Abnormal inflammation leads to maternal coagulopathies associated with placental haemostatic alterations in a rat model of foetal loss

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Summary
Spontaneous pregnancy loss is often associated with aberrant maternal inflammation and systemic coagulopathies. However, the role of inflammation in the development of obstetric coagulopathies is poorly understood. Further, questions remain as to whether systemic coagulopathies are linked to placental haemostatic alterations, and whether these local alterations contribute to a negative foetal outcome. Using a model of spontaneous foetal loss in which pregnant rats are given a single injection of bacterial lipopolysaccharide (LPS), we characterised the systemic maternal coagulation status following LPS administration using thromboelastography (TEG), a global haemostatic assay that measures the kinetics of clot formation. Systemic maternal coagulopathy was evident in 82% of LPS-treated rats. Specifically, we observed stage-I, -II, and -III disseminated intravascular coagulation (DIC) and hypercoagulability. Modulation of inflammation through inhibition of tumour necrosis factor α with etanercept resulted in a 62% reduction in the proportion of rats exhibiting coagulopathy. Moreover, inflammation-induced systemic coagulopathies were associated with placental haemostatic alterations, which included increased intravascular, decidual, and labyrinth fibrin deposition in cases of DIC-I and hypercoagulability, and an almost complete absence of fibrin deposition in cases of DIC-III. Furthermore, systemic and placental haemostatic alterations were associated with impaired utero-placental haemodynamics, and inhibition of these haemostatic alterations by etanercept was associated with maintenance of utero-placental haemodynamics. These findings indicate that modulation of maternal inflammation may be useful in the prevention of coagulopathies associated with complications of pregnancy.

Keywords
Pregnancy loss, coagulopathy, inflammation, rat, haemodynamics

Introduction
Spontaneous foetal loss is the most common complication of pregnancy, accounting for approximately 20% of all recognised pregnancies and recurring in 1–3% of afflicted women (1). While the aetiology of foetal loss is multi-factorial, with some cases attributed to chromosomal, anatomical, or endocrinological abnormalities, the precise cause in most pregnancies remains unknown (2).

Successful pregnancy requires an adequate maternal immune adaptation to a semi-allogeneic conceptus, and there is evidence that an abnormal maternal immune adaptation is linked to negative foetal outcomes such as foetal loss (3, 4). Specifically, maternal levels of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) have been shown to be elevated in cases of foetal loss in humans (5–9) and in rodent models of foetal loss (10, 11). Using a rat model, we previously demonstrated that foetal loss following a single injection of the bacterial pro-inflammatory molecule lipopolysaccharide (LPS) is mediated by TNF-α (12). We also showed in that study that LPS-induced foetal death is preceded by severe utero-placental haemostatic and haemodynamic alterations.

There is strong evidence that maternal haemostatic alterations are also associated with foetal loss as observed in acquired and inherited thrombophilias (4, 13–15), particularly those associated with anti-phospholipid antibody syndrome (APLS) (5, 16). Furthermore, disseminated intravascular coagulation (DIC), characterised by widespread fibrin deposition followed by depletion of coagulation factors and a state of hypocoagulability, has been associated with foetal death and other complications of pregnancies. Specifically, DIC characterises pregnancies afflicted with HELLP syndrome (haemolysis, elevated liver enzymes, and low platelets),
placental abruption, and severe pre-eclampsia (17–20). Interestingly, alterations in maternal coagulation have been shown to precede foetal death in women with a history of miscarriage (21, 22). Using a rat model, we determined in the present study whether abnormal maternal inflammation associated with foetal death is causally linked to the development of coagulopathies.

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. Outbred virgin female Wistar rats (Charles River Laboratories, Montreal, QC, Canada), aged 3–4 months, were kept in controlled conditions of light and humidity, fed ad libitum and had free access to tap water. Rats were mated in a 2:1 female to male ratio overnight. Vaginal smears were stained with toluidine blue and analysed by microscopy for the presence of sperm and/or the stage of oestrus cycle. The morning when spermatozoa were present in vaginal smears was designated as gestational day (GD) 0.5.

Experimental protocol

Dams were given an intra-peritoneal (i.p.) injection of E. coli serotype 0111:B4 lipopolysaccharide (LPS: 100 μg/kg in saline, for a final volume of 300–350 μl; Sigma-Aldrich, Oakville, ON, Canada) or saline (100 μl/kg). We have previously reported that this dose of LPS results in >95% foetal death between 2–3 hours (h) following administration (12). Therefore, experiments involving thromboelastography and histological analysis were performed 1 h after LPS administration. An additional group consisted of seven non-pregnant rats injected with LPS.

For studies involving modulation of inflammation, a single i.p. injection of the TNF-α inhibitor etanercept (10 mg/kg; Enbrel®; Amgen, Thousand Oaks, CA, and Wyeth Pharmaceuticals Inc., Collegeville, PA, USA) was administered six hours prior to saline or LPS injections. To confirm the role of TNF-α in the development of coagulopathies, four pregnant rats were injected i.p. with 5 μg of TNF-α (R&D Systems, Minneapolis, MN, USA) in 100 μl of sterile phosphate-buffered saline (PBS) 1 h prior to euthanasia and blood collection for thromboelastography.

Blood collection and tissue preparation

Dams were anaesthetised on GD 14.5 with an i.p. injection of sodium pentobarbital (0.5 ml of a 54.7 mg/ml solution; CEVA Santé Animale, Rutherford, NJ, USA) prior to cardiac puncture. Whole blood was drawn using a 26-gauge needle pre-flushed with 0.1 ml of trisodium citrate to attain a 9:1 ratio of blood to trisodium citrate. Collected blood was carefully dispensed into a 1.5-ml microcentrifuge tube and rapidly inverted three to four times before thromboelastographic analysis. Following blood collection, a midline laparotomy was performed. Utero-placental units were dissected, fixed in 10% formalin at 4ºC for at least 24 h, transferred to 70% ethanol and processed for histological analysis.

Assessment of circulating TNF-α levels

To confirm that LPS administration resulted in maternal inflammation, maternal plasma was obtained from blood collected by cardiac puncture at the time of euthanasia. Levels of TNF-α were measured by ELISA according to the manufacturer’s instructions (R&D Systems) for both saline and LPS-treated dams.

Thromboelastography

Thromboelastography was performed on citrated whole blood in order to assess specific haemostatic parameters in maternal blood following administration of LPS, saline, or etanercept + LPS. Blood was analysed as per manufacturer’s instructions using a TEG® 5000 Hemostasis System and TEG® Hemostasis Analyser software (Version 4.2). Three hundred forty μl of citrated blood was re-calified with 20 μl of calcium chloride (CaCl2; 0.2 M) and loaded onto a disposable plastic cuvette for analysis. Traces were recorded for approximately 1.5 h. For each trace, the clotting time [R], maximum amplitude [MA], speed of clot formation [K], rate of clot formation [k], and fibrinolysis [LY30] parameters were recorded. All TEG analyses were performed by the same individual in order to minimise operator error. Electronic quality tests on the TEG® 5000 Hemostasis System were performed prior to each analysis.

Histological analysis and immunohistochemistry

In order to visualise fibrin deposition in the implantation sites, sections of utero-placental units were stained with Periodic Acid and Schiff (PAS) reagent (Sigma-Aldrich, procedure No. 395) and counter-stained with haematoxylin. To further confirm the presence and location of fibrin deposition, formalin-fixed, paraffin-embedded sections of placentas (6-μm thick) were stained for fibrin/fibrinogen using a polyclonal rabbit anti-human fibrinogen antibody that cross-reacts with rat fibrin/fibrinogen (DakoCytomation, Code No. A0080; DAKO Canada Inc., Burlington, ON, Canada). Slides were imaged with an Aperio ImageScope microscope (Aperio; Vista, CA, USA) and analysed with ImageScope digital software (Aperio). Fibrin deposition was quantified with ImagePro® Plus version 6.0 software (Media Cybernetics Inc., Bethesda,
Figure 1: Representative thromboelastogram of normal saline-treated rat. Blood from saline-treated dams (n=6) was collected via cardiac puncture 1 hour after LPS administration. The horizontal line denoted (R) represents the time in minutes for the clot to begin forming; the rate of clot formation (α) is represented by the slope of the line, and reflects the rate of clot formation. The speed of clot formation (K) is represented by the vertical line at the point when the trace splits; indicative of the clot reaching a resistance of 2 mm. The maximum amplitude (MA) reflects clot strength/stability and is represented by the distance between the two horizontal lines of the trace. LY30 represents the percentage of clot lysis (fibrinolysis) occurring within 30 minutes.

Table 1: TEG ranges for saline-treated Wistar rats at GD 14.5.

<table>
<thead>
<tr>
<th>TEG parameter</th>
<th>Reference range</th>
<th>Mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting time (R) [min]</td>
<td>8.5–14.1</td>
<td>11.24</td>
</tr>
<tr>
<td>Clotting rate (K) [mm]</td>
<td>2–5.4</td>
<td>3.78</td>
</tr>
<tr>
<td>Clotting angle (α) [deg]</td>
<td>34.9–64</td>
<td>49.14</td>
</tr>
<tr>
<td>Maximum amplitude (MA) [mm]</td>
<td>51.3–71</td>
<td>65.02</td>
</tr>
<tr>
<td>Clotting index (CI)</td>
<td>–1 – 3.2</td>
<td>1.88</td>
</tr>
<tr>
<td>Fibrinolysis (LY30) [%]</td>
<td>0–3.7</td>
<td>0.82</td>
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</table>

MD, USA). Specifically, positive fibrin staining was electronically overlaid with red pixels. The intensity of positive fibrin stain recognised by the software was standardised for all the imaged uteroplacental units. The Image-Pro® Plus software then measured and added the surface area of each point of red pixilation on the uteroplacental units.

Ultrasound biomicroscopy

Doppler ultrasound recordings were performed to determine spiral arteriole flow velocity. Dams were imaged as reported previously by us (12). Spiral arteriole peak flow velocity of at least three randomly chosen spiral arterioles of the same implantation site were measured and averaged. In all dams, the second foetus proximal to the bifurcation of the uterine horns was imaged to avoid positional variation. Measurements were performed at 20-minute (min) intervals starting at the time of injection and consecutively for 160 min.

Statistical analysis

Values are expressed as mean ± standard error of the means (SEM). Repeated measures ANOVA was used to determine differences in spiral arteriole flow velocities between the treatment groups. To assess changes in the slopes of the flow velocities over the 160-min period following treatment, we employed a one-sample t-test using zero (no change in slope) as reference. ANOVA was used to compare the degree of fibrin deposition in the implantation sites, followed by Bonferroni post hoc test to determine significant differences between groups. F-test for variance was utilised when comparing TEG parameters between experimental groups. Chi-square analysis was utilised to compare proportions. Statistical significance was determined as p < 0.05 and a trend towards significance as p ≤ 0.15.

Results

Haemostatic profile at GD 14.5

The thromboelastographic parameters obtained from blood of saline-treated control rats (Fig. 1) were used to create reference ranges and to allow comparisons with experimental groups. Reference ranges were obtained using the lowest and highest values of each thromboelastographic parameter (Table 1), as previously reported in other animal studies (23). Blood samples were deemed to exhibit coagulopathy when any thromboelastographic parameter value fell outside the saline control range, in conjunction with an abnormal TEG trace.

LPS administration increases maternal circulating levels of TNF-α and leads to systemic coagulopathy

Blood collected by cardiac puncture one hour following LPS administration had TNF-α levels that were approximately 5,000 times higher than those in blood taken from saline-treated rats at the same time point (p < 0.001; Fig. 2).

Eighty-two percent of LPS-treated rats (9 of 11) exhibited systemic coagulopathy after 1 h of LPS administration, a proportion significantly greater than that of saline controls (1 of 6: 16.7%; p < 0.05, Chi-square analysis). Specifically, the variability of R, K, α, and MA was greater in LPS-treated rats than in saline-treated control rats, showing a trend towards significance (p < 0.15, F-variance test); LY30 values varied significantly more in LPS-treated dams than in saline-treated control rats (p < 0.05, F-variance test).

Abnormalities in TEG parameters were stratified into four specific phenotypes (Table 2). Two LPS-treated rats exhibited stage I DIC, characterised by a shortened clotting time (R) (2 ± 0.1 min) and hyperfibrinolysis (7.6 ± 0.5 % clot dissolution) as compared to saline controls (11.24 ± 1.13 min; 0.82 ± 0.7 % clot dissolution) (Fig. 3A). Two rats exhibited DIC-II, characterised by excessive
fibrinolysis (13.25 ± 8.9 % clot dissolution) and normal R time (12.05 ± 0.55 min; Fig. 3B). Two rats exhibited DIC-III, characterised by a prolonged R time (17 ± 0.3 min) and reduced rate of clot formation (α) (21.6 ± 4.9 degrees) as compared to saline-treated rats (49.14 ± 5.52 degrees; Fig. 3C). Three rats exhibited hypercoagulability, characterised by a shortened R time (6.63 ± 1.22 min; Fig. 3D). Two rats did not exhibit coagulopathy following LPS treatment.

To determine whether the observed coagulopathies were pregnancy-specific, non-pregnant age-matched controls (n=7) were sacrificed 1 h following LPS administration. Hypercoagulability was observed in three of the five non-pregnant rats, as evidenced by a shortened R time (4.47 ± 1.48 min) and increased clotting index (CI) (4.3 ± 0.37); two rats showed TEG parameters fitting within the saline ranges. Two of the non-pregnant rats treated with LPS exhibited DIC-I and DIC-II, respectively.

Table 2: TEG parameters of LPS-treated rats.

<table>
<thead>
<tr>
<th>Coagulopathy</th>
<th>R (min)</th>
<th>K (min)</th>
<th>Angle (Deg)</th>
<th>MA (mm)</th>
<th>CI</th>
<th>LY30 (%)</th>
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<tr>
<td>DIC I</td>
<td>2.1</td>
<td>2.6</td>
<td>58.2</td>
<td>46.9</td>
<td>0.9</td>
<td>8.1</td>
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<tr>
<td></td>
<td>1.9</td>
<td>1.9</td>
<td>64.1</td>
<td>48.9</td>
<td>1.1</td>
<td>7.1</td>
</tr>
<tr>
<td>DIC II</td>
<td>12.6</td>
<td>4.5</td>
<td>42.2</td>
<td>54.3</td>
<td>–0.1</td>
<td>4.3</td>
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<tr>
<td></td>
<td>11.5</td>
<td>3.6</td>
<td>44.2</td>
<td>50.4</td>
<td>–0.5</td>
<td>22.2</td>
</tr>
<tr>
<td>DIC III</td>
<td>17.3</td>
<td>7.1</td>
<td>26.5</td>
<td>64.5</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>16.7</td>
<td>12.8</td>
<td>16.7</td>
<td>51.5</td>
<td>–0.8</td>
<td>0</td>
</tr>
<tr>
<td>Hypercoagutable</td>
<td>7.8</td>
<td>2.1</td>
<td>62.3</td>
<td>52.6</td>
<td>0.3</td>
<td>0</td>
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<tr>
<td></td>
<td>7.9</td>
<td>2.2</td>
<td>60.4</td>
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<td>0</td>
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<tr>
<td></td>
<td>4.2</td>
<td>1.2</td>
<td>72.2</td>
<td>70.3</td>
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<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>9.2</td>
<td>4.0</td>
<td>45</td>
<td>56.5</td>
<td>1</td>
<td>0</td>
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<tr>
<td></td>
<td>10.3</td>
<td>3.2</td>
<td>52.3</td>
<td>63.8</td>
<td>1.8</td>
<td>0.2</td>
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Maternal inflammation is causally linked to systemic coagulopathies

To characterise the link between an LPS-induced abnormal maternal inflammatory response and development of maternal coagulopathies, we treated rats (n=5) with the TNF-α inhibitor etanercept (10 mg/kg) 6 h prior to administration of LPS (100 μg/kg). 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 (24). Only one of the five rats treated with LPS + etanercept developed systemic coagulopathies (p < 0.05 vs. LPS-treated rats, Chi-square analysis; Fig. 4A). Thromboelastography revealed a substantial degree of inter-individual variability in TEG parameters in all of the LPS-treated rats (Fig. 4B-D). An F-test for variance comparing etanercept + LPS vs. LPS-treated dams was performed and it confirmed the significant reduction in the variability of TEG parameters in etanercept-treated rats.
To further confirm that the effect of LPS on the development of coagulopathies is mediated by TNF-α, we injected four pregnant rats with TNF-α (5 μg per rat). All of the dams injected with TNF-α developed a DIC phenotype (one developed DIC-I and three developed DIC-II) within 1 h of exposure to TNF-α.

Abnormal fibrin deposition in implantation sites following LPS administration

Compared with utero-placental units from saline-treated rats (▶Fig. 5A) implantation sites of LPS-treated dams that exhibited hypercoagulability were characterised by widespread intravascular and decidual clots that were generally larger, more numerous, and more widely interspersed than in saline controls (▶Fig. 5B and C). In addition, placentas exhibited partial and complete blockage of maternal vessels and near complete obstruction of the maternal arterial channels (MAC) (▶Fig. 5D) that traverse the thickness of the placenta. Placentas of LPS-treated animals exhibiting a DIC-I phenotype were also characterised by the presence of widespread decidual clots and fibrin deposition surrounding giant cells of the junctional zone (▶Fig. 5E). Placentas of animals exhibiting DIC-III had minimal fibrin deposition, but were rather characterised by enlarged blood spaces in the labyrinth, junctional zone and decidua (▶Fig. 5F). Immunohistochemical staining for fibrin/fibrinogen confirmed the findings observed by PAS and also revealed greater labyrinth fibrin deposition in the implantation sites of LPS-treated rats exhibiting hypercoagulability (▶Fig. 6).
Etanercept-mediated inhibition of maternal systemic coagulopathy is associated with normal placental haemostasis

Compared with implantation sites from rats treated with LPS alone, histological analysis revealed fewer placental intravascular fibrin deposits in implantation sites from rats treated with LPS + etanercept (Fig. 7A). Immunohistochemical staining for fibrin/fibrinogen in the implantation sites from rats treated with LPS + etanercept revealed levels of intravascular, labyrinth,

Figure 5: Histological evidence of placental haemostatic alterations following maternal LPS administration. Utero-placental units of LPS- and saline-treated rats were dissected, formalin-fixed, paraffin-embedded, cut (6 μm) and stained with PAS and haematoxylin. Utero-placental unit of a saline-treated rat exhibiting minor decidual and intravascular fibrin deposition (dark pink stain) (A). Utero-placental units of LPS-treated dams (B-F). Utero-placental units of hypercoagulable dams were characterised by intravascular fibrin deposition; boxed inset on top right shows almost complete occlusion of maternal vessels (B). Utero-placental units of hypercoagulable dams were also characterised by substantial decidual fibrin deposition (C) and occluded maternal arterial channels (MAC) (D). Utero-placental units of dams exhibiting DIC-I revealed widespread intravascular clots as shown by the boxed inset on the top right (E). Utero-placental units of dams exhibiting DIC-III revealed minimal fibrin deposition but rather were characterised by enlarged maternal decidual sinuses (F). IZ: Junctional zone, L: Labyrinth, D: Decidua, MT: Mesometrial triangle. (n=8 LPS-treated rats, n=5 saline-treated animals; an average of 5–6 utero-placental units were studied from saline-treated dams, and 8–9 from LPS-treated dams.)

Figure 6: Immunohistochemical evidence of placental haemostatic alterations following maternal LPS administration. Utero-placental units of LPS and saline treated rats were stained for fibrin/fibrinogen. Utero-placental unit of saline-treated animal (A). Utero-placental unit of hypercoagulable dams exhibited substantial intravascular coagulation; boxed inset shows enlarged micrograph of several occluded mesometrial vessels (B). As compared with minimal decidual fibrin deposition in saline controls (C), hypercoagulable dams exhibited extensive decidual fibrin deposition (D). As compared with minor labyrinth fibrin deposition in saline controls (E), hypercoagulable dams exhibited substantial labyrinth fibrin deposition (F). Utero-placental units of dams exhibiting DIC-I dam were characterised by widespread intravascular clots (G). Dams exhibiting DIC-III showed minimal if any fibrin deposition (H). n=8 LPS-treated rats, n=5 saline-treated animals; and average of 5–6 utero-placental units were analysed from saline controls and 8–9 from LPS-treated dams.

Etanercept-mediated inhibition of maternal systemic coagulopathy is associated with normal placental haemostasis
Figure 7: Prevention of systemic coagulopathies by etanercept is associated with normal placental haemostasis. PAS staining of utero-placental units of etanercept-treated dams exhibited intravascular fibrin deposition similar to that of saline controls. Boxed inset shows enlarged micrographs of un-occluded maternal vessels (A). These findings were confirmed by IHC staining for fibrin/fibrinogen (B). In addition, the amount of decidual (C) and labyrinth fibrin deposition (D) was comparable to that of saline controls. Quantification of fibrin deposition was calculated using Image-Pro software analysis. The placental surface area covered by fibrin deposition was markedly high in LPS-treated dams exhibiting hypercoagulability, as compared with LPS-treated dams exhibiting DIC-III, saline-treated dams, and etanercept-treated dams (E). (At least three dams from each treatment group were analysed, with the exception of DIC-III, since only two dams exhibited this coagulopathy. An average of 4–7 utero-placental units were analysed.)
and decidual fibrin deposition similar to those observed in saline-treated control rats (Fig. 7B-D). Quantification of fibrin deposition revealed that utero-placental units of hypercoagulable dams exhibited significantly higher levels of fibrin deposition than utero-placental units from dams exhibiting DIC-III, saline-treated dams, and dams treated with etanercept + LPS (p < 0.05; Fig. 7E). At least three dams from each group were analyzed (except for the DIC-III groups because only two dams exhibited this type of coagulopathy) and an average of 5–6 utero-placental units per group were assessed. In order to perform ANOVA, implantation sites of dams in each individual group were pooled. Utero-placental units were chosen randomly.

**Systemic and local coagulopathies are associated with impaired utero-placental haemodynamics**

To determine whether systemic and local haemostatic alterations are linked to abnormalities in utero-placental haemodynamics, dams were imaged by means of Doppler ultrasonography at 20-min intervals beginning at the time of LPS/saline injection (denoted as time 0) for a total of 160 min. Using repeated measures ANOVA on flow velocity values over time, it was determined that the treatment groups differed significantly from each other (p<0.05). In saline-treated rats there was no change in spiral arteriole peak systolic flow velocity over the course of the ultrasound measurements (Fig. 8A) and placental perfusion was maintained, as determined by power Doppler (Fig. 8B). Administration of LPS resulted in a substantial decline in mean spiral arteriole peak systolic flow velocity over 160 minutes of LPS exposure (p=0.052; one-sample t-test; Fig. 8A) and a reduction in placental perfusion (Fig. 8C). Post-hoc analysis revealed that our study had 50% power to detect a significant effect of LPS on systolic flow velocity. The effect of LPS on spiral arteriole peak systolic flow velocity was absent in the LPS + etanercept-treated rats (Fig. 8A).

**Discussion**

Women with thrombophilia are at an increased risk of suffering pregnancy loss especially during the second half of pregnancy when foetal viability relies on the maintenance of adequate placental perfusion (25). The hypercoagulable haemostatic shift that occurs in normal pregnancy renders women increasingly vulnerable to developing coagulopathy if exposed to pathological stimuli (26, 27). Here, we showed that acute inflammation induced by LPS administration to pregnant rats is causally linked to the development of systemic and placental haemostatic and haemodynamic alterations preceding negative foetal outcomes.

While the dose of LPS given to the rats was relatively low (approximately 1/200th of the LD<sub>50</sub> for rats [28]), the fact that circulating maternal TNF-α levels were high 1 h later was evidence of a strong inflammatory response. Importantly, this study also demonstrated that TNF-α is a key mediator of LPS-induced activation of coagulation and fibrinolysis. This conclusion is based on the fact that R time, reflective of coagulation activation, and LY30, reflective of the fibrinolytic system were not altered in rats treated with LPS in combination with the TNF-α inhibitor etanercept. The pro-
tective effect of TNF-α-inhibition on the maternal systemic coagulation highlights the role of inflammation in the development of systemic coagulopathies. To our knowledge, this is the first study that establishes a causal link between TNF-α-mediated inflammation and the development of systemic maternal coagulopathies preceding foetal death.

Thromboelastography provides an accurate and real-time assessment of cellular and enzymatic components of haemostasis and, although several studies have reported aberrant elevation of pro-coagulant markers during pregnancy complications (29–31), only a few have used thromboelastography (32, 33). This study showed various inflammation-induced coagulopathies in pregnant rats as determined by TEG, including DIC stages I, II, and III, as well as a state of hypercoagulability. Despite receiving the same dose of LPS, pregnant rats exhibited various types of DIC, likely due to the genetic heterogeneity characteristic of outbred Wistar rats. It is not known whether this haemostatic variability in response to inflammation also occurs in human pregnancy. There is evidence that links inflammation associated with APLS and sepsis with various coagulopathies in human pregnancy, such as DIC and thrombophilia (16, 18, 19). It is also likely that most cases of inflammation-associated pregnancy loss in humans occur over a more gradual and longer period of exposure to pro-inflammatory molecules. Because of its acute nature, our model may more closely resemble cases of pregnancy loss associated with transient infection. Consistent with previous reports (31), we showed that LPS also induced haemostatic alterations in non-pregnant animals. It is important to note that ‘normal’ TEG parameters for the Wistar rat have not been reported. Our study provides information regarding normal TEG parameters in this rat strain and we have used this information to validate comparisons with experimental groups.

Further, this study demonstrated that inflammation-induced systemic coagulopathies are associated with observable alterations in placental haemostasis preceding foetal death. Systemic hypercoagulability reflects an increase in the levels of pro-coagulant factors leading to increased fibrin production. Accordingly, hypercoagulable dams exhibited reduced clotting time and significantly higher intravascular, decidual and labyrinth fibrin deposition than saline controls. These data support the concept that a thrombotic tendency may exert its detrimental effects on pregnancy locally, by obstructing the utero-placental vasculature. We also demonstrated that inflammation-induced DIC was associated with specific placental haemostatic alterations. In the first stage of DIC we detected a significantly reduced clotting time concomitant with widespread intravascular clots. As DIC progresses into the second and third stages, depletion of platelets and coagulation factors leads to excessive fibrinolysis and bleeding. TEG revealed these systemic changes as shown by a significant increase in fibrinolysis (LY30) in DIC-II and a significant reduction in clotting time and rate of clot formation in DIC-III phenotypes. Moreover, these systemic alterations were associated with minimal or absent placental fibrin deposition. It has previously been shown that fibrinogen-deficient mice undergo foetal loss due to severe placental haemorrhage (34). Thus, complete absence of fibrin could also be detrimental to foetal outcome. While thrombophilia has been associated with human pregnancy loss in the second and third trimesters (2, 35), because of the close sequential relationship between these processes it is possible that excessive placental haemorrhaging due to depletion of pro-coagulants also contributes to foetal death as revealed in our model. This warrants further investigation.

Our results also revealed that endotaxin-mediated inhibition of coagulopathy in pregnant rats is associated with physiological placental haemostasis, as evidenced by normal levels of decidual, intravascular, and labyrinth fibrin deposition. These placental changes could also reflect a direct effect of TNF-α on the placental endothelium and the syncytiotrophoblast. To our knowledge this is the first study that demonstrates the protective effect of TNF-α inhibition on the development of systemic and local placental coagulopathies.

The mechanism by which systemic and local coagulopathies lead to foetal death requires further investigation. We demonstrated that inflammation-induced systemic and local coagulopathies are associated with a reduction in spiral arteriole peak flow velocity and reduced placental perfusion preceding foetal death. It is likely that increased local fibrin deposition impairs the flow of blood through maternal vessels and the exchange of gases and nutrients. Most clots were observed in the mesometrial triangle, an area adjacent to the decidua basalis that contains maternal spiral arterioles and veins continuous with blood channels of the placenta. It is possible that fibrin deposits in this area impede venous drainage of the placenta, which could explain the decreased spiral arteriole flow velocity and overall reduction in placental perfusion. Concomitantly, hypocoagulation and subsequent haemorrhage could be reflected by leaks in the maternal vasculature that also ultimately impair blood flow to the fetus.

While we cannot ignore vaso-constrictive effects of TNF-α on endothelium, our data support the concept that the maintenance of utero-placental blood flow in the endotaxin-treated rats is secondary to prevention of systemic and placental haemostatic alterations. Further investigation utilising anticoagulants or inhibitors of tissue factor (TF, the main trigger of coagulation) is warranted to provide a causal link between maternal coagulopathy, impaired utero-placental blood flow and foetal death.

The results of this study provide evidence of the important role of TNF-α and maternal coagulopathies in the pathophysiology of pregnancy complications.
pregnancy loss and the value of thromboelastography as a tool for haemostatic assessment. Collectively, our findings demonstrate that abnormal maternal inflammation is causally linked to coagulopathies, which are coupled with observable haemostatic and haemodynamic alterations in the placenta. We propose that modulation of the inflammatory response may be a potential therapeutic approach to restore foetal viability via improved utero-placental blood flow secondary to prevention of coagulopathies.

Acknowledgement
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Conflicts of interest
None declared.

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