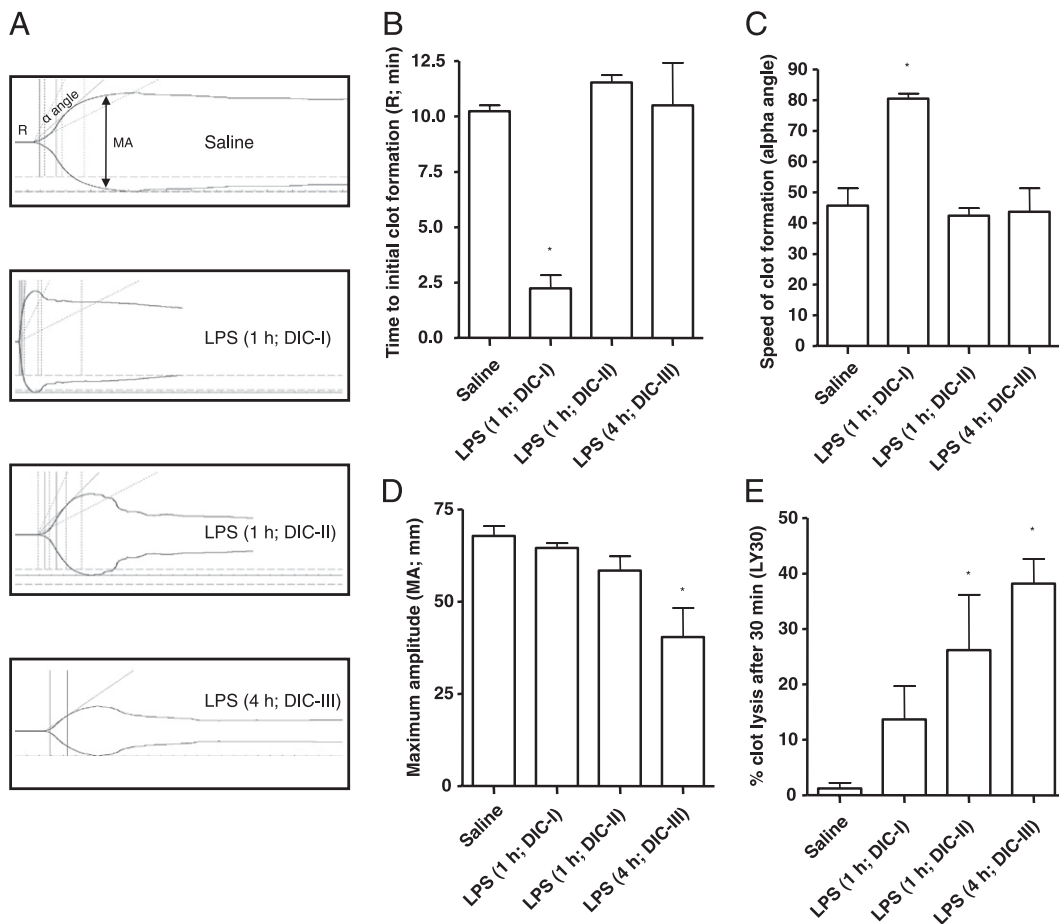


FIGURE 3. Exposure of pregnant rats to LPS causes a DIC-like condition. A, Representative TEG tracings in whole blood of pregnant rats 4 h after administration of saline ($n = 6$ rats) or 1 and 4 h after LPS administration ($n = 6$ and 3 rats, respectively). After exposure to saline or LPS, various parameters were measured using TEG. These parameters included: R (B); speed of clot formation (α angle) (C); clot strength (MA) (D); and LY30 (E). Values significantly different from saline controls are indicated by an asterisk ($*p < 0.05$). Statistically significant differences were determined by ANOVA followed by Bonferroni correction.

Histological analysis of placentas at 3 and 24 h after saline or LPS administration was performed to determine whether structural differences were present. At 3 h after LPS administration (Fig. 4A), there was no noticeable difference in overall placental architecture, invasive trophoblast, or placental size compared with 3 h after saline injection (Fig. 4B). However, in LPS-treated rats, fibrin-like deposits could be observed within uterine vessels (Fig. 4A, boxed inset). By 24 h after LPS administration (Fig. 4C), there was evidence of severe hemorrhaging and fibrin deposition in the uterus (saline administration shown in Fig. 4D). These histological findings are consistent with the aforementioned TEG results, showing a hypercoagulable tendency early after LPS injection followed by hyperfibrinolysis, as indicated by hemorrhagic evidence in the uterus.

Structural alterations in the uteroplacental vasculature after maternal LPS exposure

To further determine whether the alterations in uteroplacental hemostasis were linked to LPS-induced abnormalities in vascular structure, corrosion casts of the uteroplacental vasculature were produced. Casts made from control rats revealed the normal architecture of the arterial uteroplacental vasculature spanning the spiral arterioles, the maternal arterial channels, and the maternal labyrinth (Fig. 5A). Scanning electron microscopy showed that, compared with casts made from control rats injected with saline (Fig. 5B), casts made from rats 3 h after LPS administration revealed evidence of severe vascular structural abnormalities including aneurysms within the central arterial canal (not shown) and hemorrhaging in the spiral arterioles (extravasation of casting compound, Fig. 5C).

Maternal LPS administration results in fetal and placental hypoxia

To determine the effect of reduced uterine and placental blood flow on oxygen delivery to the placenta and the fetus, pimonidazole immunostaining was used to detect hypoxia within the conceptus. Fetuses isolated from dams injected with LPS 3 h before euthanasia showed marked pimonidazole immunoreactivity (Fig. 6A). Hyp-

oxia was detected in most fetal tissues but was especially prominent in tissues that have a high metabolic rate, including brain and liver. In contrast, fetuses isolated from saline-injected dams showed almost no pimonidazole immunoreactivity (Fig. 6B). Areas of placental tissue, particularly around giant cells at the fetal–maternal interface and areas of labyrinth, the site of maternal–fetal exchange, also stained more positively in placentas isolated from LPS-treated rats (Fig. 6C) compared with placentas isolated from saline-treated rats (Fig. 6D).

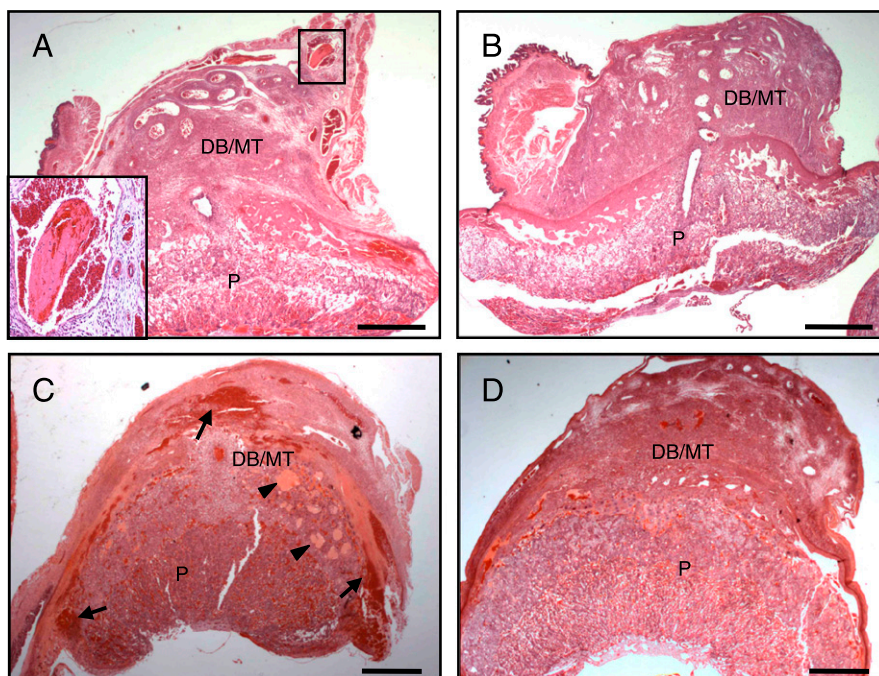
Uterine tissue (decidua + mesometrial triangle) stained positively for pimonidazole regardless of whether it was isolated from rats treated with saline or LPS. Areas of negative staining occurred circumferentially around spiral arterioles, whereas hypoxic areas were identified within the stroma distant from these vessels. Despite the propensity of uterine stromal tissue to be hypoxic regardless of exposure to saline or LPS, uterine tissue isolated from dams treated with LPS (Fig. 6C) stained much more intensely than uterine tissue isolated from saline-treated dams (Fig. 6D).

IL-10 and etanercept decrease LPS-mediated fetal death

IL-10 is an anti-inflammatory cytokine that has been postulated to play an important role in protecting the fetus from inflammation-induced fetal loss. Therefore, we sought to determine the effect of IL-10 on LPS-induced fetal demise. Injection of IL-10 subsequent to LPS administration resulted in a 62% reduction of fetal death compared with LPS injection alone, which in this set of experiments caused 100% fetal death (Fig. 7A; $p < 0.05$). Surviving fetuses were still viable up to 72 h after maternal administration of LPS. There was no effect of IL-10 alone on fetal viability or weight ($n = 43$ fetuses from 3 dams).

The reduction in fetal death by IL-10 administration was correlated with sustained spiral arteriole flow velocity in viable fetuses as determined by Doppler waveform measurements (Fig. 7B, 7C; $p < 0.05$ compared with LPS-treated rats). In addition, because studies have shown that IL-10 acts through inhibition of proinflammatory cytokine secretion, we sought to determine whether IL-10 modulated TNF- α levels in the serum of rats treated with LPS. IL-10 administration reduced LPS-induced serum TNF- α levels by 63% (Fig. 7D; $p < 0.05$).

FIGURE 4. Histological evidence of clotting and hemorrhage at the fetal–maternal interface. *A* and *B*, Uteroplacental units were isolated 3 h after maternal exposure to 100 $\mu\text{g}/\text{kg}$ LPS (*A*) or saline (*B*) at GD 14.5. Bottom left boxed inset in *A* shows the presence of a clot in the lumen of a maternal vessel located in the region of the mesometrial triangle. Micrographs shown are representative of two to three uteroplacental units processed from each of three rats in the saline group and four rats in the LPS-treated group. *C* and *D*, Uteroplacental units were isolated 24 h after maternal exposure to 100 $\mu\text{g}/\text{kg}$ LPS (*C*) or saline (*D*). *C*, Arrows indicate regions of hemorrhage, whereas arrowheads indicate the presence of fibrin deposition in the lumen of vessels within the decidua. *C* and *D*, Micrographs are representative of two to three implantation sites processed from at least six rats in each of the saline- and LPS-treated groups. Sections were stained with H&E. Scale bars, 1 mm. DB/MT, decidua basalis/mesometrial triangle; P, placenta.



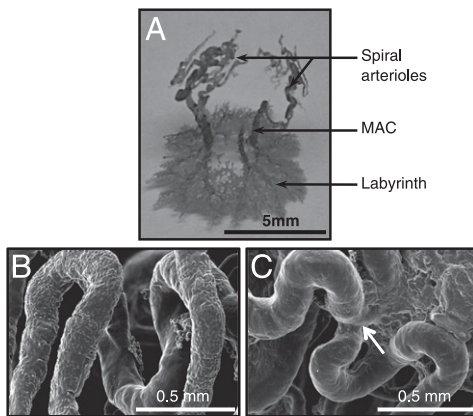


FIGURE 5. LPS administration compromises the structural integrity of the uteroplacental vasculature. Corrosion casts of the uteroplacental vasculature of control rats revealed the presence of spiral arterioles, maternal arterial channels (MAC), and the maternal sinusoids with the labyrinth (A). Scanning electron microscopy of representative casts of spiral arterioles from control rats ($n = 2$ rats) confirmed the structural integrity of these vessels (B), which was compromised by 3 h of LPS administration ($n = 2$ rats), as revealed by spillage of the casting compound into the surrounding tissues (C, arrow).

To determine whether fetal death induced by LPS was mediated by TNF- α , we conducted studies in which pregnant rats were treated with the TNF- α inhibitor etanercept (26). Results showed that etanercept administration significantly reduced LPS-induced fetal death by 63% (Fig. 7E; $p < 0.05$). Injection of etanercept alone did not have any effect on fetal viability.

Discussion

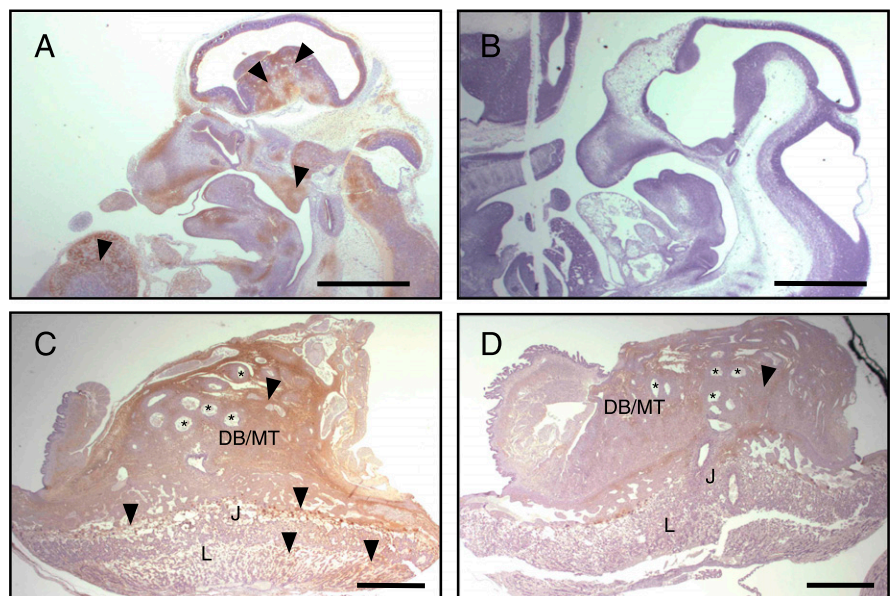
Although previous studies have shown that aberrant maternal immune activation and alterations in uteroplacental hemodynamics characterize pregnancy complications (3–5, 31–34), this study demonstrates a causal link between abnormal maternal inflammation and impaired placental perfusion rapidly leading to fetal death. The maternal immune response is, indeed, a crucial determinant of the success or failure of pregnancy. A necessary shift in immune competence has been well documented during normal pregnancy, where T cells preferentially differentiate along

a pathway promoting secretion of humoral-type cytokines and inhibition of inflammatory cytokines (35). Conversely, these immune adaptations are noticeably absent in various complications of pregnancy (4).

Inflammation-induced pregnancy loss in rodents is a well-studied phenomenon and is used as a model of spontaneous pregnancy loss (8, 36, 37). Although previous studies have observed fetal death hours or days after the initial inflammatory insult (8, 36, 37), the precise time point at which the detrimental effects of inflammation leading to fetal demise has not been elucidated. Therefore, to delineate the timing of inflammation-induced fetal death, we used ultrasound in real time to observe fetal viability after maternal exposure to LPS. Consistently, fetal cardiac arrhythmia followed by cardiac arrest occurred between 3 and 4 h after LPS administration, correlating with pronounced reductions in uteroplacental blood flow. Further experiments demonstrated decreased uterine artery flow velocity during diastole, including evidence of prominent diastolic notches that were absent in saline-treated rats. In both mice and humans, diastolic notches are features of uterine artery Doppler tracings during prepregnancy and in early pregnancy but disappear by the end of the second trimester with a corresponding increase in diastolic flow velocity (27, 38). Persistent diastolic notches during routine ultrasound examinations are frequently observed in women who subsequently experience obstetric complications (39). To our knowledge, our study is the first to observe aberrant diastolic notches immediately preceding inflammation-induced fetal demise in rats, which is consistent with events associated with adverse fetal outcomes in humans and indicates a possible shared mechanism. Therefore, a further understanding of the causes of persistent diastolic notches in rats is warranted because it may improve obstetrical management in pregnant women with irregular uterine artery Doppler tracings.

Although the causes of altered placental hemodynamics in complicated pregnancies are likely multifactorial, there is substantial evidence that pregnancy complications are closely associated with coagulopathies. For example, women who have inherited either the factor V Leiden G1691A mutation or the prothrombin G20210A mutation, the most common genetic causes of thrombophilia, have a substantially increased risk for placental thrombosis leading to second-trimester miscarriage (40, 41).

FIGURE 6. Maternal LPS administration causes fetal and placental hypoxia. Pregnant rats were injected on GD 14.5 with 100 $\mu\text{g}/\text{kg}$ LPS (A, C) or saline (B, D) 3 h before sacrifice. Ninety minutes before euthanasia, all rats were administered pimonidazole (60 mg/kg), a compound used to detect regions of hypoxia in tissues. A and B, Immunohistochemical detection (brown staining) of pimonidazole adducts in fetuses. C and D, Detection of pimonidazole adducts in uteroplacental units. Micrographs shown are representative of two to three uteroplacental units processed from each of three rats in the saline group and four rats in the LPS-treated group. Asterisks identify spiral arterioles; arrowheads highlight hypoxic areas in the fetus, placenta, and uterus. Scale bars, 1 mm. DB/MT, decidua basalis/mesometrial triangle; J, junctional zone; L, labyrinth.



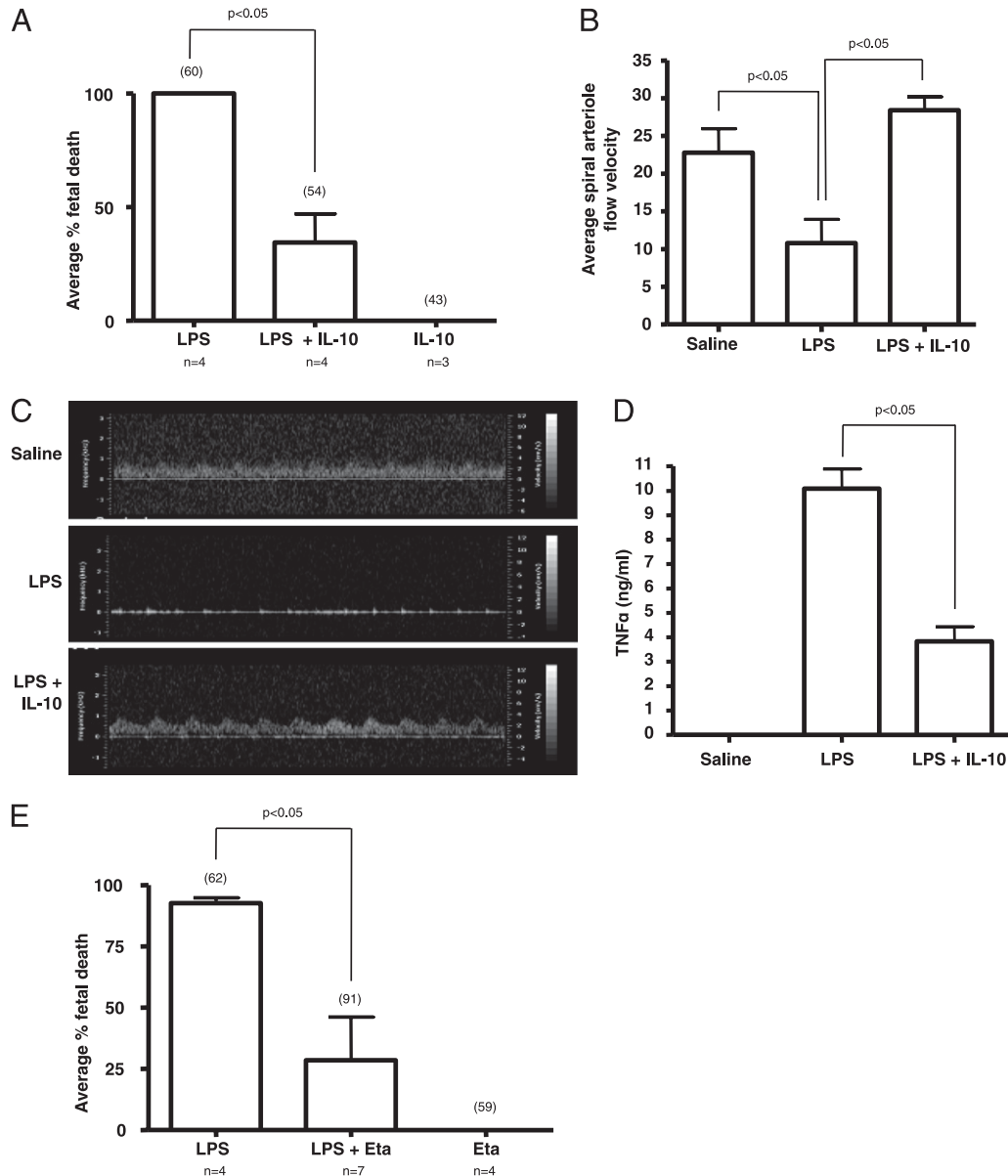


FIGURE 7. IL-10 and etanercept prevent LPS-mediated fetal demise. IL-10 (12.5 μ g) was administered i.p. to pregnant rats 20 min after LPS (100 μ g/kg) or saline injections, whereas etanercept (Eta; 10 mg/kg) was administered 6 h before LPS or saline injections. **A**, Fetal demise after administration of LPS, LPS + IL-10, or saline + IL-10. Numbers in brackets indicate the total number of implantation sites for each treatment group, whereas the numbers below the axis labels indicate the number of pregnant dams in each treatment group. **B** and **C**, Spiral arteriole flow velocity 2–3 h after administration of saline ($n = 16$ spiral arterioles from four rats), LPS ($n = 17$ spiral arterioles from four rats), or LPS + IL-10 ($n = 19$ spiral arterioles from four rats). Representative Doppler recordings are shown in **C**. **D**, TNF- α levels in maternal serum taken 4 h after exposure to saline ($n = 3$ rats), LPS ($n = 3$ rats), or LPS + IL-10 ($n = 3$ rats). **E**, Fetal demise assessed 3 d after administration of LPS, LPS + etanercept, or saline + etanercept. Statistically significant differences were determined by ANOVA followed by Bonferroni correction.

Moreover, anti-phospholipid Ab syndrome, an acquired autoimmune disorder that is highly associated with arterial and venous thromboses, has emerged as a leading cause of pregnancy loss and pregnancy-related morbidity (20, 42, 43). In our study, there was evidence of fibrinoid deposition within uterine vessels 3 h after maternal LPS administration, followed by severe hemorrhaging within the uterus. Further analyses indicated that LPS exposure triggered an acute DIC-like condition in these pregnant rats, which is consistent with the histological observations. DIC is a life-threatening condition characterized by widespread thrombophilia followed by excessive bleeding once components of the coagulation system are consumed. It has been suggested that thrombophilia exerts its detrimental effects on pregnancy by obstructing uteroplacental blood flow (44). Our study provides evidence sup-

porting this claim, as we observed clot formation within uterine vessels and systemic activation of DIC in our inflammation model of pregnancy loss. This clot formation occurred at the same time as the hemodynamic alterations observed using Doppler ultrasound recordings.

Placental and fetal hypoxia is a key pathological feature of a variety of pregnancy complications and is thought to result mainly from deficient remodeling of the uterine spiral arterioles by invasive trophoblast cells (45–47). Our study provides evidence that placental and fetal hypoxia can develop independently of impaired remodeling of the uteroplacental vasculature. This conclusion is based on the observation that there was a substantial increase in immunodetection of pimonidazole adducts in fetal and placental tissue within 3 h of LPS administration, indicative of severe hy-

poxia (45–48). Our findings indicate that proinflammatory molecules may play a more causal role in the development of placental and fetal hypoxia associated with these complications than had been previously appreciated.

Although exposure to LPS is linked to an increased production of multiple proinflammatory molecules, *in vivo* and *in vitro* evidence indicates that many of the biological effects induced by LPS are mediated by TNF- α (49). In pregnant rats, administration of TNF- α has been shown to elicit similar adverse effects on fetal outcomes as those that occur after LPS administration (50). In this study, inhibition of TNF- α bioavailability by administration of etanercept significantly inhibited fetal loss in LPS-treated dams. Likewise, administration of IL-10, an immunoregulatory cytokine whose levels are markedly decreased in women suffering recurrent spontaneous pregnancy loss (51, 52), decreased serum TNF- α levels and also prevented fetal demise after LPS exposure (53). These results implicate TNF- α as a causal factor mediating inflammation-induced fetal demise and are consistent with previous studies (7–9, 53, 54). Therefore, given the fact that increased levels of TNF- α are closely linked to recurrent pregnancy loss (4, 55, 56), our study indicates that a potential therapeutic approach to manage women susceptible to recurrent pregnancy loss may include treatment with etanercept or IL-10. Indeed, a recent study showed that treatment with TNF- α inhibitors and *i.v.* Ig may improve live birth rates in women who suffer recurrent pregnancy loss (57).

One of the difficulties in determining specific mechanisms leading to LPS-induced fetal death is identifying whether the effector proinflammatory cascades that cause fetal demise are maternal or fetal in origin. LPS does not readily cross the placenta partly because of its large m.w. and partly because of the remarkable antimicrobial, endotoxin-sequestering capabilities of placental and amniotic membranes, thereby preventing these potentially harmful substances from entering the fetal compartment (58, 59). In a study by Silver et al. (9), there was no evidence of fetal loss in C3H/HeJ (LPS-hyporesponsive) mothers after exposure to LPS even at doses 100-fold greater despite the fact that progeny were capable of responding to LPS. Likewise, in a separate study using sheep, maternal LPS administration caused significant fetal hypoxemia, increased fetal cortisol and PG secretion, and increased uterine electromyographic activity (60). However, these effects were not observed after direct amniotic exposure even at doses of LPS that were 200-fold greater (60). Collectively, these studies suggest that the maternal response to LPS is the crucial determinant leading to fetal demise. However, these studies cannot exclude the possibility that maternally generated proinflammatory cytokines gain access to the fetal compartment, thereby causing direct embryo-toxic effects. However, this study and others with similar LPS dosing regimens did not observe any increase in amniotic fluid concentrations of TNF- α or other proinflammatory cytokines at 2 or 6 h after LPS injection, despite a substantial increase of this cytokine in the maternal circulation and evidence of fetal deterioration (Supplemental Fig. 1) (61). Therefore, the possibility that maternally generated proinflammatory cytokines exert their detrimental effects on pregnancy through direct fetal cytotoxicity is unlikely.

The results of this study delineate the events that precede fetal death in an aberrant inflammatory environment. In particular, an increase in TNF- α levels 2 h after LPS administration was associated with deficient uteroplacental perfusion, the development of thrombotic events, and placental and fetal hypoxia. Of potential clinical importance was the inhibition of these effects by IL-10 and etanercept. Most significantly, this study demonstrates a direct role for proinflammatory cytokines and thrombotic events in modulating placental perfusion rather than the classical notion that

proinflammatory cytokines are aberrantly expressed in response to a poorly perfused, hypoxic placenta.

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Disclosures

The authors have no financial conflicts of interest.

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