ORAL DISEASES

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ORIGINAL ARTICLE

The effect of *Porphyromonas gingivalis* lipopolysaccharide on pregnancy in the rat

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OBJECTIVE: Periodontitis, mostly associated with *Porphyromonas gingivalis*, has frequently been related to adverse pregnancy outcomes. We therefore investigated whether lipopolysaccharides of *P. gingivalis* (Pg-LPS) induced pregnancy complications in the rat.
METHODS: Experiment 1: pregnant rats (day 14) received increasing Pg-LPS doses (0.0–50.0 μg kg^{-1} bw; $n = 2/3$ p per dose). Maternal intra-aortic blood pressure, urinary albumin excretion, placental and foetal weight and foetal resorptions were documented. Experiment 2: 10.0 μg kg^{-1} bw (which induced the highest blood pressure together with decreased foetal weight in experiment 1) or saline was infused in pregnant and non-pregnant rats ($n = 7/9$ p per group). Parameters of experiment 1 and numbers of peripheral leucocytes as well as signs of inflammation in the kidney and placenta were evaluated.
RESULTS: Pg-LPS infusion in pregnant rats increased maternal systolic blood pressure, reduced placental weight (dose dependently) and decreased foetal weight and induced foetal resorptions. It, however, did not induce proteinuria or a generalised inflammatory response. No effects of Pg-LPS were seen in non-pregnant rats.
CONCLUSION: Pg-LPS increased maternal blood pressure, induced placental and foetal growth restriction, and increased foetal resorptions, without inducing proteinuria and inflammation. Pg-LPS may therefore play a role in pregnancy complications induced by periodontitis.
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Introduction

Porphyromonas gingivalis has been identified as a major aetiological factor in the pathogenesis of periodontitis (van Winkelhoff *et al*, 2002). Periodontitis is not only a local infection, but periodontal pathogens, especially *P. gingivalis* (Hayashi *et al*, 2010), can also be found at distant sites (Haraszthy *et al*, 2000) or induce changes in systemic inflammatory parameters (Moutsopoulos and Madianos, 2006), for instance increased plasma C-reactive protein (CRP) levels (Paraskevas *et al*, 2008) as well as activated inflammatory cells and endothelial cells (Piconi *et al*, 2008). Indeed, periodontitis has been associated with various systemic diseases, such as atherosclerosis, myocardial infarction and cardiovascular diseases (CVD) (Scannapieco *et al*, 2003). The systemic nature of periodontitis can also be seen from the putative relationship between maternal periodontitis and poor obstetric outcome (Vergnes and Sixou, 2007; Xiong *et al*, 2007; Kunnen *et al*, 2010).

A large number of observational studies showed a relationship between pregnancy outcome and periodontal disease. Periodontal disease during pregnancy has been associated with preterm birth, which is defined as a delivery before 37 weeks of gestation (Xiong *et al*, 2007), low birth weight, which is defined as infants with birth weights of <2500 g (Vergnes and Sixou, 2007), miscarriage or stillbirths (Xiong *et al*, 2007) and pre-eclampsia (Kunnen *et al*, 2010). Pre-eclampsia is one of the most important pregnancy complications in the western world and is characterised by maternal hypertension and proteinuria clinically manifest during the second half of pregnancy (Walker, 2000). It has been suggested that the chronic general low-grade inflammation that is seen in patients with periodontitis may play a role in the pathogenesis of these pregnancy-related pathologies (Cetin *et al*, 2012). Although the exact relationship between periodontitis and pregnancy complications and mechanisms needs to be established, it seems likely that *P. gingivalis* is involved in inducing adverse pregnancy outcomes, as *P. gingivalis*

has the capacity to disseminate from the oral cavity to the foetoplacental unit (Barak *et al*, 2007; Leon *et al*, 2007; Katz *et al*, 2009; Swati *et al*, 2012; Chaparro *et al*, 2013). In animal experiments, *P. gingivalis* has been found in placental tissues after intravenous infection and appeared to induce lesions similar to human chorioamnionitis and placentitis (Bélanger *et al*, 2008).

Moreover, *P. gingivalis* in mice and rats was associated with foetal and placental growth restriction, increased foetal resorptions and foetal death (Lin *et al*, 2003; Michelin *et al*, 2012). *P. gingivalis* bacteria contain factors that are pro-inflammatory and can also potentially affect placental tissue. One of the most important factors is lipopolysaccharide (LPS) (Holt *et al*, 1999). Indeed, intravenous infusion with LPS of *P. gingivalis* (Pg-LPS) in pregnant animals induced foetal growth restriction and foetal death (Collins *et al*, 1994). Interestingly, pregnant individuals are extremely sensitive to LPS of *Escherichia coli* (Ec-LPS) and in recent animal studies, this LPS has been shown to induce not only placental effects (Renaud *et al*, 2011), but also peripheral effects such as hypertension, proteinuria and generalised inflammation (Faas *et al*, 1994, 2003).

The aim of the present study therefore was to investigate whether Pg-LPS in pregnant rats also induced systemic complications, such as hypertension, proteinuria and generalised inflammation next to affecting placental and foetal growth.

Materials and methods

Handling of the rats

Animal experiments were conducted under protocols approved by the Animal Ethical Committee of the University of Groningen, the Netherlands. Female Wistar outbred rats (175–200 g) were obtained from Harlan Inc., Zeist, the Netherlands. Rats were maintained in a temperature- and light-controlled room (22°C, 12:12H light/dark cycle), with free access to standard laboratory chow and water. Animals were allowed 7 days to acclimatise to the laboratory environment and were housed together until selection for experiments. After acclimatisation, vaginal smears were taken to follow oestrus cyclicity. On pro-oestrus, rats were housed overnight with two fertile Wistar outbred males. A positive vaginal smear for spermatozoa the next day was defined as day 0 of pregnancy. On day 0 of pregnancy and on dioestrus-2 in non-pregnant control rats, a permanent cannula was surgically inserted into the right jugular vein under isoflurane/oxygen anaesthesia, according to standard methods (Faas *et al*, 1999). This cannula allows stress-free infusion of either endotoxin or saline and stress-free blood sampling. Rats were then housed individually and allowed to recover from surgery for 14 days. On day 14 of pregnancy (14 days after surgery in non-pregnant rats), rats were infused with either 2.0 ml endotoxin solution (concentrations ranging from 1.0 to 50.0 μg Pg-LPS kg^{-1} bw in saline) or 2 ml saline alone during 1 h via the permanent jugular vein cannula. Ultrapure lipopolysaccharide of *Porphyromonas gingivalis* (tlrl-pglps, strain ATCC 33277; InvivoGen, San Diego, CA, USA) was used in this study.

Experimental design

Experiment 1. In this experiment, pregnant rats were infused with Pg-LPS at increasing doses from 0.0 to 50.0 μg Pg-LPS kg^{-1} bw ($n = 2/3$ per dose) in order to establish a putative dose that would induce systemic, placental and foetal signs. Twenty-four hour urinary albumin excretion was measured 1 day before infusion and at one and 5 days after infusion. On day 21, which is the end of pregnancy, and 7 days after Pg-LPS infusion (in none of the rats parturition had started), rats were anaesthetised and aortic blood pressure was measured after which the rats were sacrificed by aortic puncture. Living foetuses and their placentas were collected and weighed. Total number of foetuses and the number of foetal resorptions were also counted. The percentage of foetal resorptions was calculated.

Experiment 2. From experiment 1, it appeared that Pg-LPS induced hypertension, decreased placental weight and increased the number of foetal resorption. To further evaluate the effect of Pg-LPS on pregnant rats, in the second experiment, rats were infused with 10.0 μg kg^{-1} bw Pg-LPS, the dose that appeared to induce the highest blood pressure and also affected placental and foetal weight. The pregnant Pg-LPS (10.0 μg kg^{-1} bw) group ($n = 9$) as well as the pregnant control group was expanded, which was infused with 2.0 ml saline alone ($n = 9$). Blood pressure, albumin excretion, foetal and placental weight were measured as in experiment 1. In addition, in order to investigate possible effects of Pg-LPS on the inflammatory response, we evaluated the number of peripheral white blood cells (WBC) as well as differential WBC counts and also signs of inflammation in the placenta and the kidney were evaluated. We choose to study these organs, as these organs are usually affected in pregnancy complications, such as pre-eclampsia. To explore whether the response to Pg-LPS was specific for pregnancy, also two groups of non-pregnant rats were included that were infused with either Pg-LPS (10.0 μg kg^{-1} bw in 2 ml saline) ($n = 7$), or with 2.0 ml saline alone ($n = 7$) using an identical experimental set-up.

Blood pressure

For measuring intra-aortic blood pressure, at the end of pregnancy, a midline ventral incision was made under standard isoflurane/oxygen anaesthesia and the abdominal aorta was exposed. Immediately after exposure of the aorta, a needle connected to the probe of a pressure recorder (Lifescope 9, Bedside monitor MU-832 RK, Nihon Kohden Corporation, Tokyo, Japan) was inserted into the abdominal aorta after which the systolic and diastolic blood pressure were recorded. Rats were bled immediately afterwards by aortic puncture.

Albumin excretion

One day before the start of the infusion, as well as 1 day and 5 days after the infusion, rats were placed in metabolic cages (from 10.00 AM until 10.00 AM the next day) to collect a 24-h urine sample. Urinary volume was measured and urinary albumin levels were assayed by the

rocket-electrophoresis technique using rabbit-anti-rat albumin antiserum (Nordic Immunology, Tilburg, The Netherlands) according to the method of Laurell (Laurell, 1972).

Tissue preparations
Immediately after sacrifice, the pregnant uterus was transected longitudinally to determine the number of living foetuses and resorptions. Each living individual foetus was weighed. In order to investigate if leucocyte infiltration occurred not only in placental tissue, but also in maternal tissue, from each dam, according to standard procedures, three representative placentas (from live foetuses) with associated mesometrial triangle were harvested, cut sagittally into two halves and snap-frozen in isopentane ($-80°C$) and stored at $-80°C$ for later analysis. Remaining placentas from the living foetuses (without mesometrial triangle) were weighed individually. Also from each dam, the left kidney was removed and collected. Kidney fragments were snap-frozen immediately and stored at $-80°C$ for later analysis.

White blood cell counts
Blood samples (0.1 ml in 12% ethylenediaminetetraacetate (EDTA)) were drawn from the permanent jugular vein cannula on day 14 (just prior to the start of the infusion), on day 15, 16, 19 and on day 21 between 09.00 and 11.00 AM. Twenty microlitre of blood was used to determine total WBC count on a microcell counter (model Sysmex pocH-100i Haematology Analyser; Sysmex Corp., Kobe, Japan). In order to determine differential WBC counts from each blood sample, a smear was made on a microscope slide and stained using the Giemsa method (1:10 dilution; Merck) for 20 min (Garcia, 2001). After evaluation of 200 cells per smear, relative and absolute numbers of granulocytes, lymphocytes and monocytes were calculated according to standard methods.

Immunohistochemistry
Cryostat placenta sections (along with the mesometrial triangle) and kidney sections (5 μm) were cut using a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany). The sections were collected on silane-coated glass slides (Starfrost, Waldemar Knittel, Braunschweig, Germany) and allowed to air dry at room temperature and then stored at -20°C for later analysis.

Staining for granulocytes and monocytes/macrophages
Glomerular and placental sections were stained for granulocytes and monocytes/macrophages using primary monoclonal antibodies against rat granulocytes (His48; Becton Dickinson, Franklin Lakes, NJ, USA) and primary monoclonal antibodies against rat monocytes/macrophages (ED1/CD68, dilution 1:100; AbD Serotec, Düsseldorf, Germany) according to standard protocols. Briefly, after drying in air, sections were fixed in precooled acetone (4°C) for 10 min. Subsequently, sections were air-dried and then incubated with the first antibody for 1 h in the dark at room temperature. After washing in phosphate-buffered saline (PBS), endogenous peroxidase activity was blocked by incubation of the slides for 20 min in methanol supplemented with 3%

hydrogen peroxide (H_2O_2). Thereafter, sections were incubated with normal rabbit serum (dilution 1:10; DAKO, Glostrup, Denmark) for 30 min. Then without rinsing, sections were incubated for 30 min with a peroxydase-conjugated secondary antibody rabbit-anti-mouse (dilution 1:50; DAKO). After washing in PBS, the reaction product was visualised using 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich, St. Louis, MO, USA), counterstained with haematoxylin (1 min) and embedded in Kaiser's glycerine-gelatin. Control sections not incubated with the primary antibody were consistently negative.

Evaluation of kidney sections
Kidney sections of each individual rat were quantitatively scored by light microscopic examination. Sections stained for the presence of granulocytes and monocytes/macrophages were quantified by counting the total number of positive cells in 100 glomeruli per section, as described previously (Faas *et al*, 1995). Results were expressed as the median of number of positive cells per glomerulus.

Evaluation of placental sections
Granulocyte and monocyte/macrophage infiltration were evaluated in the placentas and their associated mesometrial triangle by light microscopic examination.

Statistics
Statistical analysis was performed using SPSS 20 software for Windows. Results are expressed as individual values or illustrated in box and whisker plots. In experiment 1, Pearson correlation coefficients were calculated to evaluate the relationship between dose of Pg-LPS and blood pressure, placental weight or foetal weight. R^2 and the slope were calculated, and it was tested whether the slope was statistically significantly different from zero. A slope that was different from zero indicated a dose-response effect. If no dose-response effect was observed, an effect of Pg-LPS was evaluated by grouping the data of all Pg-LPS-infused rats together. These grouped data of the Pg-LPS-infused rats were then compared with the saline-infused rats using the Mann–Whitney *U*-test. Before starting experiment 2, a power analysis was performed using maternal blood pressure as our main endpoint. The power analysis was based on the blood pressure data of experiment 1, with a mean systolic blood pressure of control pregnant rats of 86.3 mmHg and a variance of 12%. Based on the systolic blood pressure of Pg-LPS-infused rats (Figure 1a), a 25% increase in mean systolic blood was expected. Using a power of 0.9 and a probability level of 0.05, power analysis showed that a minimum of six rats per group should be included. In experiment 2, differences between groups were tested by using the Mann–Whitney *U*-test. Nominal pregnancy outcome data (number of resorptions and/or number of foetuses with growth restriction) were tested by using the Fisher's exact test. Postinfusion albumin excretion, total WBC counts and differential WBC counts were tested *vs* preinfusion values using the Wilcoxon signed rank test. In the case of multiple comparisons, Bonferroni correction was used. $P < 0.05$ was accepted as statistically significant.
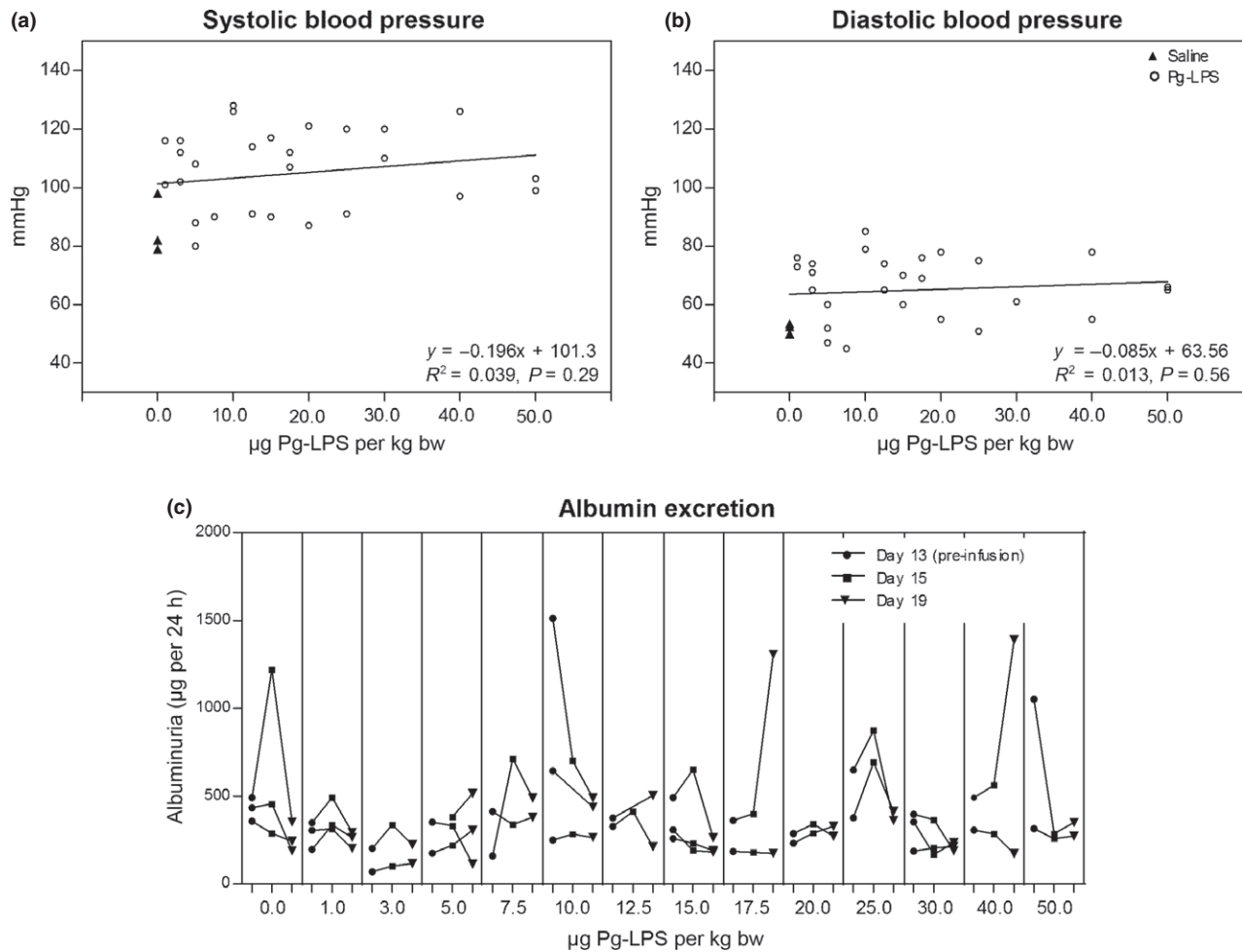
Figure 1 (**a**) Individual intra-aortic systolic blood pressure (**b**) and diastolic blood pressure of pregnant rats under standard isoflurane/oxygen anaesthesia on gestational day 21, 7 days after infusion with increasing doses of Pg-LPS (0–50.0 μg kg^{-1} bw, n = 2/3 p. dose). Closed triangles: saline-control rats; open dots: Pg-LPS-infused rats (R^2 = Pearson correlation coefficients; P < 0.05 indicates that slopes are significantly different from zero); (**c**) individual 24 h urinary albumin excretion of pregnant rats after infusion with increasing doses of Pg-LPS (0–50.0 μg kg^{-1} bw, n = 2/3 p. dose) at gestational day 13 (preinfusion values: closed dots), gestational day 15 (1 day after infusion: closed squares) and gestational day 19 (5 days after infusion: closed triangles)

Results

Experiment 1

Systolic and diastolic blood pressure and urinary albumin excretion. Figure 1a,b show the aortic systolic and diastolic blood pressure of pregnant rats at gestational day 21, which is 7 days after infusion with increasing doses of Pg-LPS (0.0–50.0 μg kg^{-1} bw Pg-LPS). No dose–response relationship between dose Pg-LPS and systolic blood pressure (R^2 = 0.039, P = 0.29) or diastolic blood pressure (R^2 = 0.013, P = 0.56) was observed. However, when all Pg-LPS-infused rats were grouped together, both diastolic and systolic blood pressure of Pg-LPS-infused rats were significantly higher as compared with saline-infused rats (P < 0.05, Mann–Whitney *U*-test). Urinary albumin excretion after infusion with increasing doses of Pg-LPS in pregnant rats is presented in Figure 1c. Pregnant rats did not exhibit increased albumin excretion after infusion with saline or Pg-LPS at any dose tested at 1 day after infusion (gestational day 15) or 5 days after

infusion (gestational day 19) as compared with preinfusion values (gestational day 13).

Placental and foetal weight. After infusion with 0.0–50.0 μg kg^{-1} bw Pg-LPS, a decreasing mean placental weight was observed with increasing Pg-LPS doses (R^2 = 0.218, P = 0.005) (Figure 2a). There was no significant dose-response effect of increasing Pg-LPS doses and mean foetal weight (R^2 = 0.015, P = 0.49) (Figure 2b). No significant differences in foetal weight were observed between saline-infused rats and all Pg-LPS-infused rats grouped together.

Foetal resorption. Table 1 shows the total number of foetuses as well as percentages of foetuses resorbed (disintegrated and assimilated dead foetuses in the uterus) in the animals infused with the different doses of Pg-LPS. As can be seen from this table, foetal resorption was exclusively found after infusion with doses of Pg-LPS at or above 7.5 μg kg^{-1} bw.
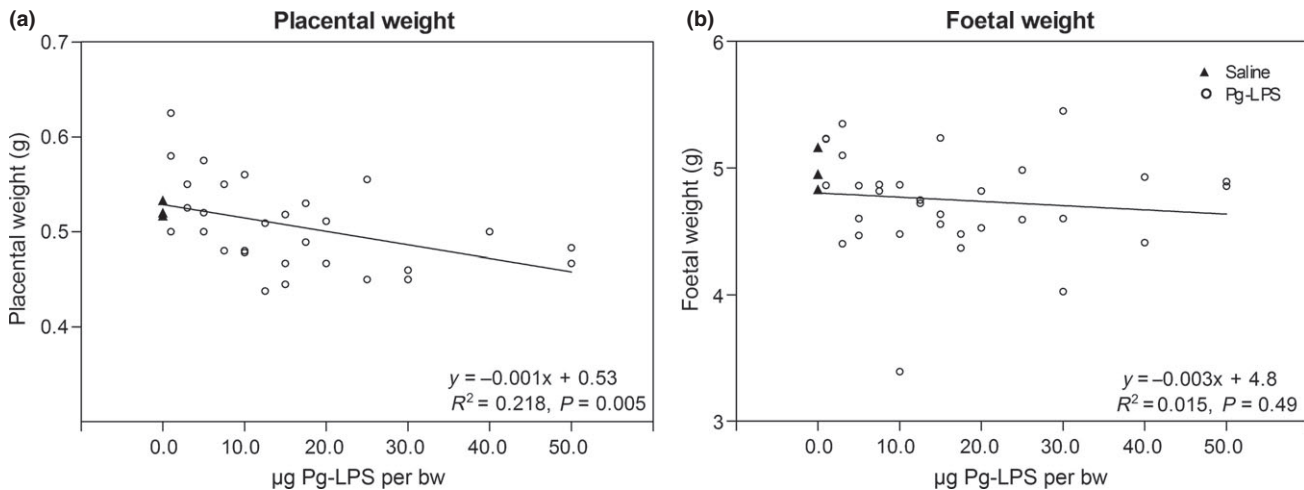
Figure 2 Placental (**a**) and foetal (**b**) weight after sacrifice at gestational day 21, 7 days after infusion with increasing doses of Pg-LPS (0–50.0 μg kg^{-1} bw, $n = 2/3$ p. dose). Each triangle/dot represents the mean placental or foetal weight per individual mother. Closed triangles: saline-infused control rats; open dots: Pg-LPS-infused rats (R^2 = Pearson correlation coefficients; $P < 0.05$ indicates that slopes are significantly different from zero)

Table 1 Foetal resorptions at gestational day 21 following infusion with saline or increasing doses of Pg-LPS

Pg-LPS (μg kg^{-1} bw)	No. of mothers	R/T	Resorptions (%)
0.0	3	0/32	0
1.0	3	0/31	0
3.0	3	0/34	0
5.0	3	0/39	0
7.5	2	2/25	8.0
10.0	3	2/35	5.7
12.5	2	1/27	3.7
15.0	3	0/39	0
17.5	2	1/26	3.8
20.0	2	1/21	4.8
25.0	2	0/25	0
30.0	3	8/29	27.6
40.0	2	0/24	0
50.0	2	1/22	4.5

R/T, number of resorptions/total number of resorbed and living foetuses.

Experiment 2
Systolic and diastolic blood pressure and urinary albumin excretion. As infusion of 10 μg kg^{-1} bw Pg-LPS appeared to induce increased blood pressure together with decreased foetal weight, we chose this dose of LPS to further evaluate the effect of this LPS on pregnant animals. Figures 3a,b show the aortic systolic and diastolic blood pressure of non-pregnant and pregnant rats after infusion with saline or 10.0 μg kg^{-1} bw Pg-LPS. It can be seen from these figures that infusion with 10.0 μg kg^{-1} bw Pg-LPS significantly increased systolic blood pressure in pregnant rats as compared with the saline-infused pregnant controls ($P < 0.05$, Mann–Whitney *U*-test). This increase in systolic blood pressure after infusion with 10.0 μg kg^{-1} bw Pg-LPS did not occur in non-pregnant rats. No significant differences were observed in diastolic blood pressure in non-pregnant or pregnant rats infused with 10.0 μg kg^{-1} bw Pg-LPS *vs* saline infusion. Figure 3c shows 24-h albumin excretion

of non-pregnant and pregnant rats after infusion with saline or 10.0 μg kg^{-1} bw Pg-LPS. There was no difference in urinary albumin excretion after infusion with saline or 10.0 μg kg^{-1} bw Pg-LPS in non-pregnant or pregnant rats 1 day after infusion (gestational day 15 in pregnant rats) or 5 days after infusion (gestational day 19) as compared with preinfusion values.

Placental and foetal weight. Infusion with 10.0 μg kg^{-1} bw Pg-LPS into day 14 pregnant rats significantly decreased placental weight as compared with the saline-infused controls (median 0.5 [interquartile range (IQR) 0.1] gr saline *vs* median 0.4 [IQR 0.1] gr Pg-LPS, $P < 0.05$, Mann–Whitney *U*-test) (Figure 4a). Although there were no differences in median foetal weight between the Pg-LPS-infused rats and the saline controls, there was a substantial amount of smaller than normal foetuses in the litter of the Pg-LPS-infused group, as can be seen from Figure 4b. We therefore analysed the numbers of foetuses that were more than two standard deviations (2 SD) from the normal mean (4.75 $\pm$ 0.50 g; i.e. <3.75 g) and defined them as foetal growth restricted. Of 102 foetuses in the Pg-LPS-infused group, 11 foetuses were growth restricted, while in the control group, only two of the 103 foetuses showed growth restriction. The number of growth restricted foetuses was significantly increased in LPS-infused pregnant rats as compared with saline-infused pregnant rats ($P < 0.05$, Fisher's exact test).

Foetal resorption. In saline-control rats, no foetal resorptions were observed. However, after infusion with 10.0 μg kg^{-1} bw Pg-LPS, foetal resorptions were observed: of the total of 109 foetuses in nine mothers, seven foetuses were resorbed ($P < 0.05$, Fisher's exact test).

Inflammatory parameters. Total WBC and granulocyte, monocyte and lymphocyte counts. Figure 5a shows total numbers of WBC as well as the differential counts for granulocytes, monocytes and lymphocytes at gestational
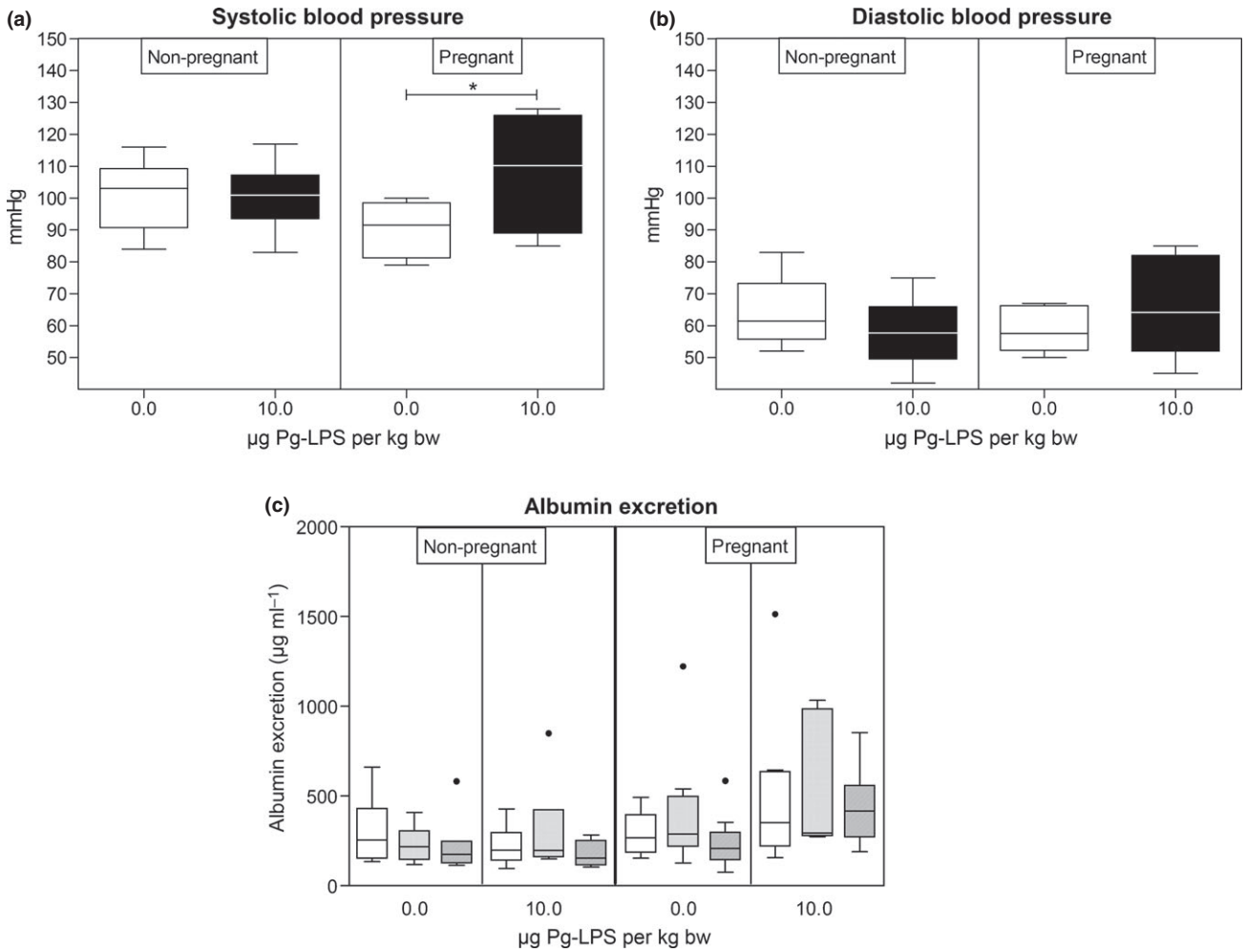
(a) Systolic blood pressure

(b) Diastolic blood pressure

(c) Albumin excretion

Figure 3 (**a**) Median and 25th and 75th percentiles of intra-aortic systolic and (**b**) diastolic blood pressure under standard isoflurane/oxygen anaesthesia of non-pregnant and pregnant rats after infusion with saline or 10.0 μg Pg-LPS kg^{-1} bw, 7 days after infusion (gestational day 21 of pregnant rats). Open bars: saline-infused control rats; black bars: Pg-LPS-infused rats; (**c**) median and 25th and 75th percentiles of 24-h albumin excretion of non-pregnant and pregnant rats after infusion with saline or 10.0 μg Pg-LPS/kg bw. Open bars: day 13 (preinfusion values); dotted bars: day 15 (1 day after infusion); striped bars: day 19 (5 days after infusion). Error bars demonstrate 1.5 interquartile range and outlier values are represented as individual black dots. * Significantly increased *vs* saline infusion (Mann–Whitney *U*-test; $P < 0.05$)
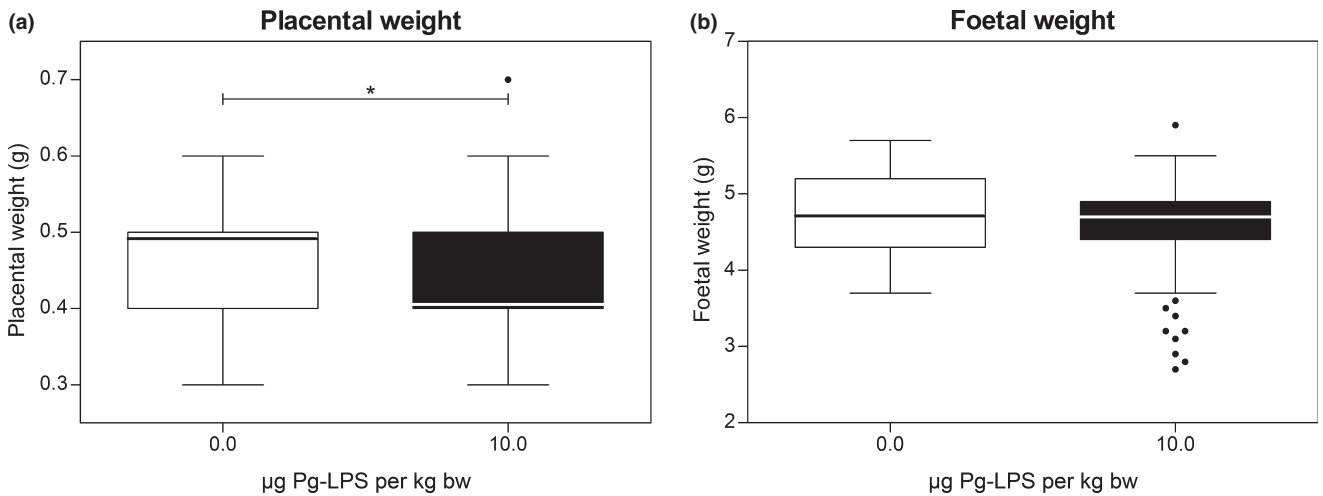
(a) Placental weight

(b) Foetal weight

Figure 4 Median and 25th and 75th percentiles of placental (**a**) and foetal (**b**) weight after sacrifice at gestational day 21, 7 days after infusion with saline or 10.0 μg Pg-LPS kg^{-1} bw. Error bars demonstrate 1.5 interquartile range and outlier values are represented as black dots. Open bars: saline-infused control rats; black bars: Pg-LPS-infused rats. * Significantly decreased *vs* saline infusion (Mann–Whitney *U*-test; $P < 0.05$)

(a) Median (interquartile range) of total and differential white blood cell (WBC) counts in non-pregnant and pregnant rats (gestational day 14), prior to infusion with saline or 10.0 µg Pg-LPS per kg bw

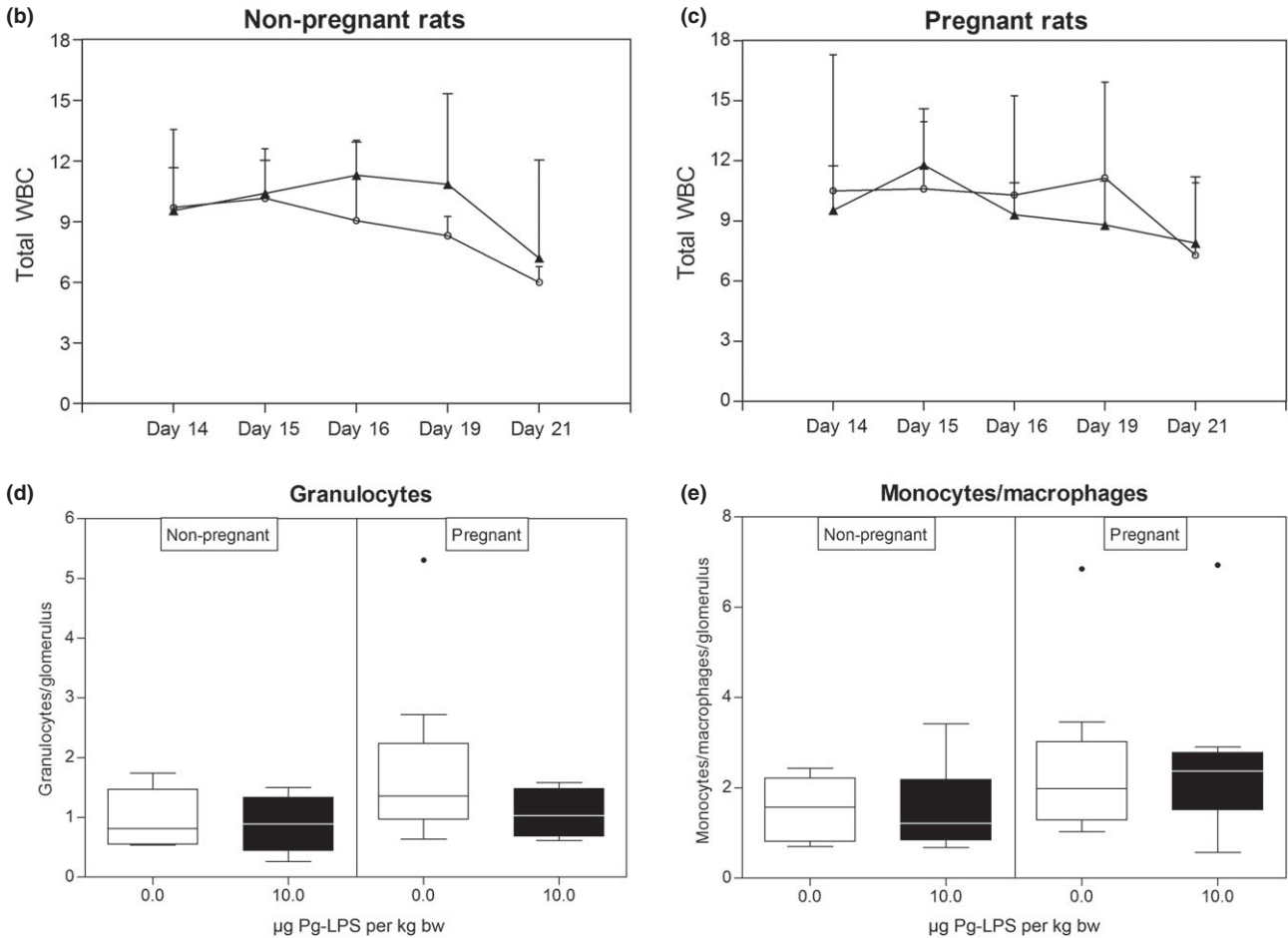| WBC	Non-pregnant rats		Pregnant rats	
	Number of cells (10^9 l^{-1})	% of WBC	Number of cells (10^9 l^{-1})	% of WBC
WBC	9.55 (8.43–12.25)		10.20 (7.70–13.70)	
Monocytes	0.35 (0.30–0.46)	3.5 (3.0–4.5)	0.48 (0.35–0.66)	5.3 (3.9–6.0)*
Lymphocytes	8.25 (6.85–9.60)	79.0 (75.5–83.5)	6.44 (4.66–7.71)*	60.8 (56.0–66.63)*
Granulocytes	1.62 (1.43–2.23)	17.0 (13.5–19.5)	2.91 (2.10–4.90)*	34.0 (28.4–37.8)*

Figure 5 (**a**) Median and 25th and 75th percentiles of total and differential white blood cell counts (WBC) in non-pregnant and pregnant rats (gestational day 14), prior to infusion with saline or 10.0 µg Pg-LPS kg^{-1} bw; (**b, c**) median and 25th and 75th percentiles of total white blood cell (WBC) counts in non-pregnant (**b**) and pregnant (**c**) rats at day 14 (preinfusion), and at days 15, 16, 19 and 21 of pregnancy (postinfusion days 1, 2, 5 and 7, respectively) after infusion of saline (closed triangles) or 10.0 µg Pg-LPS kg^{-1} bw (open dots); (**d**) median and 25th and 75th percentiles of number of granulocytes and (**e**) monocytes/macrophages per glomerulus in non-pregnant and pregnant rats, 7 days after infusion with saline or 10.0 µg Pg-LPS kg^{-1} bw (gestational day 21 of pregnant rats). Error bars demonstrate 1.5 interquartile range, and outlier values are represented as black dots. Open bars: saline-infused control rats; black bars: Pg-LPS-infused rats

day 14, that is, before the infusion with 10.0 µg kg^{-1} bw Pg-LPS or saline. Total WBC counts before infusion were not significantly different in day 14 pregnant rats as compared with non-pregnant rats. In pregnant rats, total numbers of lymphocytes were decreased while total numbers of granulocytes were increased as compared with non-pregnant controls ($P < 0.05$, Mann–Whitney *U*-test).

Figures 5b,c show the preinfusion and postinfusion total WBC counts in non-pregnant rats and pregnant rats after infusion with 10.0 µg kg^{-1} bw Pg-LPS or saline. In both non-pregnant and pregnant rats, no differences between

preinfusion (gestational day 14 in pregnant rats) WBC counts and WBC counts on days 15, 16, 19 and 21 (i.e. 1, 2, 5 and 7 days postinfusion, respectively) were observed in either the Pg-LPS-treated rats or saline-treated rats. There were no effects of infusion of either saline or 10.0 µg kg^{-1} bw Pg-LPS on the percentages of monocytes, lymphocytes and granulocytes in both non-pregnant and pregnant rats (results not shown).

Glomerular granulocyte and monocyte/macrophage infiltration. Figures 5d,e show the number of glomerular
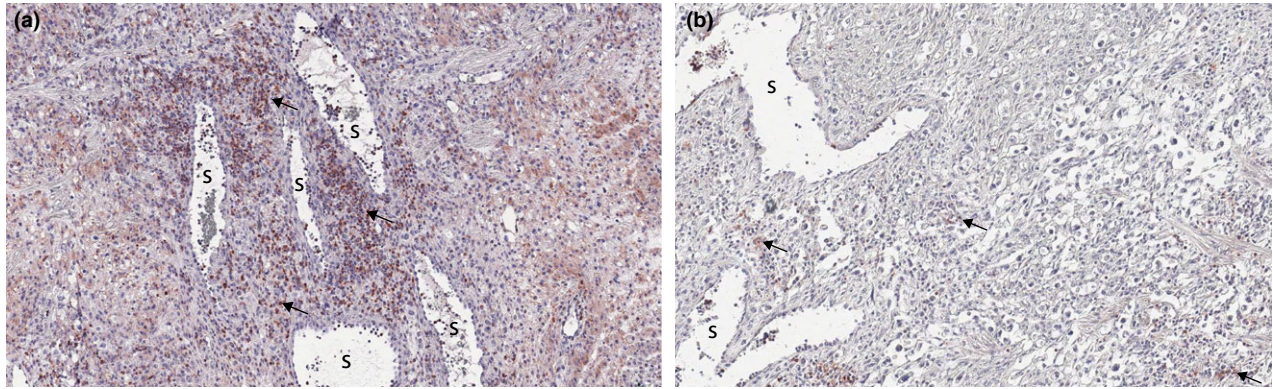
Figure 6 Photomicrographs of parts of the mesometrial triangle of a control pregnant rat at day 21 of pregnancy, stained for the presence of monocytes/macrophages (ED1; **a**) or for the presence of granulocytes (His48; **b**). ED1 positive cells were found throughout the mesometrial triangle, but especially around the spiral arteries (S). Less His48 positive cells were found in the mesometrial triangle, and they were found throughout the mesometrial triangle. S: spiral artery; arrows indicate positive cells

granulocytes and glomerular monocytes/macrophages in non-pregnant and pregnant rats after infusion with saline or with 10.0 μg kg^{-1} bw Pg-LPS. We observed no effect of Pg-LPS on glomerular granulocyte or monocyte/macrophage number.

Placental granulocyte and monocyte/macrophage infiltration. In control placental tissues, only a few scattered granulocytes and monocytes/macrophages were present in the labyrinth and in the spongiotrophoblast layer. In the decidua basalis as well as in the mesometrial triangle, in particular in the central part of the mesometrial triangle and in close proximity of the spiral arteries, ED1 positive monocytes/macrophages were observed (Figure 6a). Granulocytes were found scattered in the mesometrial triangle (Figure 6b). However, when comparing the placental tissues of the 10.0 μg kg^{-1} bw Pg-LPS-infused rats with the saline-infused controls, no differences between the groups were observed in numbers of granulocytes and monocytes/macrophages (results not shown).

Discussion

This study was set up to evaluate a causal relationship between Pg-LPS and pregnancy complications. Therefore, it was examined whether Pg-LPS infusion in pregnant rats could induce maternal hypertension, proteinuria and foetal or placental growth restriction and general inflammation. Rats were infused at day 14 of pregnancy, as this is in accordance with previous studies (Faas *et al*, 1994, 1995). For these previous studies, this time point was chosen, as at day 14 of pregnancy, the chorioallantoic placenta is fully developed, while the trophoblast invasion into the decidua and mesometrial triangle starts around that day (Caluwaerts *et al*, 2005; Vercruysse *et al*, 2006). Our results showed that Pg-LPS infusion in day 14 pregnant rats increased maternal systolic blood pressure, decreased placental weight and slightly decreased foetal weight (only in experiment 2) and also slightly increased the number of foetal resorptions, but did not induce albuminuria. Moreover, in the present study, no signs of placental or renal

inflammation were seen, and also, there were no signs of inflammation in the peripheral circulation (no increase in white blood cell counts or changes in white blood cell populations). Therefore, in this study, we observed no generalised inflammatory response in pregnant rats after infusion of Pg-LPS. The effects of Pg-LPS appeared to be specific for pregnant rats, as hypertension was not induced in non-pregnant animals.

Our findings of increased blood pressure, decreased foetal weight and increased number of foetal resorptions may originate from the placenta as placental weight was dose dependently decreased by Pg-LPS infusion. The reason for the decreased placental weight remains unclear from this study. Because we did not observe local inflammation in the placental tissues, Pg-LPS may have had a direct effect on the placenta. The fact that placental weight was dose dependently decreased may support this suggestion. Pg-LPS is a Toll-like receptor 2 (TLR2) ligand (Zhang *et al*, 2008) and placental trophoblast cells express TLR2 (Abrahams and Mor, 2005). It has been demonstrated *in vivo* and *in vitro* that activation of TLR2 receptors on trophoblast cells can evoke cell death pathways (Abrahams *et al*, 2008) and trophoblast apoptosis (Abrahams *et al*, 2004). It can be hypothesised that the reduction in placental size may be associated with an increased resistance to blood flow in the placental arteries. Increased arterial resistance may result in reduced transport of oxygen and nutrients to the foetus and in some foetuses to foetal demise or death (Renaud *et al*, 2011). It is suggested that reduced placental perfusion in humans enhances the release of pro-inflammatory factors, such as cytokines, soluble endoglin (sEng), the soluble form of the vascular endothelial growth factor (VEGF) receptor (sFlt-1) and placental growth factor (PlGF). Although these factors may be involved in endothelial dysfunction, subsequently leading to hypertension (Redman and Sargent, 2009), they are also involved in inducing generalised inflammation. Because in the present study there were no signs of systemic inflammation, it seems unlikely that the placenta released such pro-inflammatory factors into the maternal circulation.

It can be hypothesised that the increase in blood pressure in the present study may have been a compensatory

attempt to maintain perfusion in the affected placentas. Such an adaptive role for the foetus has also been suggested to occur in humans in the face of uteroplacental dysfunction (von Dadelszen *et al*, 2005). Indeed, maternal hypertension during pregnancy has been associated with improved neonatal health (McCowan *et al*, 2002; von Dadelszen *et al*, 2005). The observation that antihypertensive therapy of mild-to-moderate hypertension during pregnancy appeared to impair intrauterine foetal growth further supports this hypothesis (von Dadelszen *et al*, 2000; Magee *et al*, 2009). In the present study, even in the face of hypertension, foetal growth restriction or foetal death still occurred in some foetuses, suggesting that also other factors may play a role. Alternatively, it is also possible that the increased systolic blood pressure was the consequence of a direct effect of Pg-LPS on the endothelium, for it has recently been shown that exposure to Pg-LPS increases the sensitivity of contractile responses mediated by endothelin-1 (ET-1) in cultured rat coronary arteries (Ghorbani *et al*, 2010). Because ET-1 is a strong vasoconstrictor produced by endothelial cells that has been associated with hypertension during pregnancy in both animal and human studies (George and Granger, 2011), this may also be a possible mechanism by which Pg-LPS directly increased systolic blood pressure in pregnant rats in the present study.

Previously, our laboratory established a model for pre-eclampsia (Faas *et al*, 1994) by infusion of low doses of LPS ($1.0 \ \mu g \ kg^{-1}$ bw) of *E. coli* in pregnant rats. These rats developed not only hypertension and a slightly decreased foetal weight, but in contrast to the present study, also proteinuria and generalised inflammation (Faas *et al*, 1994, 1995, 2004). These differences in responses to both LPS species may be due to differences in signalling via TLRs. LPS from *E. coli* signals through TLR4, which leads to predominantly pro-inflammatory cytokine production (Manicassamy and Pulendran, 2009). Indeed, these animals showed a generalised inflammatory response (Faas *et al*, 1994, 1995, 2004). The detrimental effects of this pro-inflammatory response induced by *E. coli* LPS were better observed after infusion of a slightly higher dose of this LPS ($6.5 \ \mu g \ kg^{-1}$ bw), which induced septic-like signs, such as hypotension and maternal illness and increased numbers of foetal resorptions (Faas *et al*, 1994).

In contrast, Pg-LPS signals via TLR2 (Zhang *et al*, 2008), and *in vitro* stimulation of whole blood with Pg-LPS induced a lower pro-inflammatory cytokine production as compared with *in vitro* stimulation with *E. coli* LPS (Kunnen *et al*, 2012). Interestingly, increasing the dose of Pg-LPS from $10.0 \ \mu g \ kg^{-1}$ bw to $50.0 \ \mu g \ kg^{-1}$ bw did not result in a further rise in blood pressure or foetal resorptions, a further decrease in foetal weight or in other septic-like signs as compared with $10.0 \ \mu g \ kg^{-1}$ bw Pg-LPS. Since *in vitro*, this Pg-LPS induced a dose-dependent increase in cytokine production of monocytes (Kunnen *et al*, 2012), suggesting that also in this in vivo study, higher Pg-LPS doses induced higher cytokine production. This lack of dose dependency, therefore, remains to be investigated.

Although various clinical and epidemiological studies suggest a relationship between periodontitis and pregnancy complications, there are not many animal or *in vitro* studies evaluating possible causal mechanisms. The results of the present study indicate that Pg-LPS may be (one of) the causal mechanisms between periodontitis and adverse pregnancy outcomes. By affecting placental growth, Pg-LPS may be one of the contributing factors by which the periodontal infection causes unfavourable pregnancy outcomes including foetal demise and maternal hypertension. The results of the present study do not confirm a causal relationship between periodontitis and pre-eclampsia, for we did not observe albuminuria or inflammation. This study also shows that the effects of Pg-LPS appear relatively mild as compared with effects of *E. coli* LPS, suggesting that *E. coli* LPS may be more harmful for pregnancy than Pg-LPS.

In summary, this study showed that Pg-LPS infusion in pregnant rats increased maternal systolic blood pressure, impaired placental growth and slightly decreased foetal weight and also slightly increased numbers of foetal resorptions. Pg-LPS infusion did not induce albuminuria, a systemic maternal inflammatory response or local inflammation in the kidneys or placentas. The present data provide a next step in the identification of the mechanism in the relationship between periodontitis and adverse pregnancy outcomes. The study undertaken suggests that Pg-LPS may be causally involved in some pregnancy complications, such as hypertension, foetal growth restriction, foetal death or miscarriage (in this rat model represented as foetal resorptions). However, a causal relationship of Pg-LPS with pre-eclampsia seems unlikely from the present study. Further research is therefore needed to identify the exact pathogenic mechanisms by which *P. gingivalis* or its products induce pregnancy complications, including placental and foetal growth restriction or maternal hypertension.

Conflict of interest

The authors report no financial relationship related to any materials or products used in the present study.

Author contributions

A. Kunnen designed and performed experiments, analysed data and wrote the paper. M.G. van Pampus commented on and approved the final manuscript. J.G. Aarnoudse and F. Abbas are supervisors and commented and approved the final manuscript. C.P. van der Schans is supervisor, performed statistics and commented and approved the final manuscript. M.M. Faas is supervisor, designed and supervised experiments, analysed data, wrote the manuscript and approved the final manuscript.

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