Shock, Publish Ahead of Print

DOI : 10.1097/SHK.0000000000001343

LPS-induced hypotension in pregnancy: the effect of progesterone supplementation

Julia Zöllner [1,2,3], Laura G Howe [1,2,3], Lydia F Edey[1,2], Kieran P O'Dea[4], Masao Takata[4], James Leiper [3] and Mark R Johnson [1,2].

[1] Imperial College Parturition Research Group, Chelsea and Westminster Hospital, 369 Fulham Road, London, SW10 9NH, UK.

[2] Imperial College Parturition Research Group, Institute of Reproductive and Developmental Biology, Hammersmith Hospital Campus DuCane Road, London W12 0NN, UK.

[3] MRC Clinical Sciences Centre, Hammersmith Hospital Campus, DuCane Road, London W12 0NN, UK.

[4] Section of Anaesthetics, Pain Medicine, and Intensive Care, Faculty of Medicine, Chelsea and Westminster Hospital, London SW10 9NH, UK

JZ and LH contributed equally to the paper and are joint first authors

Funding: This work was supported by a grant from Imperial College Trust. LH was supported by a studentship from the MRC.

Correspondence to: Dr MR Johnson MBBS, MRCP, MRCOG, PhD, Imperial College Parturition Research Group, Department of Obstetrics and Gynaecology, Imperial College School of Medicine, Chelsea and Westminster Hospital, 369 Fulham Road, London, SW10 9NH, UK.

Telephone: 44 20 88 46 78 78 Fax: 44 20 88 46 77 96

Email: mark.johnson@imperial.ac.uk

Conflict of Interest: none declared.

Abstract

Our previous work has shown that pregnancy exacerbates the hypotensive response to both infection and LPS. The high levels of progesterone (P4) associated with pregnancy have been suggested to be responsible for the pregnancy-induced changes in the cardiovascular response to infection. Here, we test the hypothesis that P4 supplementation exacerbates the hypotensive response of the maternal cardiovascular to LPS.

Female CD1 mice had radiotelemetry probes implanted to measure haemodynamic function non-invasively and were time-mated. From day 14 of pregnancy, mice received either 10mg of P4 or vehicle alone per day and on day 16, intraperitoneal LPS (10μg of serotype 0111:B4) was injected. In two identically treated cohorts of mice, tissue and serum (for RNA, protein studies) were collected at 6 and 12 hours.

Administration of LPS resulted in a fall in blood pressure in vehicle treated, but not P4 supplemented mice. This occurred with similar changes in the circulating levels of cytokines, vasoactive factors and in both circulating and tissue inflammatory cell numbers, but with reduced left ventricular expression of cytokines in P4-supplemented mice. However, left ventricular expression of markers of cardiac dysfunction and apoptosis were similar.

This study demonstrates that P4 supplementation prevented LPS-induced hypotension in pregnant mice in association with reduced myocardial inflammatory cytokine gene expression. These observations suggest that rather than being detrimental, P4 supplementation has a protective effect on the maternal cardiovascular response to sepsis.

Key words: Sepsis, Blood Pressure, Innate Immune System, parturition, pup death,

Introduction

Pregnant women are more vulnerable to infection, with a three times greater risk of dying from flu or malaria [1] and with genital tract sepsis being the leading cause of direct maternal mortality in the recent CSMED report [2]. This may relate to pregnancy-induced changes in the cardiovascular system or to an excessive response of the innate immune system to infection. Previously, we reported that the administration of the bacterial wall component, lipopolysaccharide (LPS), to pregnant mice resulted in marked hypotension, but had no effect in non-pregnant mice [3]. We extended these findings using caecal ligation and puncture (CLP), showing that pregnant mice had a more marked hypotensive response and a higher mortality [4]. Earlier, in a mouse model of inflammation-induced preterm labour (PTL) based on an intrauterine injection of LPS, Elovitz and Mrinalini reported that progesterone (P4) supplementation, in the form of 17-alpha hydroxyprogesterone caproate [17-OHPC], was associated with a dose dependent increase in maternal mortality of between 50-75% [5]. These data suggest that the greater susceptibility to sepsis may be related to a progesterone-induced effect on the cardiovascular or immune response to infection.

Hormonal control of blood pressure during pregnancy is relatively poorly understood. Gonadal steroids affect blood vessel function, oestrogens promoting vasodilatation through the production of nitric oxide, prostacyclin and prostaglandin E2 (PGE_2) [6-8] and P4 having inconsistent effects depending on the tissue and the level of exposure [9-11]. However, P4 seems to predominantly lower blood pressure as shown by Barbagallo *et al* (2001), who demonstrated that intravenous P4 blunted the hypertensive response to noradrenaline infusion in anaesthetised rats and reduced agonist-induced vessel constriction in rat aorta and tail artery strips [12]. In hypertensive subjects, oral P4 treatment reduced blood pressure [13].

These studies were conducted in non-pregnant animals/subjects and it is possible that in the context of pregnancy, where progesterone levels are already high, that the administration of exogenous P4 may modulate the cardiovascular response to LPS.

In terms of the inflammatory response, P4 has also been shown to directly influence the shift in immunity to Th2-type responses at the maternal-fetal interface by suppression of T-cell differentiation into Th1 cells, thus contributing to the development of maternal immune tolerance of the feto-placental unit [14]. P4 supplementation, given to reduce the risk of spontaneous PTL, appears to repress the response to recall antigens [15]. Further, we have shown that P4 represses inflammation in human myometrial cultures, acting via the glucocorticoid receptor (GR) [16]. Consequently, the greater maternal mortality seen in mice pre-treated with 17-OHPC was surprising, particularly its association with higher circulating levels of IL-6 [5].

Currently, 17-OHPC is recommended by the American College of Obstetricians and Gynecologists for the prevention of PTL in singleton pregnancies, but doubts remain about the potential adverse effects of 17-OHPC [17]. In contrast, natural progestogens have not been reported to have adverse effects in animal or human studies and are widely used for the prevention of PTL in high-risk women, despite inconsistent evidence. However, since P4 alters both the maternal immune and cardiovascular systems, it is possible that it could change the maternal response to infection, potentially making women more susceptible to sepsis. In an earlier study, we reported that LPS administration induced a greater hypotensive response in pregnant compared to non-pregnant mice [3]. In this study, we have tested the hypothesis that P4 supplementation exacerbates this hypotensive response in pregnant mice and investigated potential causative mechanisms, studying aspects of the innate immune and the cardiac responses to LPS. The results of this study may help us understand the greater

susceptibility to sepsis exhibited by pregnant women and identify a potential causative mechanism. They may also provide a further reason to withhold P4 supplementation in women at risk of PTL, given the failure of the Opptimum study, the largest randomised study to date, to show any benefit [18].

Materials and Methods

Female CD1 mice (Charles River, UK) aged 8-12 weeks were used for all experiments. All animal procedures were carried out in accordance with the Home Office Animals (Scientific Procedures) Act, 1986 (PPL 70/7372), and were previously approved by the Animal Welfare and Ethical Review Board (AWERB) at Imperial College London. All mice were housed in individually ventilated cages with access to normal rodent chow and water ad libitum, and maintained on a 12:12 light-dark cycle.

Time-mating: Female mice were placed overnight into cages with male studs. The following morning the female mice were inspected for a copulatory plug. The day the plug was detected was designated as gestational day 0 (E0). From previous work in our group, we defined E16 to be equivalent to 34 weeks gestation of a human pregnancy. All experiments in pregnant animals were conducted on day 16 or pregnancy (E16).

Mouse blood pressure telemetry: Telemetry probes (PA-C10, Data Sciences International) were surgically implanted. The technique allows continuous measurements of blood pressure, heart rate and activity to be made. Mice were anaesthetised using isoflurane (5% for induction and 2% maintenance of isoflurane, 2L/min O2 flow rate), and surgically cleaned and draped. Pre-operatively, mice received 5% enrofloxacin and 0.3mg/mL buprenorphine. A small ventral incision on the neck was made and the left common carotid artery isolated prior

to cannulation. The catheter was advanced until placed in the aortic arch and secured in place with sutures. The transmitter body was then advanced subcutaneously to lie over the right flank before the incision was closed. Mice were then placed into a heated recovery chamber (30^{o}C). Buprenorphine was given the day after surgery and animals were allowed to recover for a minimum of one week prior to any experiments.

All experiments included light and dark periods to account for diurnal variations. Following recovery, continuous 24 hour recording (data collection) began using the Dataquest ART Acquisition System (Data Sciences International, v4.1) for at least 48 hours to collect data in non pregnant (NP) animals (NP baseline). These data allowed animals to act as their own controls. Radio-telemeters were magnetically activated and deactivated. Whilst recording, animals were kept in their standard cages with access to food and water ad libitum. These cages were placed in a separate room to ensure a calm environment for recording.

For this study, data were collected for 24 hours after LPS administration from NP and pregnant mice at E16 and compared. The data collected included mean arterial pressure (MAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), heart rate (HR), and activity. Haemodynamic data were collected for 10 seconds every 30 seconds. Labour time (latency to labour after intraperitoneal (i.p.) injection of LPS or control) and litter sizes were also recorded. Accurate labour time was enabled by the use of infrared cameras in conjunction with the AVerDiGi (NV3000 Series) HYBRID Surveillance Platform (RF Concepts, Dundonald, UK).

LPS administration: LPS endotoxin (serotype 0111:B4, Sigma-Aldrich) or sterile vehicle control phosphate buffered saline (PBS) were administered i.p. on day 16 of gestation (E16).

The dose of LPS was firstly determined using a dose-response study in CD1 females on E16 using a range of doses indicated by the intrauterine work in our group and current literature. A single injection of 10μg LPS per dam (in 100μL PBS) gave the most consistent outcomes in time to labour from LPS and causing no maternal mortality (see supplementary data Fig. 1, http://links.lww.com/SHK/A858). All pregnant mice were anaesthetised under isoflurane for i.p. injection to minimise potential harm to fetuses and carried out between 8am and 10am.

Progesterone Treatment: In those experiments examining the role of progesterone, P4 (Sigma-Aldrich, Poole, UK) was dissolved in peanut oil (Sigma-Aldrich, Poole, UK) at a stock concentration of 250mg/mL. A final concentration of 10mg per mouse (in 40μL peanut oil) was injected subcutaneously (s.c.) into the right flank. P4 or vehicle (peanut oil) injections were carried out once a day from E14 to E16 between 8am-10am.

Time points: To avoid effects of handling animals during telemetry recordings, serum and other tissues were taken from separate groups of animals at two time points, 6 hours and 12 hours post i.p. LPS injection or vehicle control. Mice were terminally anaesthetised using 5% isoflurane (2L/min O2 flow rate) and a cardiac puncture was performed to collect plasma or serum samples. Subsequent to schedule 1 culling, lung, heart, left ventricle, myometrium and placenta were collected and snap frozen on dry ice. These samples were then stored at -80^{o}C until RNA and protein extraction.

Flow cytometry: Fluorescence-activated cell sorting (FACS) technique was used to further investigate the impact of P4 supplementation on the innate immune response to LPS. Monocyte and neutrophil numbers were analysed in the blood, myometrium, placenta, lung and liver. Macrophage numbers were studied in uterus and placenta only. The detailed

methods have been described previous [4] and the antibodies used listed in Supplementary table 1, http://links.lww.com/SHK/A861.

Multiplex Assay: Mouse cytokine/chemokine or angiogenesis/growth factor magnetic bead panel (Merck Millipore) were used according to manufacturer's protocol. Analytes were quantified using a Luminex MAGPIX instrument with xPonent 4.2 software (Luminex Corp).

RNA extraction and quantitative RT-PCR: RNeasy mini kit by Qiagen Ltd. (Crawley) was used to extract RNA from the whole heart and from a second series of animals from the left ventricle only following the manufacturer's instruction. Less than 20mg of tissue was used. To convert the extracted RNA to cDNA, 6μg of the RNA was reverse transcribed using oligo dT random primers using MuLV transcriptase (Applied Biosystems Ltd.). A unique set of primers (such as *Anp*, *Bnp*, *Vegf*, *eNos* etc.) specific to mouse were designed and obtained from Life Technologies Ltd. (Paisley, UK, see supplementary table 2, http://links.lww.com/SHK/A862). These were validated prior to their use. Quantitative real-time PCR was done using SYBR Green (Roche Diagnostics Ltd.) and amplicon yield monitored by Rotor Gene R-G 3000 (Corbett research Ltd.). The results of the PCR analysis were expressed relative to the constitutively expressed ribosomal protein s18.

Statistical Analysis

Data collected in the 24 hour period after LPS injections were shown as hourly mean values ± SEM as a change (Δ) from baseline alongside area under the curve (AUC) analysis, maximum increase and maximum decrease (where appropriate). Normally distributed data were analysed using t test or ANOVA with correction for multiple comparisons as indicated. Where the data were not normally distributed as determined by Kolmogorov-Smirnoff tests,

non-parametric equivalent tests such as Kruskal-Wallis followed by Dunn's post-hoc test, or Mann-Whitney U were employed. Differences in the cardiovascular parameters at different time points throughout pregnancy were analysed by repeated measures ANOVA followed by Bonferroni correction for multiple comparisons between the means. Two-way ANOVA followed by Bonferroni correction was used to compare pup survival and latency to delivery after LPS.

Results

Labour Time and Pup Survival: To explore the effect of P4 supplementation on maternal haemodynamic and inflammatory pathways, a dose-response using increasing concentrations of P4 was performed. Previous work in the group (Lei *et al*. unpublished data) demonstrated that progesterone levels could be elevated 10-fold (from approximately 400nmol/L on E16) by daily subcutaneous administration of 10mg P4 in pregnant CD1 mice. Also, in a previous paper, we measure circulating P4 levels in response to 2mg of P4 administered on alternate days, this was able to maintain blood levels at approximately stable levels vs. E13 levels [19] and prevent the mice from labouring if daily injections were continued through to E18. In this study, daily subcutaneous injections on gestational days E14-E16 of peanut oil (control: vehicle), 5mg or 10mg P4 were given to raise P4 concentrations for 48hr prior to LPS administration. In the initial experiments, 5mg/day P4 delayed the onset of labour after LPS administration on E16 compared to controls (52.92 ± 13.21 hours vs. 22.46 ± 1.47 hours respectively), however latency to labour was only significantly increased compared to controls using 10mg/day P4 (95.33 ± 7.76 hours, $p<0.001$; Supplementary Figure 1A, http://links.lww.com/SHK/A858). Pup survival was increased using 5mg/day P4 compared to vehicle (61.54 ± 31.09 % vs. 0 %), but fell to 10 ± 10 % using 10mg/day P4 (Supplementary Figure 1B, http://links.lww.com/SHK/A858). As 10mg/day was able to significantly delay latency to labour this dose was chosen for use in all subsequent experiments.

Latency to labour was recorded throughout the experiments using telemetered mice to monitor blood pressure after supplementation with P4 (Supplementary Figure 1C, http://links.lww.com/SHK/A858). P4 supplementation reproducibly increased latency to labour after LPS ($p<0.001$, 98 ± 11.8 hours vs. 21.5 ± 2.07 hours). However, in this cohort of animals, no pup survival was recorded at labour in either control or P4 treatment groups (Supplementary Figure 1D, http://links.lww.com/SHK/A858). At the intermediate time-points after LPS administration, pup survival significantly decreased from 56.9 ± 13.5 % and 50.5 ± 15.8 % for control and P4 respectively at 6 hours, to 6.67 ± 6.67 % and 7.5 ± 7.5 % respectively at 12 hours ($p<0.001$). P4 treatment had no impact on pup survival (Supplementary Figure 1D, http://links.lww.com/SHK/A858). The study design is shown in supplementary figure 2, http://links.lww.com/SHK/A859 illustrating how the design mimics the management of a woman at high risk of preterm labour treated with supplementary P4 (Supplementary 2, http://links.lww.com/SHK/A859).

The Haemodynamic Response to LPS-induced: Haemodynamic and activity data were collected from telemetered CD1 mice. Mice were given 10mg P4 or vehicle (peanut oil control) via the subcutaneous route daily from E14-E16 followed by i.p. LPS on E16. A decline in MAP was observed in animals treated with vehicle in the first 12 hours post-LPS (n=3, Figure 1A) and consistent with our previous work [3]. A nadir was observed at 9 hours post-LPS when MAP dropped to 101.09 ± 9.9 mmHg from a pre-LPS baseline of 115.54 ± 6.1 mmHg (Δ-14.5 ± 6.2). P4-treated animals maintained their MAP without any hypotensive response (n=4, Δ-1.56 ± 4.44 at 9 hours post-LPS). However, analysis using 2-way ANOVA did not indicate any significant differences between the treatment groups. Similar changes in blood pressure were also reflected in SAP (Figure 1B). In vehicle-treated animals a nadir was observed at 11 hours post-LPS when SAP dropped to 118.2 ± 15.7 mmHg from a pre-LPS

baseline of 131.37 ± 13.9 mmHg (Δ-13.18 ± 7.9). No hypotensive response was seen in P4-treated animals (Δ-1.51 ± 4.47 at 11 hours post-LPS). Less marked changes were observed in DAP (Figure 1C). From 12-24 hours post-LPS there appeared to be a gradual return to baseline in all three parameteres in the vehicle-treated group, however P4-treated animals exhibited a slight increase in blood pressure above baseline pre-LPS levels. This was more pronounced in DAP where the peak pressure was 89.79 ± 2.8 mmHg compared to pre-LPS baseline of 76.78 ± 2.8 mmHg (Δ13.01 ± 3.39) at 19 hours post-LPS. No rise above baseline was observed in the vehicle-treated group (99.3 ± 7.4 mmHg at 19 hours vs. 99.59 ± 1.1 at baseline). The peanut oil vehicle itself was not thought to affect the haemodynamic profile of E16 mice as a similar response was observed in mice treated with LPS alone [3]. In the vehicle-treated group, there was a small reduction in HR, however due to the large variability of HR between animals this did not reach significance, therefore there was no signficiant change in heart rate in either treatment group after LPS (Figure 1D). P4-treatment had no significant effect on the %TSI and activity displayed by CD1 mice in the 24 hours after LPS administration (Figure 1E). There was no significant change from baseline observed in either treatment group in the %TSI, although there was a slight reduction in the last three hours observed (Figure 1E). As labour would have occurred in the PO-treated animals, this could account for the increase in activity at this point, however, as this was not true of P4-treated animals, it may be that the effects of the LPS was wearing off (Figure 1E). These results are also reflected in a slight increase in activity at 18-23 hours post-LPS, although the increase was not significant (Figure 1E). The intenstity of activity, as described by activity >0, also remained unchanged from baseline despite a sharp and unexplained drop in activity >0 in both groups at 3 hours post-LPS (Figure 1E). These results indicate that altered activity are not likely to be responsible for any differences in blood pressure in P4-treated mice. Area under the curve (AUC) analyses showed no significant changes between P4- and vehicle-

treated animals for either MAP, SAP, DAP or HR (Supplementary Figure 3A, D, G & H, http://links.lww.com/SHK/A860). However, the maximum decrease in SAP was significantly reduced in P4 treated mice ($p<0.05$; Supplementary Figure 3E, http://links.lww.com/SHK/A860).

Circulating Cytokine, Nitric Oxide, Asymmetric Dimethylarginine and VEGF levels: The maternal serum levels of cytokines were measured in samples obtained from vehicle and P4-treated mice at 6 and 12 hours after LPS i.p (n=6 in each group). There were no significant differences in any of the pro- or anti-inflammatory cytokines measured between P4 and vehicle-treated mice (Figure 2A-C), suggesting that P4 supplementation did not change the response to LPS in pregnant mice. Similar results were found for the chemokines (CCL2, CCL5, CXCL1, CXCL2) and anti-inflammatory cytokines measured in these samples (Figure 2D-I). Circulating NO, ADMA and VEGF levels were not significantly different between P4-treated mice and controls (Figure 3A-C).

Left ventricular mRNA expression of cytokines, markers of cardiac dysfunction and apoptosis: In contrast to the lack of change in circulating cytokine levels, cytokine mRNA expression in left ventricular tissue of *IL-1β*, at 6 hours, but not 12 hours ($p<0.05$, Figure 4A) and *TNF-α*, at 6 hours and 12 hours ($p<0.01$ and $p<0.001$, Figure 4C), was significantly reduced after P4-supplementation. There were no differences in *IL-6* or *IL-10* expression in any treatment group (Figure 4B & D). Markers of myocardial dysfunction were not significantly different after P4 supplementation (Figure 4E-G). The expression of *Nkx2.5*, a transcription factor implicated in cardiac development and function [20], was significantly greater after P4 supplementation ($p<0.05$, Figure 4H). The expression of the markers of apoptosis was unchanged after P4-supplementation (Figure 4I-K). We studied the expression of LPS-binding protein cysteine-rich secretory protein containing LCCL domain 2 (*Crispld2*)

in myocardial samples as recent evidence suggests that P4 up-regulates its expression and that it protects mice from LPS-induced mortality [21-23]. Surprisingly, *Crispld2* expression was significantly reduced in P4 treated compared to control mice ($p<0.05$; Figure 4L).

Leukocyte densities: Baseline levels of leukocytes were similar in the blood and all tissues in E16 mice with and without P4 supplementation (Fig.5). The changes in response to LPS were also similar in each group and to those we have reported before [3]. In summary, circulating neutrophils increased and monocytes declined, while tissue levels of both cell types increased (Fig.5).

Discussion

P4 supplementation prevented the LPS-induced hypotensive response observed in control animals and although there appeared to be no differences in the systemic cytokine and inflammatory cellular response to LPS, left ventricle mRNA expression of *IL-1β* and *TNF-α* were significantly down regulated after P4-treatment, suggesting that the hypotensive effect of LPS may be mediated through a negative effect on cardiac function and that P4 ameliorates this. These data suggest that P4 supplementation in pregnant mice certainly does not exacerbate the hypotensive response to LPS observed in pregnant animals [3] and, actually contrary to our hypothesis that P4 would exacerbate the haemodynamic response to LPS, it appears to exert a protective effect on LPS-induced hypotension.

P4 supplementation did not influence the response of the innate immune system either in terms of leukocyte dynamics or in terms of the circulating levels of pro- or anti-inflammatory cytokines. This is surprising given the well-documented immune-modulatory and anti-inflammatory effects of P4, for example in the shift from Th1 to Th2 dominance in pregnancy [24-27] and the repression of macrophage LPS-induced TNFα release [28]. However,

although we found no significant difference in the circulating inflammatory cytokine levels, in the left ventricle, P4 supplementation reduced the LPS-induced increase in *IL-1β* and *TNFα* mRNA expression. In macrophages, P4-repression of LPS-induced TNFα release was mediated through an increase in IκBα levels, an endogenous inhibitor of NFκB activation [28] and we have shown previously that P4 represses both NFκB and AP-1 activation in human myometrial explants [29]. Collectively, these data, suggest that P4 may exert a differential effect in tissues and inflammatory cells. Indeed, we have observed a similar repressive effect using a lower dose of P4 on LPS-induced myometrial inflammation (Herbert et al, unpublished observation), suggesting that P4 does repress LPS-induced inflammation at the tissue level. Perhaps inflammatory cells are less responsive to P4 during pregnancy. Interestingly, Jain *et al* (2004) showed that P4 treatment of a human pro-monocytic cell line (U937) actually induced a two-fold increase in basal TNF-α secretion [30]. Interestingly, Elovitz and Wang found that 2mg progesterone (per mouse) did not significantly delay the onset of labour induced by intra-uterine LPS, while 1mg MPA (per mouse), which has both glucocorticoid and progestational effects, was able to delay labour onset and reduce pup mortality [31]. These data suggest that P4 may exert only a limited anti-inflammatory effect. However, when we used 5mg of P4 in the same model, although we did not see any delay in LPS-induced labour onset, we did see a reduction in inflammatory cytokine mRNA and protein levels (Herbert et al, unpublished observation), suggesting that P4 does exert some anti-inflammatory effect at the tissue level.

Since Elovitz and Mrinalini reported that the administration of 17-OHPC, but not medroxyprogesterone acetate (MPA) was associated with increased maternal mortality using the IU LPS model of PTL [5], we chose to assess the affect of the widely prescribed "natural" progesterone in the current study. Neither 17-OHPC or MPA exactly resemble progesterone,

17-OHPC is a purely synthetic compound, that has been associated with an increase risk of gestational diabetes and stillbirth [32], and MPA has both glucocorticoid and progestotogenic effects. Further, while vaginal progesterone has been shown to have anti-inflammatory effects at the choriodecidual interface in a murine model, sub-cutaneous 17-OHPC did not [33]. Nor did 17-OHPC repress basal or LPS-stimulated TNFα or COX-2 production in human myometrial explants [34]. In terms of *in vitro* contractility studies on strips of human myometrium, 17-OHPC enhanced oxytocin-induced contractions in one study [35] and to have no effect in several other studies [36-38]. These data suggest that there are marked differences between 17-OHPC and progesterone and question how 17-OHPC might act to reduce the risk of PTL.

The ability of P4 to prevent LPS-induced hypotension in association with a reduction in myocardial inflammatory cytokine expression suggests that LPS may cause hypotensive through a repressive effect on cardiac function as has been described in septic shock [39]. However, in our earlier study, where we first described the marked hypotensive effect of LPS in pregnant as compared to non-pregnant mice, we found that LPS-induced increased inflammatory cytokine gene expression in LV samples [3] and, in this study, although we observed differences in cytokine mRNA levels, they were not associated with a reduction in markers of apoptosis or cardiac dysfunction. These data question whether cardiac dysfunction is the cause of the hypotension. Cardiac echo may have helped to clarify whether there was any evidence of cardiac dysfunction, but we did not have access to this.

Circulating NO levels were not different between groups, this may be because P4 has opposing actions on NO production in different sites. In macrophages, P4 appears to repress NO production [40], however, these experiments were undertaken in macrophage cell lines and mouse bone marrow culture-derived macrophages *in vitro*. While in endothelial cells, P4

alone and incombination with oestradiol enhances NO production [41]. These opposing effects may explain the lack of change in circulating NO levels after P4 supplementation.

The expression of *Crispld2* in P4-treated mice was signficantly decreased compared to PO controls. This result was surprising, as in previous studies, performed in mice treated with P4 for 6 hours, *Crispld2* expression in the uterus was increased [23]. This group also demonstrated that P4 regulation of *Crispld2* is regulated by the P4 receptor (PR) as *Crispld2* was signficantly increased upon treatment with P4 only in WT mice and not in PR knockouts. However, the expression of *Crispld2* may be regulated differently in the heart or prolonged P4 stimulation may reduce PR levels and/or increase PR chaperone proteins altering the response to P4 [42]. Indeed, there is limited evidence to suggest that the classic nuclear PR (nPR) is downregulated at very high concentrations of P4, but the mPRα has been shown to undergo endocytosis after P4 stimulation via a clathrin-mediated pathway [43, 44]. Further work to define which PR are expressed in the heart would help to clarify P4 action on cardiac *Crispld2* expression.

Taken together, these results are reassuring that P4 supplementation does not exaccerbate the LPS-induced hypotension reported in our previous study [3]. Consequently, they suggest that P4 supplementation is not likely to increase maternal mortality or morbidity in the context of infection. However, we used progesterone in this study and not 17-OHPC, which may have a very different effect as suggested by the work of Elovitz et al [5]. Further, these data suggest that P4 supplementation may afford some haemodynamic protection to pregnant individuals, perhaps through a repressive effect on cardiac inflammation.

References

1. Damber MG, von Schoultz B, Stigbrand T: The immunological paradox of pregnancy. *Acta Obstet Gynecol Scand Suppl* 66:39-47, 1977.
2. Cantwell R, Clutton-Brock T, Cooper G, Dawson A, Drife J, Garrod D, Harper A, Hulbert D, Lucas S, McClure J, Millward-Sadler H, Neilson J, et al: Saving Mothers' Lives: Reviewing maternal deaths to make motherhood safer: 2006-2008. The Eighth Report of the Confidential Enquiries into Maternal Deaths in the United Kingdom. *BJOG* 118 Suppl 1:1-203, 2011.
3. Zollner J, Howe LG, Edey LF, O'Dea KP, Takata M, Gordon F, Leiper J, Johnson MR: The response of the innate immune and cardiovascular systems to LPS in pregnant and nonpregnant mice. *Biol Reprod* 2017; 97:258-272.
4. Zollner J, Lambden S, Nasri NM, Leiper J, Johnson MR: Rapid onset of severe septic shock in the pregnant mouse. *Biol Reprod* 100:505-513.
5. Elovitz MA, Mrinalini C: The use of progestational agents for preterm birth: lessons from a mouse model. *Am J Obstet Gynecol* 195:1004-1010, 2006.
6. Cannoletta M, Cagnacci A: Modification of blood pressure in postmenopausal women: role of hormone replacement therapy. *Int J Womens Health* 6:745-757, 2014.
7. Chakrabarti S, Morton JS, Davidge ST: Mechanisms of estrogen effects on the endothelium: an overview. *Can J Cardiol* 30:705-712, 2014.
8. Darblade B, Pendaries C, Krust A, Dupont S, Fouque MJ, Rami J, Chambon P, Bayard F, Arnal JF: Estradiol alters nitric oxide production in the mouse aorta through the alpha-, but not beta-, estrogen receptor. *Circ Res* 90:413-419, 2002.
9. Jiang CW, Sarrel PM, Lindsay DC, Poole-Wilson PA, Collins P: Progesterone induces endothelium-independent relaxation of rabbit coronary artery in vitro. *Eur J Pharmacol* 211:163-167, 1992.
10. Omar HA, Ramirez R, Gibson M: Properties of a progesterone-induced relaxation in human placental arteries and veins. *J Clin Endocrinol Metab* 80:370-373, 1995.
11. Wenner MM, Stachenfeld NS: Blood pressure and water regulation: understanding sex hormone effects within and between men and women. *J Physiol* 590:5949-5961, 2012.
12. Barbagallo M, Dominguez LJ, Licata G, Shan J, Bing L, Karpinski E, Pang PK, Resnick LM: Vascular Effects of Progesterone : Role of Cellular Calcium Regulation. *Hypertension* 37:142-147, 2001.
13. Rylance PB, Brincat M, Lafferty K, De Trafford JC, Brincat S, Parsons V, Studd JW: Natural progesterone and antihypertensive action. *Br Med J* (Clin Res Ed) 290:13-14, 1985.
14. Miyaura H, Iwata M: Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. *J Immunol* 168:1087-1094, 2002.
15. Shah NM, Imami N, Johnson MR: Progesterone Modulation of Pregnancy-Related Immune Responses. *Front Immunol* 9:1293, 2018.
16. Lei K, Georgiou EX, Chen L, Yulia A, Sooranna SR, Brosens JJ, Bennett PR, Johnson MR: Progesterone and the Repression of Myometrial Inflammation: The Roles of MKP-1 and the AP-1 System. *Mol Endocrinol* 29:1454-1467, 2015.
17. Calda P: Safety signals of 17-OHP-C use in pregnancy and efficacy in the prevention of preterm birth. *J Matern Fetal Neonatal Med* 22:540-542, 2009.
18. Norman JE, Marlow N, Messow CM, Shennan A, Bennett PR, Thornton S, Robson SC, McConnachie A, Petrou S, Sebire NJ, Lavender T, Whyte S, et al: Vaginal

progesterone prophylaxis for preterm birth (the OPPTIMUM study): a multicentre, randomised, double-blind trial. *Lancet* 387:2106-2116, 2016.
19. Edey LF, Georgiou H, O'Dea KP, Mesiano S, Herbert BR, Lei K, Hua R, Markovic D, Waddington SN, MacIntyre D, Bennett P, Takata M, et al: Progesterone, the maternal immune system and the onset of parturition in the mouse. *Biol Reprod* 98:376-395, 2018.
20. Zakariyah AF, Rajgara RF, Veinot JP, Skerjanc IS, Burgon PG: Congenital heart defect causing mutation in Nkx2.5 displays in vivo functional deficit. *J Mol Cell Cardiol* 105:89-98, 2017.
21. Wang T, Wang ZQ, Wang L, Yan L, Wan J, Zhang S, Jiang HQ, Li WF, Lin ZF: CRISPLD2 is expressed at low levels during septic shock and is associated with procalcitonin. *PLoS One* 8:e65743, 2013.
22. Wang ZQ, Xing WM, Fan HH, Wang KS, Zhang HK, Wang QW, Qi J, Yang HM, Yang J, Ren YN, Cui SJ, Zhang X, et al: The novel lipopolysaccharide-binding protein CRISPLD2 is a critical serum protein to regulate endotoxin function. *J Immunol* 183:6646-6656, 2009.
23. Yoo JY, Shin H, Kim TH, Choi WS, Ferguson SD, Fazleabas AT, Young SL, Lessey BA, Ha UH, Jeong JW: CRISPLD2 is a target of progesterone receptor and its expression is decreased in women with endometriosis. *PLoS One* 9:e100481, 2014.
24. Piccinni MP, Giudizi MG, Biagiotti R, Beloni L, Giannarini L, Sampognaro S, Parronchi P, Manetti R, Annunziato F, Livi C, et al: Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *J Immunol* 155:128-133, 1995.
25. Piccinni MP, Scaletti C, Maggi E, Romagnani S: Role of hormone-controlled Th1- and Th2-type cytokines in successful pregnancy. *J Neuroimmunol* 109:30-33, 2000.
26. Raghupathy R: Pregnancy: success and failure within the Th1/Th2/Th3 paradigm. *Semin Immunol* 13:219-227, 2001.
27. Romagnani S: The Th1/Th2 paradigm. *Immunol Today* 18:263-266, 1997.
28. Miller L, Hunt JS: Regulation of TNF-alpha production in activated mouse macrophages by progesterone. *J Immunol* 160:5098-5104, 1998.
29. Georgiou EX, Lei K, Lai PF, Yulia A, Herbert BR, Castellanos M, May ST, Sooranna SR, Johnson MR: The study of progesterone action in human myometrial explants. *Mol Hum Reprod* 22:877-889, 2016.
30. Jain SK, Kannan K, Prouty L, Jain SK: Progesterone, but not 17beta-estradiol, increases TNF-alpha secretion in U937 monocytes. *Cytokine* 26:102-105, 2004.
31. Elovitz M, Wang Z: Medroxyprogesterone acetate, but not progesterone, protects against inflammation-induced parturition and intrauterine fetal demise. *Am J Obstet Gynecol* 190:693-701, 2004.
32. Romero R, Stanczyk FZ: Progesterone is not the same as 17alpha-hydroxyprogesterone caproate: implications for obstetrical practice. *Am J Obstet Gynecol* 208:421-426, 2013.
33. Furcron AE, Romero R, Plazyo O, Unkel R, Xu Y, Hassan SS, Chaemsaithong P, Mahajan A, Gomez-Lopez N: Vaginal progesterone, but not 17alpha-hydroxyprogesterone caproate, has antiinflammatory effects at the murine maternal-fetal interface. *Am J Obstet Gynecol* 213:846.e841-846.e819, 2015.
34. Patel S, Li A, Goodwin TM, Brower M, Blitz M, Minoo P, Felix JC, Lee RH: Effect of 17-alpha hydroxyprogesterone caproate on the production of tumor necrosis factor-

alpha and the expression of cyclooxygenase-2 in lipopolysaccharide-treated gravid human myometrial explants. *J Perinatol* 30:584-589, 2010.
35. O'Sullivan MD, Hehir MP, O'Brien YM, Morrison JJ: 17 alpha-hydroxyprogesterone caproate vehicle, castor oil, enhances the contractile effect of oxytocin in human myometrium in pregnancy. *Am J Obstet Gynecol* 202:453.e451-454, 2010.
36. Anderson L, Martin W, Higgins C, Nelson SM, Norman JE: The effect of progesterone on myometrial contractility, potassium channels, and tocolytic efficacy. *Reprod Sci* 16:1052-1061; 2009.
37. Ruddock NK, Shi SQ, Jain S, Moore G, Hankins GD, Romero R, Garfield RE: Progesterone, but not 17-alpha-hydroxyprogesterone caproate, inhibits human myometrial contractions. *Am J Obstet Gynecol* 199:391.e391-397, 2008.
38. Sexton DJ, O'Reilly MW, Friel AM, Morrison JJ: Functional effects of 17alpha-hydroxyprogesterone caproate (17P) on human myometrial contractility in vitro. *Reprod Biol Endocrinol* 2:80, 2004.
39. Pulido JN, Afessa B, Masaki M, Yuasa T, Gillespie S, Herasevich V, Brown DR, Oh JK: Clinical spectrum, frequency, and significance of myocardial dysfunction in severe sepsis and septic shock. *Mayo Clin Proc* 87:620-628, 2012.
40. Miller L, Alley EW, Murphy WJ, Russell SW, Hunt JS: Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. *J Leukoc Biol* 59:442-450, 1996.
41. Pang Y, Thomas P: Additive effects of low concentrations of estradiol-17beta and progesterone on nitric oxide production by human vascular endothelial cells through shared signaling pathways. *J Steroid Biochem Mol Biol* 165:258-267, 2017.
42. Echeverria PC, Picard D: Molecular chaperones, essential partners of steroid hormone receptors for activity and mobility. *Biochim Biophys Acta* 1803:641-649, 2010.
43. Foster H, Reynolds A, Stenbeck G, Dong J, Thomas P, Karteris E: Internalisation of membrane progesterone receptor-alpha after treatment with progesterone: Potential involvement of a clathrin-dependent pathway. *Mol Med Rep* 3:27-35, 2010.
44. Gellersen B, Fernandes MS, Brosens JJ: Non-genomic progesterone actions in female reproduction. *Hum Reprod Update* 15:119-138, 2009.

Legends

Figure 1 (A-E): The haemodynamic response to LPS-induced inflammation after progesterone supplementation. The haemodynamic response in CD1 mice recorded 24-hours after LPS administration following either vehicle or P4 treatment daily from E14. (A) ΔMAP, (B) ΔSAP, (C) ΔDAP, (D) ΔHR and (E) Δ%TSI. Data are expressed as mean change from pre-LPS baseline (pre) ± SEM (n=3-4). Veh- vehicle, peanut oil; P4- progesterone.

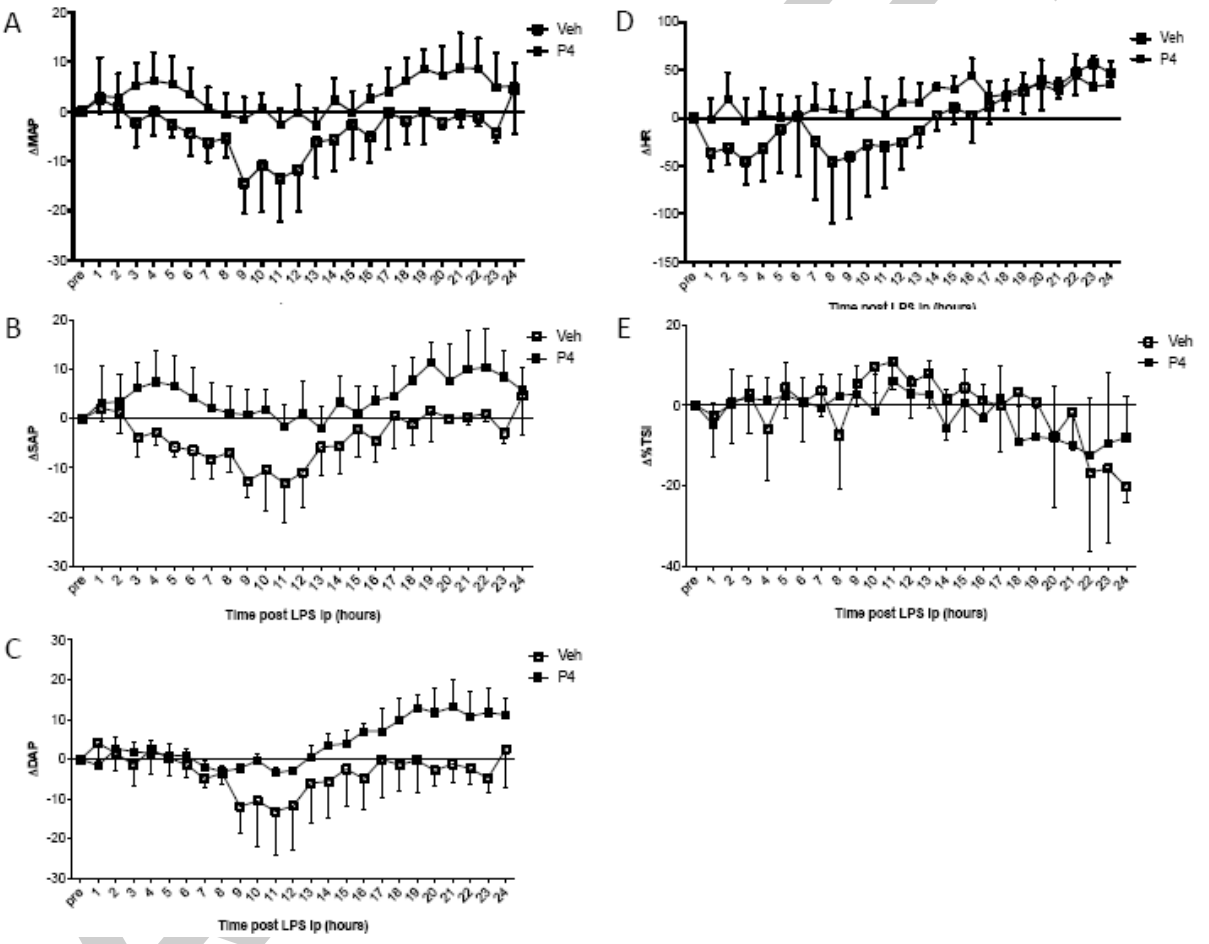

Figure 2: **Circulating concentrations of cytokines in the serum of pregnant mice treated with LPS after supplementation with progesterone (P4) or vehicle (Veh)**. Measurements were made in serum samples taken 6 or 12 hours after LPS in PO- or P4-treated mice. (A) IL-1β (B) TNF-α, (C) IL-6, (D) CCL2 (E) CCL5 (F) CXCL1 (G) CXCL2, (H) IL-4 and (I) IL-10. Data are expressed as median ± interquartile range (n=6) and were compared with a paired t test or Willcoxon matched pairs test depending on the data distribution. PO- vehicle, peanut oil; P4- progesterone

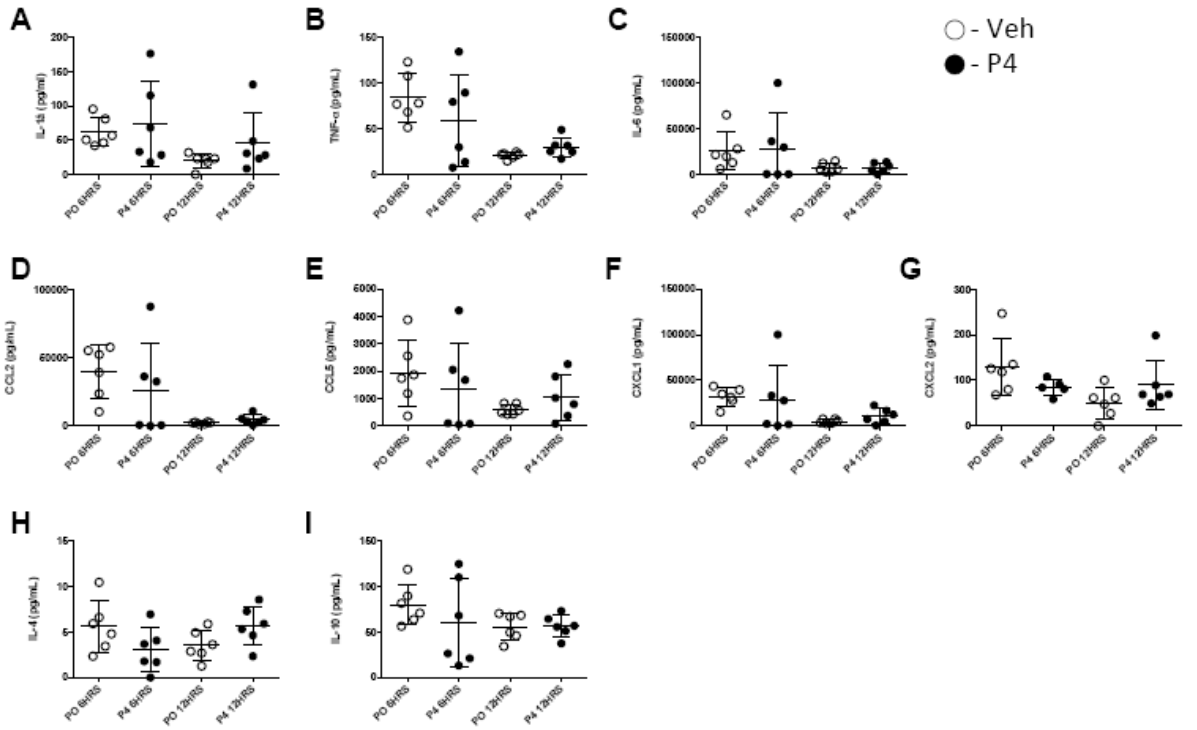

Figure 3: **Circulating concentrations of nitric oxide, asymmetric dimethylarginine and VEGF in the serum of pregnant mice treated with LPS after supplementation with progesterone or vehicle**. Measurements were made in serum samples taken 6 or 12 hours after LPS in vehicle or P4-treated mice. (A) Concentration of total nitrates and nitrites (NOx) as measured by nitric oxide analyser (B) ADMA as measured by mass spectrometry and (C) VEGF measured in a multiplex assay. Data are expressed as median ± interquartile range (n=6) and were compared with a paired t test or Willcoxon matched pairs test depending on the data distribution. PO- vehicle, peanut oil; P4- progesterone.

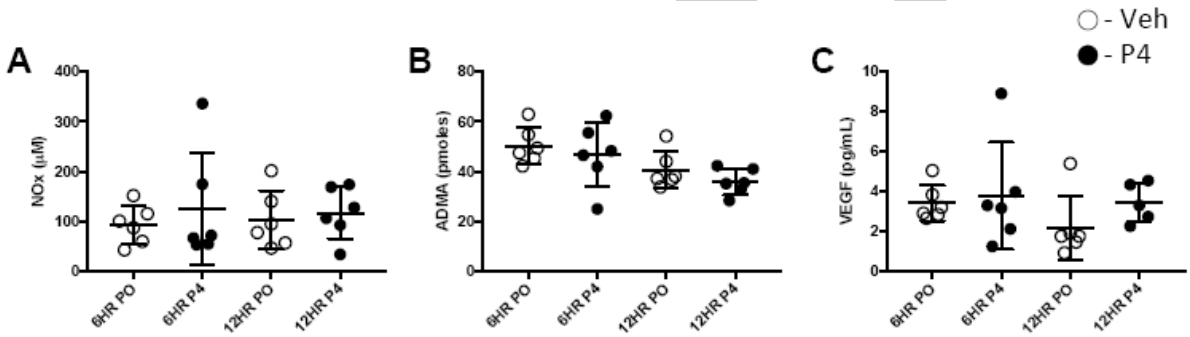

Figure 4 mRNA expression of cytokines, markers of cardiac dysfunction and apoptosis in left ventricular tissue taken from pregnant mice treated with LPS after supplementation with progesterone or vehicle. (A) *Il-1β* (B) *Il-6* (C) *Tnf-α* (D) *Il-10*, (E) *Anp* (F) *Bnp* (G) *c-Troponin I* (H) *Nkx2.5*, (I) *Bcl-2* (J) *Bax* (K) *Bad* and (L) *Crispld2*. Data are expressed as median ± interquartile range (n=6) and were compared with a paired t test or Willcoxon matched pairs test depending on the data distribution. *p<0.05, **p<0.01, ***p<0.001. Vehicle, peanut oil; P4- progesterone.

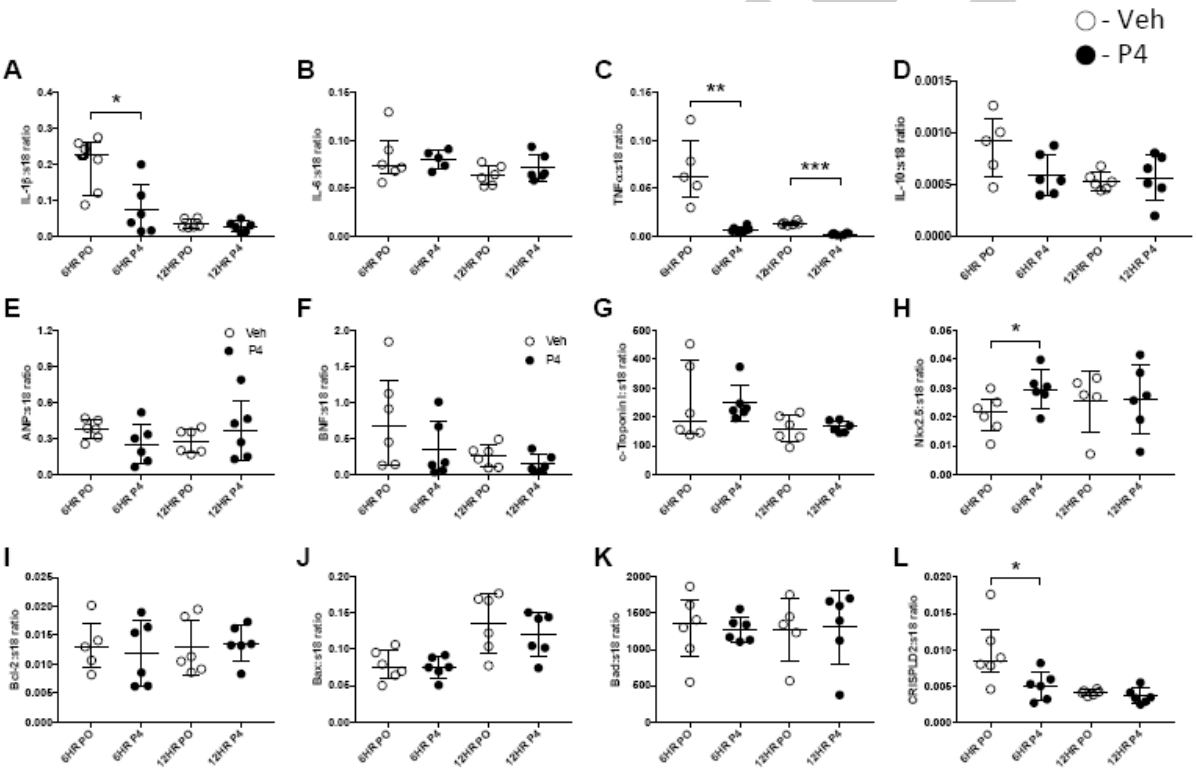

Figure 5: Leucocyte counts after LPS treatment with supplementation with progesterone or vehicle. Neutrophils and monocyte subsets in Blood (A-C), Uterus (D-F), Lungs (G-I), Liver (J&K) and Placenta (M-O). Samples were collected from pregnant (E16) mice after vehicle control (peanut oil) or at 6 or 12 hours after 10 μg LPS. Comparisons are made between progesterone and vehicle treated mice at each point using an unpaired student's t-test or Mann Whitney U tests depending on the data distribution. Data are expressed as median ± interquartile range (n=6-10). E16-pregnant.