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SHORT REPORT

Male gender promotes an increased inflammatory response to lipopolysaccharide in umbilical vein blood

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Objectives: **To establish gender-specific differences in maternal and fetal immune response in healthy human fetuses at term.** ***Methods:*** **Forty-five women with elective caesarean sections for uncomplicated singleton pregnancies were recruited for two studies. Using a multiplex biomarker immunoassay system, unstimulated maternal and fetal plasma concentrations of interleukin (IL)-1β, IL-1ra, IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, and tumor necrosis factor (TNF)-α were measured from one study population. Lipopolysaccharide (LPS)-stimulated cytokine response was measured in a second study.** ***Results:*** **There were no significant gender differences in either maternal or fetal unstimulated plasma cytokine concentrations, but concentrations of the proinflammatory cytokines IL-1β and IL-6 were significantly greater in male fetal LPS-stimulated samples than in female fetal samples.** ***Conclusions:*** **Blood of male fetuses mounts a larger pro-inflammatory response to lipopolysaccharide (LPS). This heightened response could be a critical pathway in promoting premature rupture of membranes (PPROM) and may be associated with life long differential gender response to infection.**

Keywords: Gender, fetal, blood, cytokines, pregnancy, LPS, IL-6, IL-1β, multiplex biomarker

Introduction

Gender differences have been associated with differences in mortality and morbidity of the fetus with the male fetus experiencing worse outcome following complications such as preterm delivery and infection [1]. Women carrying male fetuses have a higher incidence of pregnancy complications such as spontaneous abortions, preterm birth and preterm premature rupture of membranes (PPROM) [1–3]. These differences in outcomes have also been observed in adults following trauma or major surgery [4]. Several studies have demonstrated a higher incidence of sepsis or multiple organ failure in males and noted that male gender was an independent risk factor for major infection [4,5]. These differences have been attributed to increased cytokine response in males [6].

The maternal–fetal–placental unit is able to combat an infection through an adaptive immune response via several inflammatory cytokines. Cytokines are a diverse group of signaling proteins that are transiently produced in response to an immune challenge [7]. Their biological effects include activating and recruiting immune cells causing inflammation. Physiologically, interleukin(IL)-1β and tumor necrosis factor (TNF)-α play a role in causing uterine contractions and cervical ripening by producing prostaglandins and recruiting leukocytes. Further, IL-6, IL-8, TNF-α, and macrophage inflammatory protein (MIP)-1α have been implicated in the pathogenesis of the fetal inflammatory response syndrome (FIRS) and play a predominant role in term and preterm delivery, independent of the presence of infection [8,9].

It has been observed that a greater inflammatory response in males could lead to early preterm labour [10,11], which may avoid the opportunity for other infection-related pregnancy complications. While the female fetus would display signs of infection of placenta and membranes, the male fetus would, through an increased cytokine response, initiate labour before signs of infection become apparent. This has led us to postulate that gender-specific differences exist in fetal plasma, in the adaptive immune response of proinflammatory cytokines to bacterial challenge. The objectives of this study were to establish these differences in immune response, providing further insight into gender-specific immunological responses in adverse pregnancy outcomes.

Materials and methods

The study was approved by The University of Western Ontario Research Ethics Board for the review of Health Sciences Research involving Human Subjects (HSREB). The study comprised of 45 pregnant women and their single neonate attending St Joseph's Health Care London (SJHC), London, Ontario, Canada. All pregnant women had scheduled (unlaboured) elective caesarean sections, following uncomplicated pregnancies. Inclusion criteria were growth appropriate for gestational age, Apgar scores of >7 at 1 and 5 min and a normal placenta in appearance and weight. After informed consent was obtained, 5 mL of maternal venous blood was collected from the antecubital fossa into sterile tubes, approximately 2 h prior to caesarean section. Immediately after birth 5 mL of fetal venous blood was drawn into sterile tubes from the umbilical cord. Blood samples were either centrifuged for 15 min at 500×*g* after sampling and plasma frozen at −80°C for later

Shunaha Kim-Fine and Timothy R. H. Regnault contributed equally to this work

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assay as unstimulated samples or processed prior to centrifugation as below for lipopolysaccharide (LPS)-stimulated studies.

Determination of basal maternal and fetal cytokine concentrations

Cytokine levels in the supernatants of unstimulated collections (n = 8 female, n = 11 male) were measured using a Multiplex Immunoassay (Linco*plex*, Millipore Corp, MA, USA), with a Bio-Plex™ 200 Readout System (Bio-Rad Laboratories, Hercules, CA, USA), which utilizes Luminex® xMAP™ fluorescent bead-based technology (Luminex Corp., Austin, TX, USA). Cytokine levels were automatically calculated from standard curves using Bio-Plex Manager software (v.4.1.1, Bio-Rad Laboratories, Hercules, CA, USA).

Lipopolysaccharide-stimulated cytokine response of maternal and fetal blood

Immediately following blood collection (n = 9 female fetuses, n = 15 male fetuses), maternal and fetal blood samples were diluted 1:10 with RPMI-1640 medium, enriched with 2 mM L-glutamine. Diluted blood was then placed into wells (1 mL) and *Escherichiae coli K12–LCD25* lipopolysaccharide (LPS) was added to the treatment group at a dose of 100 ng/mL [12]. After 4 h of incubation at 37°C/21% O_2, well contents were collected and centrifuged at 3600 rpm for 10 min and the supernatant then collected and stored at −80°C until assay. The 4-h incubation was used based on previous fetal cytokine response studies [13] and our observation of attainment of differential cytokine (IL-6) responses at this time point in a limited number of samples (data not shown).The cytokines, IL- 1β, IL-1ra, IL-6, IL-8, MIP-1α and TNF-α were measured in unstimulated and LPS-stimulated supernatant after 4 h of incubation using the Multiplex Biomarker Immunoassay system as described above.

Statistical analysis

Unstimulated maternal and fetal samples gender comparisons were analyzed separately by Mann–Whitney test and are presented as mean ± SEM. LPS-stimulated data displayed unequal variance and were transformed and Kruskal–Wallis with Dunns post test ANOVA applied and are presented as mean ± SEM. A p value <0.05 was considered statistically significant.

Results

Patient population

There were no significant differences in base demographics of the study population of patients undergoing elective caesarean section giving birth to males or females in either the unstimulated or the LPS-stimulated study. Likewise, there were no significant differences between genders in the demographics of the neonatal population (Table I).

Determination of basal maternal and fetal cytokine concentrations

Basal levels of IL-1ra, IL-6, IL-8, MIP-1α, and TNF-α, but not IL-1β were detected in fetal and maternal plasma collected from elective caesarean section. There were no significant differences in either maternal or fetal plasma concentrations of the various cytokines between pregnancies bearing male and female fetuses (Table II).

Table I. Demographic data of participating mothers and neonates.

	Female fetus	Male fetus
Unstimulated baseline cytokine study		
Maternal age (years)	35.3 ± 1.7	31.6 ± 1.5
Gestational age (weeks)	38.7 ±.03	39.1 ± 0.1
Placental weight (g)	636.0 ± 58.1	690.2 ± 48.3
Birth weight (g)	3219.5 ± 191.6	3629.6 ± 135.6
Predelivery maternal leukocyte count($\times 10^9$ units/L)	8.4 ± 0.5	9.6 ± 0.6
Unstimulated and LPS-stimulated study		
Maternal age (yrs)	28.7 ± 4.3	31.8 ± 3.2
Gestational age (wks)	37.9 ±.0.9	38.1 ± 0.8
Placental weight (g)	906.0 ± 188.9	767.5 ± 156.4
Birth weight (g)	3651.0 ± 526.8	3545.7 ± 525.1
Predelivery maternal leukocyte count ($\times 10^9$ units/L)	10.4 ± 2.8	10.2 ± 1.7

Data are mean ± SEM
Unstimulated baseline cytokine study (female = 8, male = 11).
Unstimulated and LPS-stimulated study (female = 9, male = 15).

Table II. Unstimulated maternal cytokine concentrations (pg/mL) for pregnancies carrying female or male fetuses.

	Maternal cytokine concentrations		Fetal cytokine concentrations	
	Female fetus	Male fetus	Female fetus	Male fetus
IL-1ra	397.48 ± 185.04	230.478 ± 122.28	102.14 ± 33.89	72.98 ± 12.11
IL-6	53.88 ± 22.59	58.06 ± 24.61	10.19 ± 3.78	26.73 ± 14.50
IL-8	21.92 ± 7.07	25.00 ± 7.72	9.33 ± 1.56	11.48 ± 2.91
TNF-α	15.75 ± 5.66	10.79 ± 1.66	20.59 ± 2.65	19.46 ± 2.13
MIP-1α	35.01 ± 12.15	28.37 ± 7.35	7.46 ± 1.47	9.33 ± 2.65

Mean ± SEM (female = 8, male = 11).
IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

Lipopolysaccharide-stimulated cytokine response of maternal and fetal blood

Following a 4 h LPS exposure, there was a significant increase in all cytokine levels in both maternal and fetal samples from baseline, however there were no significant gender differences in maternal cytokine levels (Table III). Cytokine levels in fetal blood + LPS samples compared with the control unstimulated states were also significantly increased (Table IV). There were no significant gender differences in LPS-stimulated cytokine levels for IL-1ra, IL-8, MIP-1α, and TNF-α levels (Table IV). However, levels of the proinflammatory cytokines IL-1β and IL-6 were significantly greater in male fetal LPS-stimulated samples than in female fetal samples (Figures 1 and 2).

Discussion

By examining healthy, non-laboured fetuses at term, we were able to establish the gender-specific baseline levels of several inflammatory cytokines, which are known to contribute to the adaptive fetal immune response, and observed the effect of stimulation with lipopolysaccharide (LPS). We established that all cytokines analyzed show an increase in response to a LPS challenge. We did not observe a gender-specific difference in maternal or fetal plasma cytokines at baseline, however, we did find a gender-specific difference in cytokine response in fetal plasma, but not in maternal plasma, after LPS exposure. Specifically, there was a significantly greater response in IL-1β and IL-6 in male compared to female fetal blood.

Table III. Cytokine concentrations (pg/mL) following a 4 h incubation of maternal (female and male pregnancies) blood with 100 ng/mL LPS (stimulated).

	Female fetus +LPS	Male fetus +LPS
IL-1β	39.62 ± 49.93	94.87 ± 76.70
IL-1ra	374.79 ± 273.58	299.11 ± 65.53
IL-6	804.00 ± 505.58	1335.28 ± 904.00
IL-8	208.38 ± 192.73	158.63 ± 116.87
TNF-α	267.32 ± 179.02	232.16 ± 139.16
MIP-1α	1741.22 ± 2136.92	1509.32 ± 2579.93

Mean ± SEM. Female ($n = 9$) + LPS and male ($n = 15$) + LPS.
LPS, lipopolysaccharide; IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

Table IV. Cytokine concentrations (pg/mL) following a 4 h incubation of fetal (female and male pregnancies) blood without (unstimulated) or with 100 ng/mL LPS (stimulated).

	Female unstimulated	Female + LPS	Male unstimulated	Male + LPS
IL-1β	0.24 ± 0.02	33.60 12.05	0.93 ± 0.40	136.09 ± 26.96#
IL-1ra	8.25 ± 2.49	420.32 ± 112.31	10.80 ± 4.29	470.26 ± 134.62
IL-6	1.12 ± 0.46	927.33 ± 126.90	2.43 ± 0.70	2229.25 ± 378.93*
IL-8	1.76 ± 0.48	489.63 ± 126.52	9.86 ± 7.26	628.12 ± 105.73
MIP-1α	6.76 ± 2.79	1318.18 ± 226.30	8.80 ± 1.72	2231.33 ± 520.61
TNF-α	1.06 ± 0.32	246.86 ± 49.59	3.83 ± 2.76	245.95 ± 36.39

Mean ± SEM. Significant differences between female ($n = 9$) + LPS and male ($n = 15$) + LPS are noted.
LPS, lipopolysaccharide; IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.
#$p < 0.01$ and *$p < 0.01$.

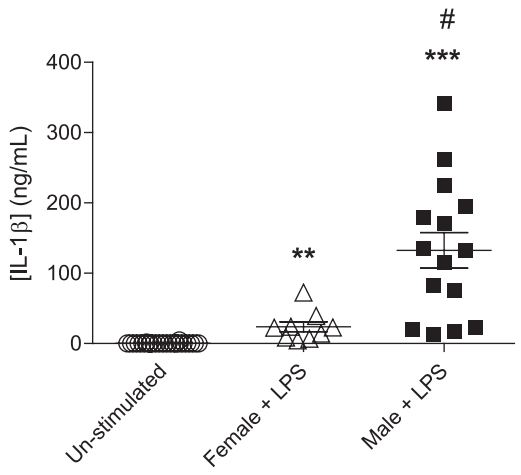

Figure 1. Media interleukin (IL)-1β concentration following a 4 h incubation of fetal female and male blood without (unstimulated) and with 100 ng/mL lipopolysaccharide (LPS). Unstimulated female and male samples were combined. Data were analyzed with Kruskal–Wallis ANOVA with Dunns post test. Female + LPS and male + LPS were significantly elevated (**$p < 0.01$ and ***$p < 0.001$, respectively) compared with pooled unstimulated samples. Male + LPS concentrations were significantly elevated compared to female + LPS (#$p < 0.01$).

During pregnancy, the maternal and fetal immune systems are intricately linked through the placenta, and proper functioning of both systems is necessary for a healthy, successful pregnancy. The onset of term or preterm labour is associated with a cytokine-activated immune response [14] and progesterone withdrawal removes constraints on the immune system and permits inflammation and labour [15]. IL-6, TNF-α, and

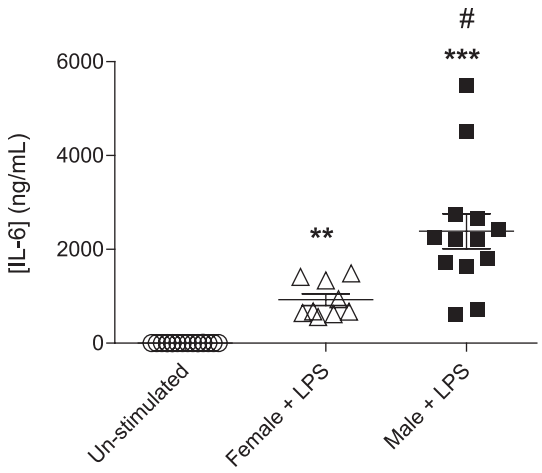

Figure 2. Media interleukin (IL)-6 concentration following a 4 h incubation of fetal female and male blood without (unstimulated) and with 100 ng/mL lipopolysaccharide (LPS). Unstimulated female and male samples were combined. Data were analyzed with Kruskal–Wallis ANOVA with Dunns post test. Female + LPS and male + LPS were significantly elevated (**$p < 0.01$ and ***$p < 0.001$, respectively) compared with pooled unstimulated samples. Male + LPS concentrations were significantly elevated compared to female +LPS (#$p < 0.01$).

MIP-1α have been implicated in the pathogenesis of FIRS, which is considered the fetal counterpart of the systemic inflammatory response syndrome observed in adults. FIRS has been described in association with intra-amniotic infection in fetuses, regardless of membrane rupture, and it is an independent risk factor for impending preterm labor and delivery, perinatal morbidity and/or mortality [9].

Bacterial infections, leading to a premature inflammation, pose a significant threat to pregnancy and the proper development of the fetus. Inflammatory changes have been associated with PPROM, chorioamnionitis, and funisitis [11]. Likewise, infections have been reported as responsible for up to 40% of preterm labour, and 80% of preterm deliveries show signs of infection. Proinflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, and MIP-1α have all been increased in neonatal cord blood following pregnancies involving these inflammatory complications [16].

Given the critical role cytokines play in the induction of partition, increases in the cytokines such as IL-1β and IL-6 would be associated with a predisposition to preterm labour and the sequelae of prematurity in neonates. Indeed, concentrations of the proinflammatory cytokine IL-6 are increased in amniotic fluid of pregnancies affected by PPROM compared to amniotic fluid obtained from pregnancies with intact membranes [17]. These changes are found in concert with high concentrations of IL-1ra in amniotic fluid and umbilical venous blood suggesting that the presence of this anti-inflammatory cytokine serves to prevent the development of overt inflammation [17]. Our results concur with the findings of increased IL-6 expression in the setting of an infection, through *ex-vivo* stimulation with LPS. The gender-specific difference in IL-6 in fetal plasma observed in our study provides a possible explanation for the increased incidence of PPROM and preterm labour occurring in male fetuses compared to female fetuses.

Systemic administration of IL-1 induces preterm labor and delivery in mice [18], highlighting IL-1 as an important contributor in modulation of inflammation related to chorioamnionitis and preterm labour in the fetoplacental compartment. In our study, incubation of fetal male blood with LPS induced a greater response of IL-1β, a response similar to the previously described

increased levels of IL-1β in adult males after significant trauma or infection [19]. The actions of IL-1 are modulated by the anti-inflammatory cytokine, Interleukin-1 receptor antagonist (IL-1ra), commonly present in amniotic fluid. IL-1ra inhibits the effects of IL-1 and can prevent preterm delivery induced by IL-1 [20]. Although our current study found no gender associated differences in the levels of IL-1ra, the concentration of IL-1ra in amniotic fluid has been reportedly significantly higher in female fetuses than male fetuses both at preterm and term gestation [11]. While the presence of gender differences in IL-1ra could explain improved outcomes in pregnancies bearing female fetuses in complications of preterm labour or chorioamnionitis, we did not observe this role for IL-1ra, either in the basal or LPS-stimulated situation.

The expression of the cytokines, MIP-α and IL-8 is increased in term placental syncytiotrophoblast cells when stimulated by bacterial LPS [21], which ultimately increases the response of the immune system [22]. Specifically, MIP-1α recruits leukocytes to the site of infection, resulting in a local inflammatory response and IL-8 recruits neutrophils to phagocytose an infectious agent. The immune response of fetal blood in our study appears to follow the same activation pattern as observed in term placental syncytrophoblast studies [21], and both genders mount a similar significant response. While the current study is adequately powered to detect gender differences after stimulation in fetal IL-1β (power 0.96) and IL-6 (0.98), the study is limited in power to detect possible smaller gender differences in some of the other cytokines, such as MIP-α (sample size 49, for $\alpha = 0.05$ and power = 0.80) and IL-8 (sample size 123). The interactions between the different cytokines are therefore likely more complex than demonstrated.

Overall, our data provides support for the important concept that gender difference in immune response is established *in utero* and remain constant throughout one's lifetime. In adulthood, it has been shown that males and females respond differently to a bacterial infection in both total immune response and steepness of the response curve [23]. Specifically, adult females have been found to have a 30% lower immune response compared to males [24] and it is suggested that following an infectious challenge, elevated plasma cytokine levels are inversely correlated to positive outcome [23]. Our results provide support for a parallel between the fetal and adult immune response [23], with a significantly greater release of proinflammatory cytokines following bacterial stimulation in male fetal plasma. One potential explanation for this gender difference is the sexual dimorphism in hormones influencing the LPS-stimulated signaling pathway [24]. Studies have also shown that testosterone, which is predominant in males, contributes to a weaker immune response, whereas estrogen seems to be protective [24]. Preterm females have significantly higher catecholamine levels than males, which may explain the better outcome in females after a hypoxic event [1].

This study provides an important insight into gender associated cytokine response to a bacterial challenge in cord blood and forms the basis for ongoing studies into the relation between fetal gender and pregnancy outcome. We report that IL-1β and IL-6, important inflammatory cytokines in the labour pathway, display differential inflammatory responses to LPS, based on gender. This heightened response in male fetuses could be a critical pathway in promoting PPROM or preterm labour and may be associated with lifelong differential gender response to infection. Clinically, this study demonstrates that gender differences in immune response exist as early as in fetal life, are likely to affect pregnancy outcome and should be taken into account in all investigations regarding cytokine response in pregnancy.

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