FACTORS DRIVING TNF-α EXPRESSION IN THE PERFUSION MODEL

IDENTIFYING FACTORS DRIVING TNF-α EXPRESSION IN THE DUAL

CLOSED LOOP *EX-VIVO* PLACENTAL PERFUSION MODEL: A

METHODOLOGICAL STUDY

By

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ABSTRACT

The pathophysiology of how a maternal infection induces fetal inflammation and subsequently premature birth is a growing area of research. The *ex-vivo* dual closed-loop placental perfusion model has been widely used to study placental physiology. To address the association between bacterial chorioamnionitis and fetal inflammation, TNF-α induction following lipopolysaccharide (LPS) challenge – a pyrogen of Gram-negative origin – was measured in the perfusion model. Preliminary analysis of perfusates unexpectedly revealed markedly elevated levels of TNF-α in control and LPS-treated groups indicating contamination of material(s) capable of activating innate immune responses.

To identify source(s) driving high background TNF-α expression in perfusates, bovine serum albumin (BSA) – the chief component of the perfusion media – the perfusion system and the materno-feto-placental unit were independently examined. To validate a cleaning protocol effective in LPS removal, acid-base and oxidative depyrogenation techniques were also additionally assessed in the perfusion system.

Using TNF-α as a surrogate marker of contamination, high background TNF-α expression in previously conducted placental perfusions were attributed to (1) LPS contaminated perfusion media and (2) LPS build up in the perfusion system. Additionally, results from depyrogenation experiments revealed both

acid-base and oxidative techniques effectively reduced LPS buildup in the perfusion system to levels that were in accordance with FDA guidelines for medical equipment (< 0.5 EU/mL). Thus, to circumvent LPS-derived contamination placentas should be perfused using endotoxin-free perfusion media and the perfusion system should be cleaned with acid-base or oxidative depyrogenation techniques prior to its use.

TABLE OF CONTENTS

LIST OF FIGRUES

LIST OF TABLES

LIST OF ABBREVIATIONS

AB- Acid-Base Cleaning

BCA- Bicinchoninic acid

BMI- Body Mass Index

BPD- Bronchopulmonary Dysplasia

BSA- Bovine Serum Albumin

BW- Birth Weight

CBC- Complete Blood Count

CD14- Cluster of Differentiation 14

D' Water- De-ionized water

DP- Diastolic Pressure

DS- Diabetic Status

ELISA- Enzyme-linked Immunosorbent Assay

EU- Endotoxin Units

FDA- Food and Drug Administration

FIRS-Fetal Inflammatory Response Syndrome

GA- Gestational Age

g- Gravitational

HDL- High Density Lipoproteins

H_2O_2- Hydrogen Peroxide

HRLMP- Hamilton Regional Lab Medicine Program

HSA –Human Serum Albumin

IL-1β- Interleukin 1-beta

IL-6- Interleukin 6

IL-8- Interleukin 8

KDO- 2-keto-3-deoxy-D-mano-octonate

K_2 EDTA- Ethylenediaminetetraacetic acid

LAL- Limulus Amebocyte Lysate

LBPs- LPS-binding Proteins

LE- Low Endotoxin

LPS- Lipopolysaccharide

mCD14- Membrane CD14

MMP-8- Matrix Metalloproteinase-8

MyD88- Myeloid Differentiation Primary Response Gene (88)

NaOH- Sodium Hydroxide

NCTC- National Collection of Type Cultures

OX- Oxidative Cleaning

PM- Perfusion Media

PS - Perfusion System

RDS – Respiratory Distress Syndrome

RPM- Revolutions per Minute

SARM- Sterile-alpha- and Armadillo-motif-containing Protein

sCD14- Soluble CD14

SD- Standard Deviation

SE - Standard Error

SP- Systolic Pressure

SS-Smoking Status

TLR4- Toll- like Receptor 4

TNF-α- Tumor Necrosis Factor Alpha

TRAM- TRIF-related adaptor molecule

TRIF- TIR-domain-containing Adapter-inducing Interferon-β

USP- United States Pharmacopoeia

WBC- White Blood Cell

PURPOSE OF STUDY

To address the association between bacterial chorioamnionitis and fetal inflammation, we measured the induction of the pro-inflammatory cytokine TNF-α following lipopolysaccharide (LPS) challenge of term placentas. Placental cytokine response was examined in the dual closed loop *ex-vivo* placental perfusion model. Perfusions were performed either in the presence or absence of LPS.

Preliminary analysis of perfusates unexpectedly revealed markedly elevated levels of the cytokine in both control and LPS-treated groups, with no difference between the two groups implying contamination of the system with material(s) capable of activating innate immune responses (*Chapter 1*). Thus, prior to embarking on the initial question of how bacterial chorioamnionitis elicits a fetal inflammatory response, the reason(s) for high background TNF-α expression in the placental perfusion model needed to be identified and resolved.

Therefore, the modified objective of the thesis was to determine the factors driving TNF-α expression and to develop methods to minimize their effects in the placental perfusion system. Thus, to address background TNF-α expression, as a surrogate marker of contamination, in the system, the following aims were addressed:

Aim I: Identify substances driving TNF-α expression in the placental perfusion system (*Chapter 2*)

AIM II: Validate a cleaning protocol for effective decontamination of the perfusion system (*Chapter 3*)

AIM III: Profile cytokine makeup in the materno-feto-placental unit to determine if the placenta is driving TNF-α expression in the perfusion system (*Chapter 4*).

CHAPTER 1:

CHORIOAMNIONITIS AND FETAL INFLAMMATION

1.1 Chorioamnionitis

Commonly presented as an intra-amniotic infection, chorioamnionitis is defined as an acute inflammation of the chorion, amnion and placenta (Galinsky, 2013). Approximately 40-70% of preterm births – the leading cause of neonatal morbidity and mortality – are complicated by a chorioamnionitic infection (Tita, 2012). Risk factors for chorioamnionitis include prolonged labor, nulliparty, maternal smoking, substance abuse and the presence of genital tract infections such as group B streptococcus, vaginal colonization of *Ureaplasma Urealyticum* as well as bacterial vaginosis (Titia and Andrews, 2010).

Chorioamnionitis can be sub-grouped as a clinical or subclinical (histological) condition. Clinical presentations of chorioamnionitis include maternal fever (>38°C), tenderness of the uterine cavity, maternal leukocytosis, maternal tachycardia (>100 beats/min), fetal tachycardia (>160 beats/min) and foul amniotic fluid odor (Galinsky, 2013; Tita, 2012). Contrary to clinical chorioamnionitis, subclinical presentation of chorioamnionitis occurs more frequently accounting for more than 50% of preterm deliveries (Edwards, 2005). Subclinical chorioamnionitis, however, does not present the above mentioned clinical symptoms, instead, it is histologically characterized by inflammation of the fetal (chorion and amnion) and placental membranes (Galinsky, 2013).

1.2 Pathogenesis of Chorioamnionitis

The pathogenesis of chorioamnionitis can occur in the presence or absence of infectious microorganisms, the former of which is predominantly associated

with the condition. Chorioamnionitis is marked by the ascension of microorganisms that migrate from the endocervical canal to the amniotic cavity (Faro and Soper, 2001). Pathogens commonly associated with chorioamnionitis are *Ureaplasma urealyticum* (47%), *Mycoplasma hominis* (30%), *Gardnerella vaginalis* (25%) and *Escherichia coli* (8%) (Tita and Andrews, 2010). As microorganisms ascend to the decidua they can trigger a localized inflammatory reaction resulting in the production of inflammatory mediators like cytokines (Romero, 2003). If the inflammatory response is not strong enough to incur labor, microorganisms can cross over into the amniotic fluid to evoke a second inflammatory response. Inflammatory response in the presence of a bacterial infection is only beneficial if it is tightly regulated. Benefits of an inflammatory response include (1) the release of inflammatory mediators to suppress the infection (2) containment of the infection and (3) allow repair of injured tissues (Romero, 2006). A prolonged state of inflammation, however, poses complication to the mother and immature fetus.

1.3 Maternal and Fetal Complications

Maternal complications of chorioamnionitis include increased risked for cesarean delivery, bacteremia and increased incidence of postpartum hemorrhaging (Tita and Andrews, 2010). Wound infection and pelvic abscess however, occurs less frequently. Moreover, maternal respiratory distress syndrome (RDS), septic shock and fatality are extremely rare complications

among women presenting with chorioamnionitis (Edwards, 2005; Tita and Andrews, 2010).

Fetal and neonatal complications associated with chorioamnionitis are far more severe. Fetuses exposed to chorioamnionitis develop a condition known as fetal inflammatory response syndrome (FIRS) - defined as the systematic activation of the fetal innate immune system (Bieghs, 2013). FIRS occurs via direct fetal contact with infected amniotic fluid and/ or exposure to inflammatory mediators from the uteroplacental (maternal) circulation (Galinsky, 2013). Thus, chorioamnionitis is a common precursor for FIRS, a condition that has potent pleiotropic effects on the development of the fetus (Beighs, 2013).

FIRS can be sub-grouped as clinical or sub-clinical. Clinical FIRS is characterized by elevated levels of interleukin (IL)-6 in fetal plasma (>11 pg/mL), while sub-clinical FIRS is histologically characterized by funisitis - inflammation of the umbilical cord (Galinsky, 2013). The incidence of neonatal morbidity is significantly greater among fetuses affected with FIRS. This is largely due to multiple organ dysfunction and septic shock - both of which progress with FIRS (Gotsch, 2007; Romero, 2006). Complications of FIRS include: respiratory distress, bronchopulmonary dysplasia (BPD), intraventricular hemorrhaging, necrotizing enterocolitis and fetal death if left untreated (Romero, 2006; Gotsch, 2007).

Thus, upregulation of inflammatory mediators seen in chorioamnionitis places the fetus at an increased risk for postnatal complications. A better understanding of how inflammatory mediators come in contact with the fetal compartment is therefore of great importance. To mitigate the above complications, molecular mechanism(s) of chorioamnionitis, in particular the role of inflammatory mediators contributing to fetal inflammation, need to be further elucidated.

1.4 Role of Cytokines

Pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1-beta (IL-1β) are thought to play a central role in preterm parturition. IL-1β and TNF-α are capable of stimulating prostaglandin (F2α and E2) production - a labor inducing hormone-like substance - by the amnion and chorion (Romero, 2003; Blackburn, 2007)). Moreover, in the presence of bacterial pathogens, IL-1β and TNF-α are produced by the human decidua. Studies have also reported an increase in amniotic fluid IL-1β and TNF-α levels of women with intra-amniotic infection and among those who experience premature labor (Romero, 2003). Additionally induction of preterm labor by IL-1β and TNF-α has been further described in animal models where independent administration of the two cytokines triggered early onset of preterm labor (Romero, 2006).

As primary cytokines, IL-1β and TNF-α are capable of initiating inflammatory cascades (Malerich, 2006). Secondary cytokines like IL-6 and IL-8 are produced later on by this cascade. IL-6 and IL-8 also play a role in intra-amniotic infection. IL-6 has been found to be a useful diagnostic tool for screening sub-clinical

chorioamnionitis (>17 ng/mL) (Dudley, 1997). Placental IL-8 production has been shown to be elevated in chorioamnionitis (Shimoya, 1992). Independently, IL-1β and TNF-α can further enhance IL-6 and IL-8 production. Moreover TNF-α can also induce IL-1β production. Thus, as a multidimensional cytokine, the onset and maintenance of an inflammatory response can be upheld by TNF-α (Malerich, 2006). Given the ability of TNF-α to orchestrate an inflammatory response and to induce the production of other inflammatory mediators, the role of TNF-α in chorioamnionitis needs to be further examined.

1.5 Animal Models to Study Chorioamnionitis

Animal models have been and continue to be useful for studying chorioamnionitis. Clinical chorioamnionitis can be reproduced in pregnant rabbits, sheep, mice and other rodents via intra-cervical administration of bacterial lipopolysaccharide (LPS) (Galinsky, 2013). LPS is a bacterial pyrogen of Gram-negative origin capable of inducing an inflammatory cascade similar to that which is observed during infection. Intravenous and intra-peritoneal administration of LPS has also produced clinical chorioamnionitis in animal models (Galinsky, 2013). Although many animal models are available, their uses are not without limitation (Mathiesan, 2010). A major drawback in using animal models to study chorioamnionitis is that the placenta which mediates the transfer between the maternal and fetal compartments is morphologically distinct among species. Aside from differences in gestation, factors contributing to species variation include placental structure, organization of the tissue layers and differences in

placental cell types (Chuong, 2013). Therefore, extrapolating results from animal models for the purpose of human application is difficult (Mathiesan, 2010).

1.6 Placental Perfusion Model

Thus far, acquisition of human data pertaining to fetal exposure have only come from terminated pregnancies or restricted clinical studies (Mathiesan, 2010). The dual closed loop *ex-vivo* placental perfusion model overcomes both the ethical dilemma and clinical limitation. Panigel was the first to describe the simultaneous perfusion of fetal vessels and maternal intervillous space of an isolated placental lobule; a technique that was further refined by Schnieder and Huch (1985). The dual closed loop placental perfusion model has assisted in the study of placental metabolism, placental drug transfer and the production and release of placental hormones like leptin (Haugel, 1983; May 2008; Linnemann, 2006). The model has also been used to investigate interactions between pro-inflammatory cytokines as well as aided in the study of preeclampsia (Holcberg, 2007; Di Santo, 2007). Today, the placental perfusion model has become a widely used tool in placentology, permitting the study of placental physiology.

Thus, to address the association between bacterial chorioamnionitis and fetal inflammation, the induction of pro-inflammatory cytokine TNF-α was measured following LPS challenge using the placental perfusion model.

1.7 MATERIALS AND METHODS

Placental TNF-α response was previously examined in the dual closed loop *ex-vivo* placental perfusion model (see Fig 1). The perfusion method was conducted as described by Bachmaier *et al.* (2007). Placental perfusions were performed on term (≥ 37 weeks of completed gestation) placentas from singleton births that were delivered via an elective cesarean section. All mothers enrolled in the study were recruited from McMaster Hospital, Labor and Delivery Ward. The study was approved by Hamilton Health Sciences/ Faculty of Health Science Research Ethics Board (REB # 11-117-T)

A suitable cotyledon for perfusion was carefully selected by examining the chorionic (fetal) and decidual (maternal) surface for possible tissue injury and/or rupture. Using olive top catheters, a chorionic artery and its corresponding vein were cannulated to establish the fetal circulation. To confirm successful perfusion of fetal vessels, heparinized (3 IU/mL) saline solution (0.9%) was perfused through the cannulated fetal vessels. Following confirmation of the fetal circulation, the cotyledon was mounted over the center of a holding ring with the fetal surface focusing upward. The cotyledon was subsequently secured in place and excess tissue surrounding the cotyledon was excised and discarded. With the maternal surface facing upwards, the ring was mounted onto an insulated (37°C) perfusion chamber. To establish the maternal circulation, three olive top catheters were carefully pressed through the basal plate into the intervillous space corresponding to the cannulated chorionic vessels. To drain the maternal

perfusate, an additional tube was introduced near the intervillous space of the cannulated cotyledon.

Maternal and fetal circulations were independent of one another such that each had its own perfusion circuit and reservoir. Peristaltic pumps were used to regulate maternal (8-12 mL/min) and fetal (4-6 mL/min) flow rates. Additionally a thermometer was placed in close proximity to the cotyledon to ensure the temperature of the perfused cotyledon was maintained at normathermic levels (37°C). A pressure sensor was built into the system to ensure fetal pressure did not exceed 60 mmHg (high pressure exerts strain on fetal vessels resulting in the loss of circulation and/or leakage). To achieve normoxic conditions maternal circulation was equilibrated with atmospheric gas (O_2) while the fetal circulation was equilibrated with 95% N_2 and 5% CO_2. Temperature, pressure and flow rates were recorded at each sample collection.

Cotyledons were perfused with media that consisted of tissue culture medium NCTC-135 (9.3 grams; Sigma-Aldrich; N3262), dextran T-40 (40 grams; Dextran Products Limited; Lot # D-4445 AM), bovine serum albumin (BSA; 160 grams; PAA; lot # K00109-2537) and heparin (1 mL). The pH of the medium was adjusted to physiological pH (7.4) prior to the perfusion. Placentas were perfused for a total of 4.5 hours and consisted of 2 phases. In Phase I, the placenta was perfused for 30 minutes in an open circulation to eliminate any residual blood in the system. In Phase II, the placenta was perfused for 4 hours in a closed configuration and samples were collected at 30 minute intervals. LPS (1 ng/mL)

was introduced into the maternal circulation at the beginning of Phase II in treatment groups; LPS was not added to control groups. Samples were collected in 1.5 mL polypropylene vials and subsequently stored at -80°C for analysis. TNF-α levels in perfusates were measured via TNF-α ELISAs (e - Bioscience). Sensitivity of the ELISA was 4 pg/mL; standard curve range was from 4 – 500 pg/mL.
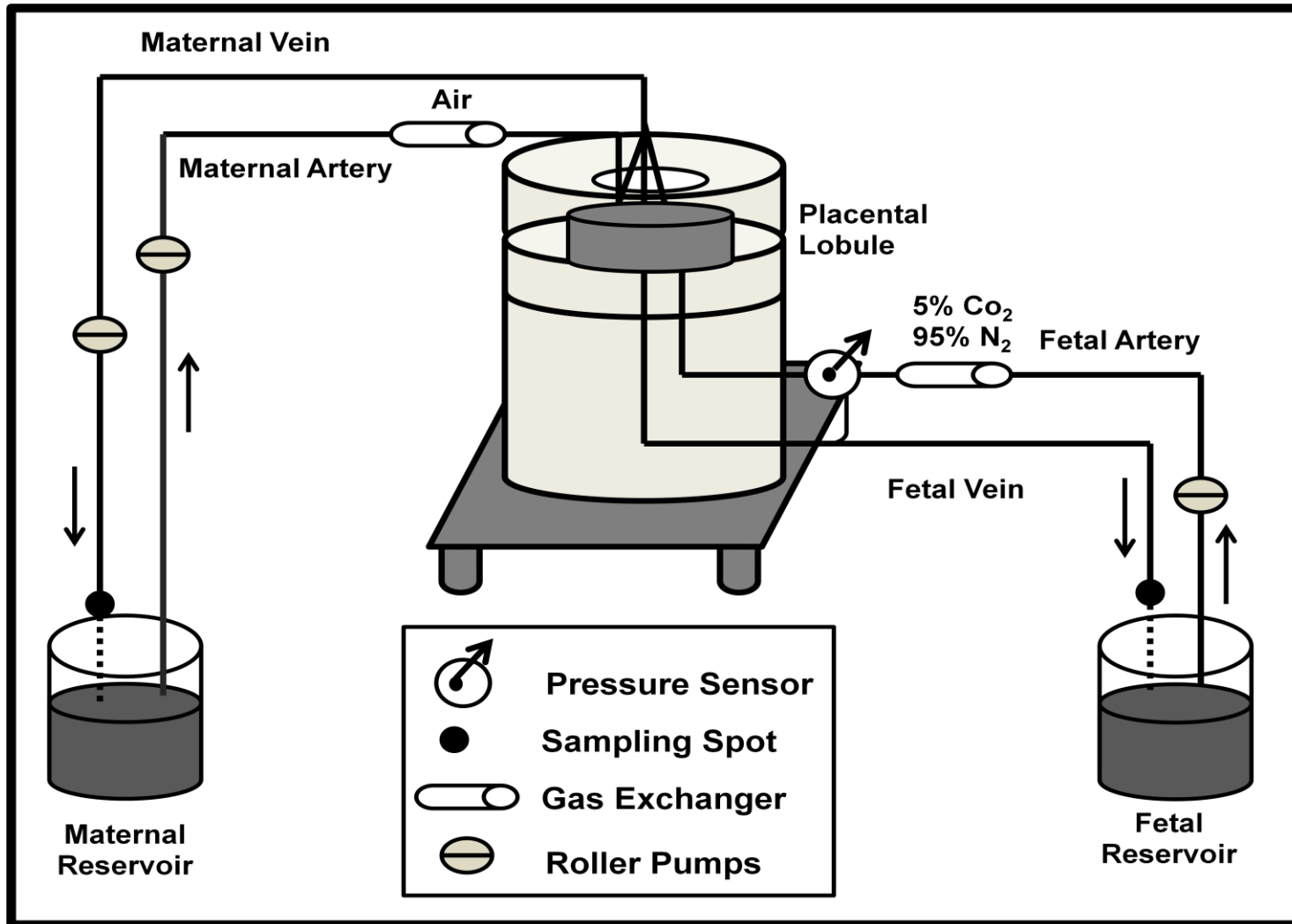
Fig 1. Experimental set-up of the dual closed loop *ex-vivo* placental perfusion model

1.8 RESULTS

TNF-α expression was detectable in all perfusate samples during Phase II of the perfusion (0 to 240 min). A temporal increase in TNF-α expression was observed in maternal and fetal perfusates with maternal perfusates containing higher levels of the measured cytokine. When perfused with medium alone (control), TNF-α expression in maternal perfusates ranged from 411-6235 pg/mL while TNF-α expression in fetal perfusates ranged from and 278-6233 pg/mL. No significant increase in TNF-α expression was observed in maternal perfusates following LPS challenge (treatment), however a slight increase in TNF-α expression was observed (60 to 120 min) in fetal perfusates following LPS exposure (treatment). TNF-α expression of maternal and fetal perfusate samples are shown in Figures 1.1 and 1.2.
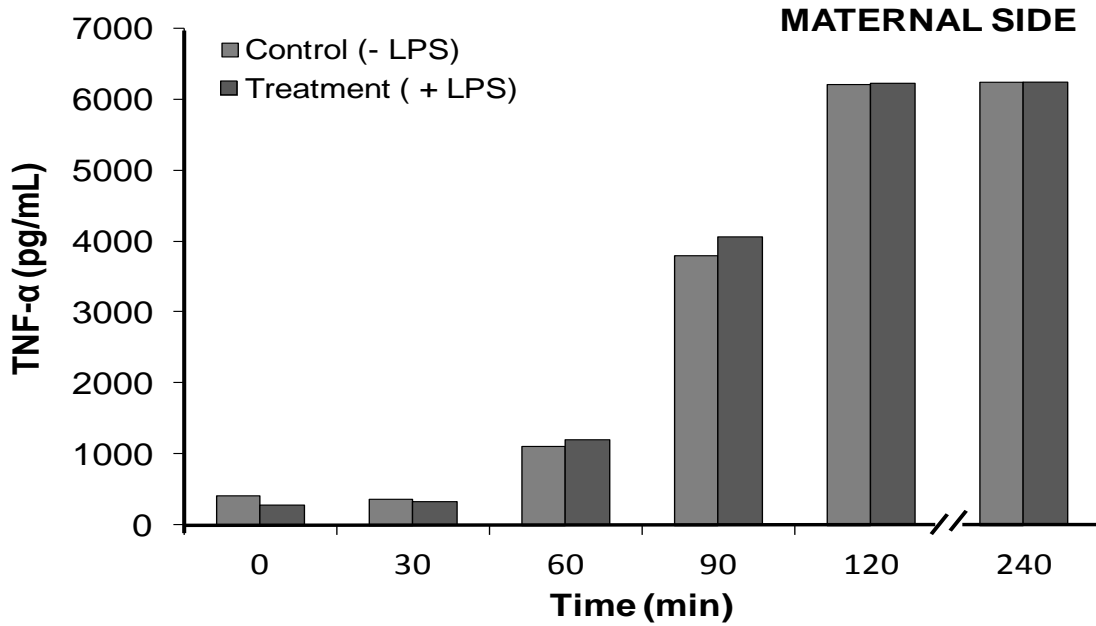
Fig 1.1 TNF-α levels in the maternal side of term placentas following perfusion with medium (control; n=1) and LPS (1 ng/mL) exposure (treatment; n=1) for 4 hrs
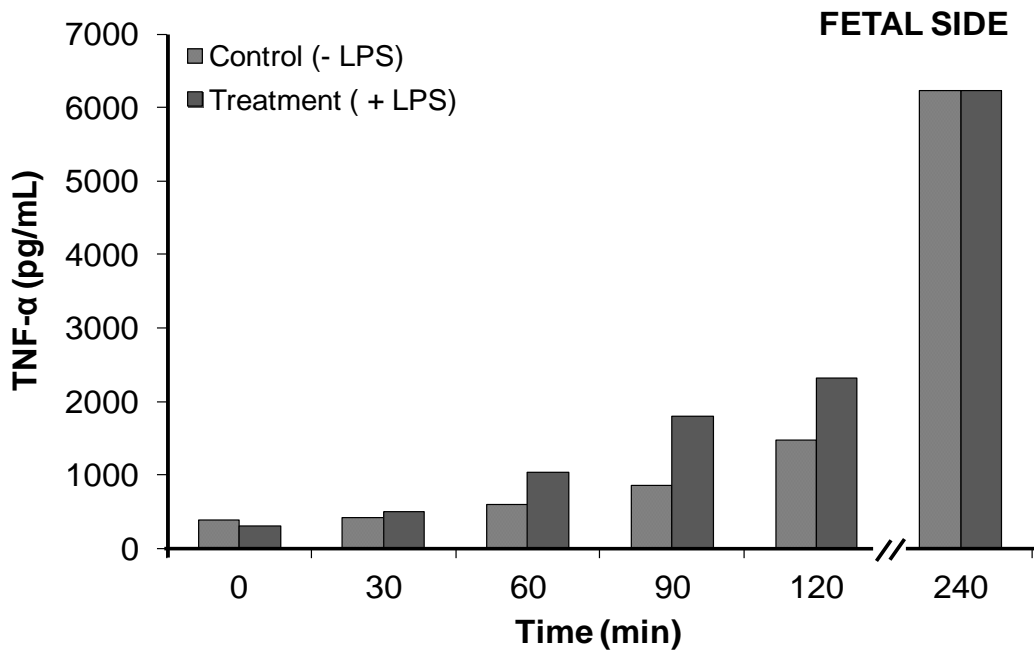
Fig 1.2 TNF-α levels in the fetal side of term placentas following perfusion with medium (control; n=1) and LPS (1 ng/mL) exposure (treatment; n=1) for 4 hrs

1.9 DISCUSSION

Placenta lobule perfused with medium alone (control) had TNF-α levels analogous to the placental lobule perfused with exogenous LPS (treatment), thus the addition of exogenous LPS did not affect placental TNF-α expression. Findings from these experiments however, do not coincide with those reported by Holcberg *et al.* who demonstrated a significant increase in placental TNF-α expression following LPS stimulation (2007). Moreover, Holcberg reported detectable levels of the cytokine following 180-200 min of perfusion, whereas TNF-α levels in our samples were already detected at 30 min into the perfusion (Phase II). Furthermore, despite demonstrating a temporal increase in TNF-α expression, the magnitude and rate at which TNF-α increased is markedly greater in our samples compared to Holcberg *et al.* (2007).

Elevated levels of the cytokine imply an activation of the innate immune response that cannot be solely attributed to the added LPS. With no clear difference in TNF-α levels among the control and treatment group, the possibility of a contaminated system with materials capable of inducing an innate immune response exists. Contamination of materials that come in direct contact with the placental lobule (i.e. perfusion media and/or perfusion system) may explain the discrepancies between the above findings and those reported by Holcberg *et al.* (2007). Thus, prior to examining how bacterial chorioamnionitis elicits fetal inflammation, factors driving high background TNF-α expression needed to be first identified and resolved.

CHAPTER 2:

IDENTIFYING SOURCES OF CONTAMINATION

2.1 Introduction

The *ex-vivo* dual closed-loop perfusion model of the human placenta has been widely used to study placental physiology. Bacterial endotoxin like LPS – a pyrogen of Gram-negative origin – is often applied in the perfusion model to evoke placental inflammation. Traces of LPS found in commercially prepared BSA – the chief component of the perfusion media – and inadequate cleaning of the perfusion system can drastically affect the outcome of experiments. Thus, to accurately measure placental inflammatory responses, the perfusion system and the perfusion media need to be pyrogen-free. The first aim of the thesis was to identify sources for TNF-α activation by assessing the perfusion system and perfusion media for LPS contamination.

2.2 LPS Structure and Function

LPS are amphipathic molecules – consisting of both hydrophobic and hydrophilic domains – that constitute the outer-most membrane of Gram-negative bacteria. The three moieties of LPS – O-antigen, core oligosaccharide and lipid A – are independently synthesized and transported to the outer membrane of the bacterial cell (Williams, 2007). LPS not only maintains the structural integrity of Gram-negative bacteria but also plays an essential role in their physiological, immunogenic and pathogenic functions. Thus, a Gram-negative bacterium that lacks its LPS structure cannot survive. As a tripartite molecule, LPS has three functional domains – the O-antigen chain is responsible for eliciting an immunogenic response; the core oligosaccharide gives LPS its overall negative charge needed for biological activity; and the lipid A moiety functions in the molecule's endotoxic activity (Williams, 2007). The O-antigen domain and the majority of the core oligosaccharide domain are not necessary for *in-vitro* survival, however lipid A is.

2.2.1 O-Antigen

The O-antigen chain consists of repeating oligosaccharide units that are 3 to 8 monosaccharides in length. The O-antigen is structurally diverse among Gram-negative species; thus, the serologic identity of each Gram-negative bacterium can be traced back to its O-antigen sequence (Erridge, 2002). Given its network of long polymer chains, the O-antigen domain also serves as a barrier for the bacteria against foreign substances (Williams, 2007).

2.2.2 Core Oligosaccharide

The core oligosaccharide domain of LPS can be divided into two sub-domains – the outer and inner. The outer core consists of D-glucose, D-galactose, N-acetyl-D-glucosamine and N-acetyl-D- galactosamine (Williams, 2007). The inner sub-domain is structurally more conserved and consists of two sugars, KDO (2-keto-3-deoxy-D-mano-octonate) and heptose. KDO bridges the core oligosaccharide to the carbohydrate backbone of lipid A (Williams, 2007). The KDO sugar is structurally important as it permits the solubilization of LPS in aqueous systems. Moreover, bacterial reproductive capability is highly dependent on the presence of at least one KDO residue linked to a lipid A moiety (Erridge, 2002). Thus, appropriate biosynthesis of the KDO sugar is essential for the growth and survival of Gram-negative bacteria.

2.2.3 Lipid A

The lipid A moiety of LPS is the most highly conserved domain of Gram-negative bacteria and is essential in promoting bacterial growth and survival. Lipid A serves as the attachment site for LPS molecules, enabling LPS to anchor on to host proteins (Raetz, 2002). Characterized as the bioactive centre of LPS, endotoxic properties of lipid A has become the focus of many researchers (Williams, 2007).   The potency of a LPS molecule is dictated by the phosphorylation, acylation and fatty acid chain length of its lipid A moiety. Nevertheless, LPS can only retain its endotoxic properties following bacterial release, if it is solubilized with carriers like HSA (human serum albumin) or BSA.

2.3 Endotoxicity of LPS

Due to its pyrogenic properties – ability to induce a febrile response – LPS molecules are also commonly referred to as endotoxins (Magalhaes, 2007). Endotoxin exposure from a Gram-negative infection can induce a wide range of pathophysiological effects, including septic shock, tissue injury and fatality (Magalhaes, 2007). Unlike most bacterial toxins, which directly interrupt the function of host cells, LPS carries out its toxic effects indirectly by exacerbating the host immune response (Ding, 2010). The onset of an endotoxic response is initiated by the release of LPS molecules, which can occur in one of two ways, either upon bacterial cell death or via bacterial shedding during cell growth and/or division (Williams, 2007).

Following a Gram-negative infection, LPS can initiate a cascade of host inflammatory responses. Activation of an inflammatory response is only beneficial in the eradication of a bacterial invasion if it is closely regulated by the host immune system. Inflammatory responses that are not tightly controlled result in the over production of pro-inflammatory mediators, leading to an uncontrolled inflammatory state that has potent effects on the host.

When LPS molecules are released by Gram-negative bacteria, they cluster together to form large LPS aggregates (Heumann, 2002). The disassociation and removal of LPS aggregates is facilitated by LPS-binding proteins (LBPs) – a lipid transfer protein. LBP is a 60 kDa glycoprotein that is produced primarily by hepatocytes and is responsible for mobilizing LPS

monomers (Miyake, 2004). LPS is additionally mobilized by CD14 (cluster of differentiation 14), another glycoprotein that exists in two forms (Miyake, 2004). CD14 that is expressed on the cell surface of macrophages, neutrophils and dendritic cells are referred to as membrane CD14 (mCD14), while CD14 present in the circulation is referred to as soluble CD14 (sCD14) (Miyake, 2004). Together, CD14 and LBP have dual functionalities, capable of augmenting and inhibiting LPS induced immune responses (Kitchens, 2005).

LPS neutralization or inhibition – one that does not lead to cell activation – occurs via LPS clearance. Both LBP and sCD14 can promote LPS clearance by mobilizing LPS monomers to high-density lipoproteins (HDL) which are synthesized by the liver (Ding, 2010). HDL neutralizes LPS monomers by reducing its bioactive ability, upon which, the monomer is excreted via the hepatobiliary pathway. Additionally, LPS that is still bound to Gram-negative bacteria can be subjected to clearance by LBP, which functions as an opsonin – a molecule that promotes phagocytosis – by enhancing whole bacterial phagocytosis (Ding, 2010).

LPS-induced cell activation is triggered by the mobilization of LPS from LBP-LPS complex to mCD14. Localized on the cell membrane of myeloid cells, mCD14 is a 53 kDa glycoprotein that functions as a receptor and scavenger for LPS (Heumann, 2002). While mCD14 is a receptor that is capable of binding to LPS, it is not involved in the signal transduction process, as this pathway can

only be triggered by proteins that contain both a transmembrane region and an intracellular domain – two features that mCD14 lacks (Heumann, 2002).

Toll-like receptor 4 (TLR4), found on the cell surface of immune cells, has been identified as the receptor responsible for LPS-induced activation (Triantafilou, 2002). mCD14 transfers LPS molecules to TLR4 which forms a complex with MD-2 – a protein that assists in the signal transduction pathway of LPS (Lu, 2008). TLR4 is the only receptor known to utilize all 5 adaptor proteins – MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule) and SARM (sterile-alpha and armadillo-motif-containing protein) – for downstream signaling (Lu, 2008). TLR4 signaling can be divided into two pathways, MyD88-dependent or independent pathway. MyD88-dependent pathway leads to activation of pro-inflammatory cytokines; MyD88 independent pathway results in the activation of Type I interferon genes which induces an antiviral or antibacterial response (Lu, 2008).

2.4 LPS Contamination

Given its endotoxic properties and ability to elicit a strong immune response, contamination of biologically active substances by LPS can pose serious complications in the study of immunology (Magalhaes, 2007). The difficulty in controlling for endotoxin contamination stems from its unique features – its ubiquity in nature, its toxic potency and its stability under harsh conditions – all of which owe to its continued existence (Williams, 2007). Furthermore, as an

amphiphatic molecule, the lipophilic domain of LPS can easily adhere to hydrophobic surfaces like glassware and plastics, resulting in the carryover of the contaminant from one experiment to another. Thus, once LPS has been introduced to laboratory equipment, the likelihood of its reoccurrence is relatively high if not adequately removed initially.

2.5 LPS Guidelines

LPS is measured in endotoxin units (EUs), which represents the biological activity of each LPS strain (Magalhaes, 2007). Pharmacopoeias have set the threshold for solutions intended for intravenous applications to 5 EU/kg/hr. In addition, FDA (Food Drug and Administration) guidelines for medical devices that come into contact with the cardiovascular or lymphatic system has a limit of 0.5 EU/mL or 20 EU/apparatus (2012). FDA guidelines for devices that come into contact with cerebrospinal fluid are even lower with the limit set at 0.06 EU/mL or 2.15 EU/apparatus (2012). The task of meeting the recommended threshold however, can become problematic due to the nature of LPS.

2.6 MATERIALS AND METHODS

To determine if solutions used during placental perfusions were sources for TNF-α activation, the three most frequently used solutions - water, deionized water (D'Water) and perfusion media - were sampled for analysis. To asses if the perfusion system, itself, was a source for TNF-α activation, the above three solutions were independently circulated (45 min) through the perfusion system and subsequently sampled for further testing. To determine if BSA alone contributed to TNF-α activation, the following samples were additionally sampled: perfusion media made with commercially prepared BSA Fraction V (PAA; lot# K00109-2537), perfusion media made with low-endotoxin (LE) BSA Fraction V (PAA; lot# K00111-0424) and perfusion media without BSA.

All samples were collected in 1.5 mL polypropylene vials and subsequently stored at -20°C prior to analysis. To measure TNF-α expression, samples were incubated in mouse macrophage cell lines (RAW 264.7; ATCC) overnight (12 hrs). Following incubation, cell supernatants were extracted and stored in 0.5 mL polypropylene vials and stored at -20°C for analysis. TNF-α expression was measured using a mouse TNF-α ELISA (R & D; DY410). The standard curve range of the kit was 31.2 - 2,000 pg/mL.

2.7 RESULTS

Water and D'Water that were not circulated through the perfusion system had TNF-α concentrations similar to that of the negative control which was free of LPS. A 10-fold increase in TNF-α levels were observed when water was circulated though the perfusion system. A 23-fold increase in TNF-α levels were observed when D'water was circulated through the perfusion system. Perfusion media that was not circulated through the perfusion system had TNF-α levels that were comparable in concentration to that of the positive control (100 ng/mL of LPS). TNF-α levels did not increase in the perfusion media following circulation through the perfusion system. Results are shown in Fig 2.

TNF-α concentrations in perfusion media containing commercially prepared BSA (PM + BSA) were similar in concentration to that of the positive control. There was a 4-fold decrease in TNF-α levels from perfusion media that was prepared using low endotoxin BSA (PM + LE BSA) compared to PM + BSA. Moreover, a 27-fold decrease in TNF-α levels were observed in perfusion media (PM) that was prepared in the absence of BSA. Results are shown in Fig 2.1.
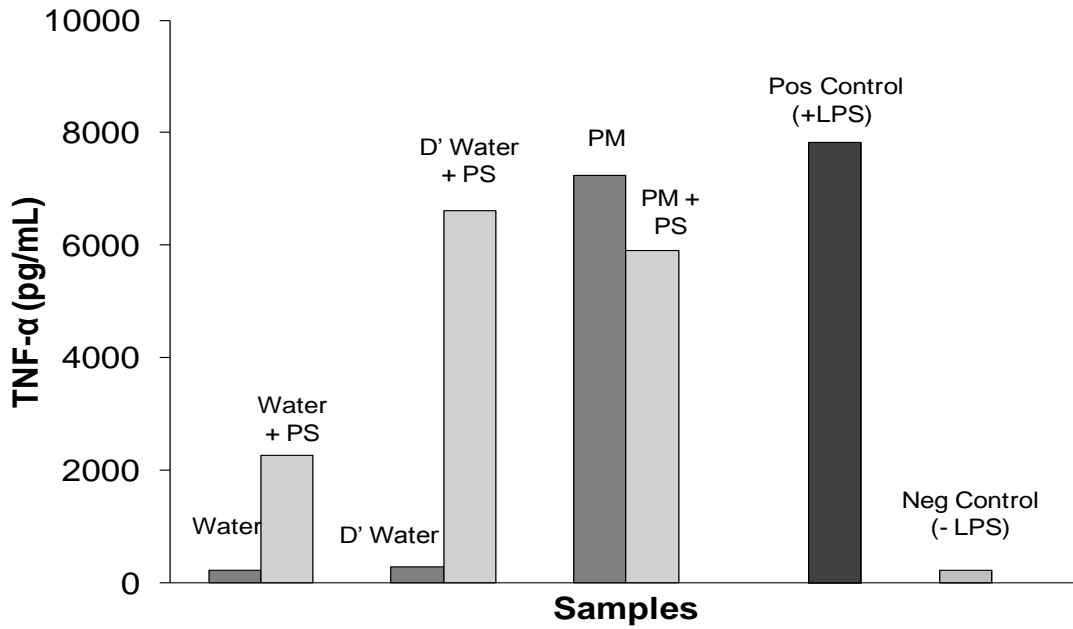
Fig 2. TNF-α levels in water, deionized water (D'Water) and perfusion media (PM) before and after circulation through the perfusion system (PS).
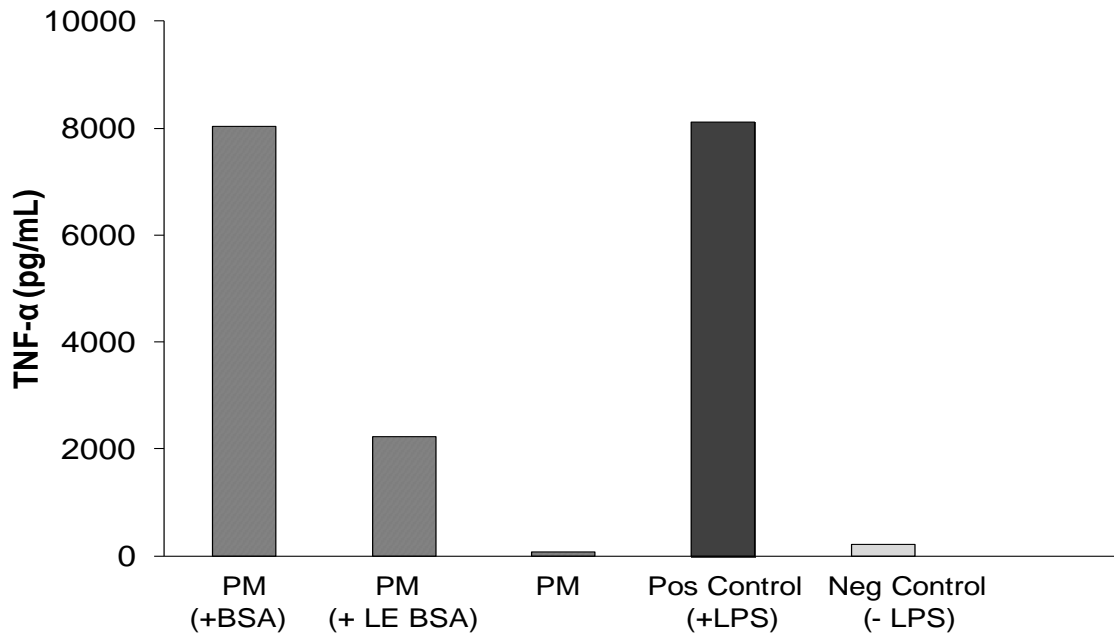
Fig 2.1 TNF-α levels in perfusion media in the absence of BSA (PM), in presence of BSA (PM + BSA) and in low endotoxin BSA (PM + LE BSA)

2.8 DISCUSSION

Water and D'water had TNF-α levels similar to that of the negative control that was free of LPS, indicating that the two solutions were also free of LPS; however upon circulation of water and D'water through the perfusion system, TNF-α levels spiked dramatically. These findings implicate the perfusion system as a source of contamination. Following each perfusion, the system was cleaned using an acid-base protocol that may not be able to effectively remove LPS buildup in the system. These findings demonstrate the importance of maintaining equipment pyrogen-free following LPS introduction as the buildup of LPS in a system can greatly alter the outcome of subsequent experiments. Therefore, to circumvent LPS contamination and its carry over in laboratory equipment, a cleaning protocol that can effectively reduce LPS buildup to levels that are in accordance with FDA guidelines should be investigated and implemented.

Perfusion media made with commercially prepared BSA (PM + BSA) had increased TNF-α levels in comparison to media that was made with low-endotoxin BSA (PM + LE BSA). Media prepared in the absence of BSA had decreased TNF-α expression in comparison to PM + BSA and PM + LE BSA. These findings implicate commercially prepared BSA as another source of contamination. Albumin is the primary protein found in serum, constituting approximately 60% of the total protein (Francis, 2010). Serum albumin regulates oncotic blood pressure, pH and also has antioxidative properties. Serum albumin also functions as a carrier protein, facilitating the transfer of lipids, metal ions and

amino acids (Francis, 2010). For these reasons serum albumin can have many biochemical applications in research. BSA is widely used in immunology due to its low cost and increased availability compared to HSA (Phillips, 1966; Francis, 2010). The quality of commercially prepared BSA, however, has undergone a lot of scrutiny due its reoccurring issues with LPS contamination. Traces of LPS in BSA can drastically affect the outcome of results during experimentation. Therefore, it is important to assess the quality of BSA prior to its use in order to avoid misinterpretation of results.

CHAPTER 3:

DEPYROGENATION

3.1 Introduction

Given the problems that surround LPS contamination in research, it is important to effectively remove LPS from laboratory equipment such that LPS contaminants do not carry over. LPS is often applied in the placental perfusion model to evoke placental inflammation. Inadequate cleaning of the perfusion system can augment cytokine expression (as shown in Chapter 2). Thus, to accurately measure placental inflammatory responses, the perfusion system needs to be pyrogen-free. Although the technique of how to dually perfuse a placental lobule has been extensively described, a cleaning protocol for the model has not. Thus, the second aim of this thesis was to validate a cleaning protocol effective in LPS removal to prevent the carryover of contaminants between subsequent perfusions.

3.2 Depyrogenation

Depyrogenation is a term used to define the removal of pyrogens – substances that elicit a febrile response – like LPS, from solutions (Williams, 2007). To date, conventional sterilization procedures for endotoxin removal are not sufficient. LPS molecules retain their endotoxic nature even under extreme conditions. They are heat-stable, resistant to normal desiccation techniques and unaffected by steam sterilization (i.e. autoclaving). For effective depyrogenation of laboratory equipment that are manufactured from heat resistant material like glassware, a minimum temperature of 250°C for at least half an hour needs to be implemented (Williams, 2007). Unfortunately, not all laboratory equipment can withstand high temperatures for such a long duration (i.e. plastic ware) without yielding deformation of the material. Thus, the application of dry heat in endotoxin removal is not universally applicable for all laboratory equipment posing a challenge for LPS removal (Williams, 2007).

3.3 Depyrogenation via Acid-Base Hydrolysis

Acid-base hydrolysis is a form of non-heat inactivation of LPS and has shown to be effective in reducing endotoxic activity by way of lipid A detachment (Williams, 2007). Inactivation by acid hydrolysis occurs due to the removal of the KDO residue – the eight carbon sugar which connects the lipid A moiety to its core polysaccharide. Free lipid A is insoluble in aqueous solutions resulting, in the reduction or elimination of endotoxic activity (Williams, 2007). Contrary to these findings however, Galanos *et al.* have demonstrated that lipid A in its free

form combined with BSA has analogous pyrogenic features to that of an intact LPS molecule due to its ability to solubilize lipid A (1972). This is problematic for equipment and solutions that repeatedly come in contact with BSA. Therefore, endotoxin removal by acid-base hydrolysis to a system that is under constant exposure to BSA, like the placenta perfusion model, is not effective, as residual lipid A in its free form has the possibility of reverting back into its functional parent form.

3.4 Oxidative Depyrogenation

Oxidative depyrogenation is another practice that has long been used for endotoxin removal. In 1912, both Hort and Penfold demonstrated the inability of *Salmonella typhosa* cells to produce a febrile response when washed with hydrogen peroxide (H_2O_2). The oxidative degradation of fatty acids found in lipid A is believed to be the mechanism upon which LPS is rendered inactive (Williams, 2007). As demonstrated by Novitsky, the magnitude at which endotoxin levels are reduced by hydrogen peroxide is dependent on the pH, concentration and the duration at which the peroxide is exposed to LPS (1985). In a span of one hour, Novitsky was able to reduce endotoxin levels by 100% using 27% H_2O_2 at 65°C (1985). The primary advantage of using hydrogen peroxide is its ability to effectively remove LPS molecules without the use of high temperatures – this is of great importance to equipment that cannot tolerate extreme conditions of heat (Williams, 2007).

3.5 LPS Detection

Developed in the 1920s, the only official test capable of endotoxin detection for approximately 37 years was the rabbit pyrogen assay (Magalhaes, 2007; Williams, 2007). The assay was performed by intravenously administering rabbits with a sample of the test solution and subsequently measuring the rise in body temperature (Magalhaes, 2007). Although capable of endotoxin detection, the rabbit pyrogen test lacked efficiency given its high cost and lengthy turnaround time (Magalhaes, 2007). Today, the Limulus Amebocyte Lysate (LAL) assay which utilizes the lysate acquired from the *Limulus polyphemus* horseshoe crab species, has become the most widely used diagnostic test for screening endotoxin contamination of intravenous drugs and medical devices. The activation of the blood coagulation pathway initiated by amebocytes of the horseshoe crab is the basis upon which the LAL assay was developed (Ding, 2010).

Amebocytes of the horseshoe crab contain two granular components – a small and large granule. Following a Gram-negative infection, the granules release clotting factors, proteins and antimicrobials into the plasma, during a process called degranulation which results in the activation of the blood coagulation cascade (Ding, 2010). The large granule has been identified as the compartment that contains all the coagulation factors while the antimicrobial peptides have been found to be localized in the small granular compartment (Ding, 2010).

Three serine protease zymogens are involved in the entrapment of the invading bacteria – Factor C, Factor B and proclotting enzyme (Ding, 2010). Exclusively found in the horseshoe crab, Factor C serves as the LPS-binding protein that becomes autocatalytically activated into its active form in the presence of LPS. The activated Factor C, in turn, activates Factor B, which converts soluble proclotting enzyme into its active form – an insoluble coagulin gel clot (Ding, 2010). The coagulin gel clot then entraps and kills the invading bacteria. An in-depth understanding of the horseshoe crab blood coagulation cascade has led to the development of the LAL gel clot, turbidimetric and chromogenic assays, all of which allow researchers to quantitatively measure LPS levels in test solutions (Magalhaes, 2007).

3.6 METHODS AND MATERIALS

A total of 46 experiments were conducted in new tubing that were contaminated, cleaned and analyzed for the effectiveness of LPS removal. Experiments were divided into three groups. Tubing in group A were perfused with LPS-treated perfusion media and rinsed either with perfusion media (no added LPS; n=3) or non–pyrogenic Sterile Water for injection USP (United States Pharmacopeia; Baxter Corp; n=3). Tubing in group B were perfused with placental perfusion media with (n=10) or without LPS (n=10), cleaned and discarded after single use. Tubing in group C were perfused with placental perfusion media with (n=10) or without LPS (n=10), cleaned and reused 5 times. Tubing from Group B and C were cleaned using either an acid-base or oxidative technique. Endotoxin-free perfusion media (pH 7.4) was used as the test solution for circulation in these two groups.   Prior to cleaning, conditions of each experiment were maintained identical to that of a placental perfusion (4hrs; 37°C) in the absence of a placenta.

Group A

To demonstrate the effect of not having a cleaning protocol in place or that flushing the system with water alone cannot effectively remove LPS, perfusion media in the presence of exogenous LPS (30 EU/mL) was circulated through the perfusion system (4hrs) and subsequently rinsed with either endotoxin-free

perfusion media (40 mL; 1hr) or non-pyrogenic Sterile Water (40 mL; 1hr). Samples were collected at the end of the 1 hr period for analysis.

Group B and C

Non-pyrogenic Sterile Water for Injection USP (40 mL.) was circulated in a closed circuit for 10 minutes and sampled (1 mL) prior to the addition of the perfusion media for baseline measures. The system was then circulated with perfusion media for 4 hours in a closed circuit. Treatment groups were contaminated with 30 EU/mL (3 ng/mL) of LPS (*E.coli* 055:B5; Sigma Aldrich) which was added to the perfusion media at the beginning of the 4 hr circulation; LPS was not added to the perfusion media in control groups. Perfusion media was sampled after 4 hours of circulation (1 mL). The system was then cleaned using one of two cleaning methods, acid-base or oxidative. Acid-base cleaning was modified from that of Living Systems Instrumentation (St. Albans, Vermont, USA) by the addition of 70% ethyl alcohol. Oxidative cleaning was modified from the protocol of Novitisky *et al.* by using exposing the tubing to a temperature of 45°C instead of 65°C (65°C in the presence of concentrated hydrogen peroxide (30%) resulted in the oxidation of connectors and tubing). Details of the two cleaning methods are depicted in Table 1.

Acid-Base Cleaning			
Cleaning Reagent	**Temperature**	**Duration**	**Circulation**
De-ionized H_2O	Room Temperature	5 min	Open
1 % NaOH	45°C	30 min	Open
1% Citric Acid	Room Temperature	10 min	Open
De-ionized H_2O	Room Temperature	10 min	Open
70% Ethyl Alcohol	Room Temperature	10 min	Open
Oxidative Cleaning			
30% H_2O_2	45°C	1 hour	Closed

Table 1: Acid- Base and Oxidative Cleaning Procedures

Following acid-base or oxidative cleaning, the system was aerated for 3 minutes to dry all surfaces. Non-pyrogenic Sterile Water for Injection USP (40 mL) was then circulated through the system in a closed circuit for 10 minutes and sampled (1 mL) to test the efficacy of the cleaning. The time course of the experiment is illustrated in Figure 3.
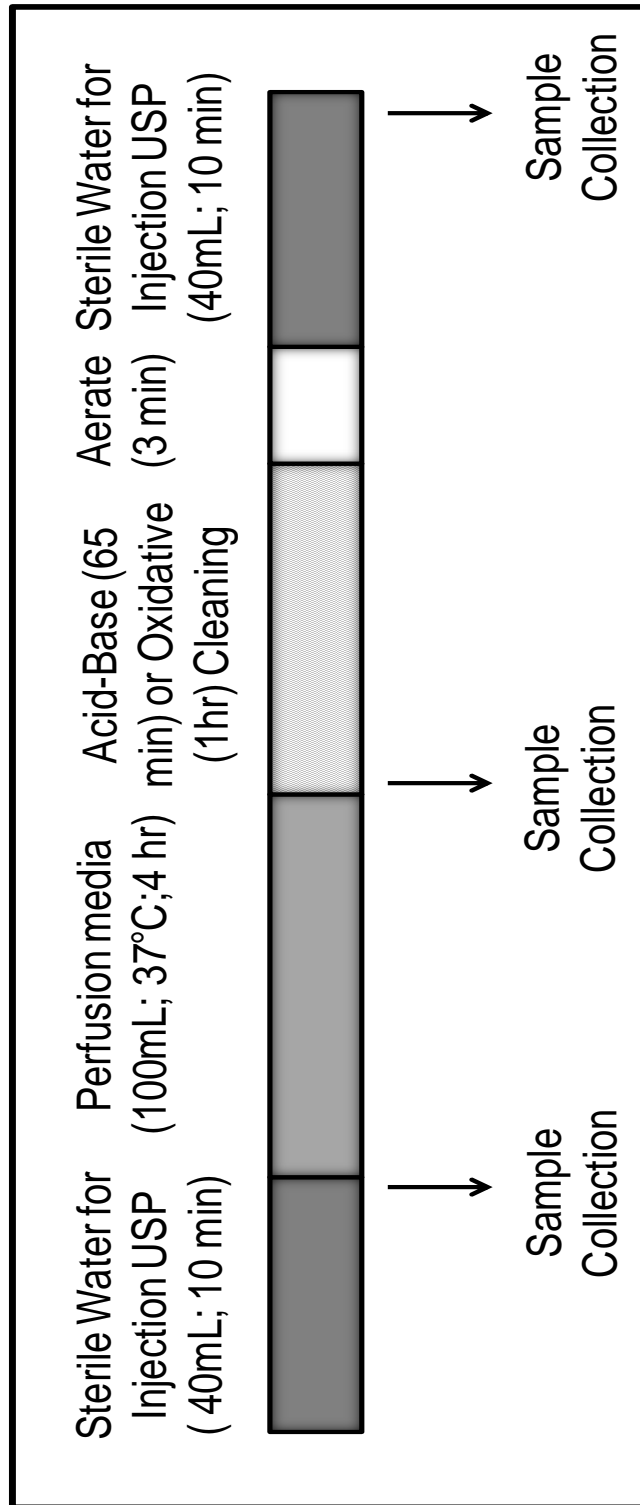
Fig 3. Time course of acid-base and oxidative cleaning (not to scale)

All solutions were perfused through the system at a flow rate of 12 mL/min. Non-pyrogenic Sterile Water for Injection USP and perfusion media were collected using non-pyrogenic pipette tips and stored in pyrogen-free polypropylene tubes (1.5 mL). Immediately following sample collection all vials were stored at -80°C for analysis. Solutions that had a LPS concentration of ≥ 0.5 EU/mL threshold level were deemed as contaminated. Aseptic techniques were practiced throughout each cleaning experiment.

LPS levels were detected using the Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation Kit (Pierce Thermo Scientific). In brief, all samples were thawed at room temperature and diluted accordingly in pyrogen-free polypropylene tubes (1.5 mL) using endotoxin free water to achieve concentrations within the standard curve of the LAL assay (0.01-1 EU/mL). Standards and samples were subsequently vortexed for 1 minute prior to sample loading as per guidelines of the kit. The microplate was equilibrated on a heating block for 10 minutes at 37°C ± 1. With the microplate maintained at 37°C ± 1, standards, blanks and samples (50 µL) were individually dispensed into each well of the microplate. The microplate was then incubated on a heating block for 5 minutes. Following incubation, 50 µL of the reconstituted LAL reagent was dispensed into each well and incubated for 10 minutes. After 10 minutes, 100 µL of the reconstituted chromogenic substrate was dispensed into each well of the microplate and incubated for an additional 6 minutes. To stop the reaction, 100 µL of the stop reagent (25% acetic acid) was dispensed into each well. All

standards, samples, blanks and reagents were dispensed using a non-pyrogenic

pipette tips. Furthermore, following the addition of each reagent and prior to

incubation, the microplate was shaken on a plate shaker for 30 seconds at 100

rpm to ensure appropriate mixing. The plate was read at 405 nm using a plate

reader (Victor[3]; Perkin Elmer). LPS levels are expressed as mean ± standard

error (SE).

3.7 RESULTS

3.7.1 Group A: Tubing contamination

LPS-treated perfusion media used to contaminate tubing had 27.4 ± 6.1 and 28.6 ± 5.2 EU/mL of LPS. Subsequently, circulated endotoxin-free perfusion media and Sterile Water used to flush the tubing had 6.8 ± 0.8 and 5.2 ± 2.5 EU/mL of LPS, respectively. LPS levels for Group A are shown in Figure 3.1.

3.7.2 Group B: Depyrogenation in Tubing Intended for Single Use

Sterile Water for Injection USP that was initially circulated to attain baseline measures of the perfusion system had minimal to no LPS in control and treatment groups. Perfusion media that was circulated for 4 hours with no added LPS contained on average 3.9 ± 0.5 and 2.2 ± 0.7 EU/mL of LPS in tubing intended for acid-base and oxidative cleaning, respectively. Perfusion media, which was circulated through the perfusion system containing the endotoxin, had average LPS levels of 36.9 ± 7.5 and 35.0 ± 3.7 EU/mL in tubing intended for acid-base and oxidative cleaning, respectively.. Post cleaning, Sterile Water for Injection USP that was circulated contained LPS levels that were well below the set threshold of 0.5 EU/mL in control and treatment groups. LPS levels for Group B are shown in Figure 3.2.

3.7.3 Group C: Depyrogenation in Tubing Intended for Reuse

Minimal to no LPS was detected in Sterile Water for Injection USP that was circulated to attain baseline measures of the perfusion system throughout the five days for both control and treatment groups. Perfusion media that was circulated for 4 hours with no added LPS contained on average 3.6 ± 0.5 and 3.8 ± 0.5 EU/mL of LPS in tubing intended for acid-base and oxidative cleaning, respectively. Perfusion media that was circulated through the perfusion system containing 30 EU/mL of the endotoxin had average LPS levels of 58.8 ± 4.4 and 34.9 ± 1.3 EU/mL in tubing intended for acid-base and oxidative cleaning, respectively. Following acid-base cleaning in control and treatment groups, 8 % of the samples contained LPS levels above the set threshold limit of 0.5 EU/mL while no LPS levels were detected post-oxidative cleaning. LPS levels in Group C are shown in Figure 3.2.
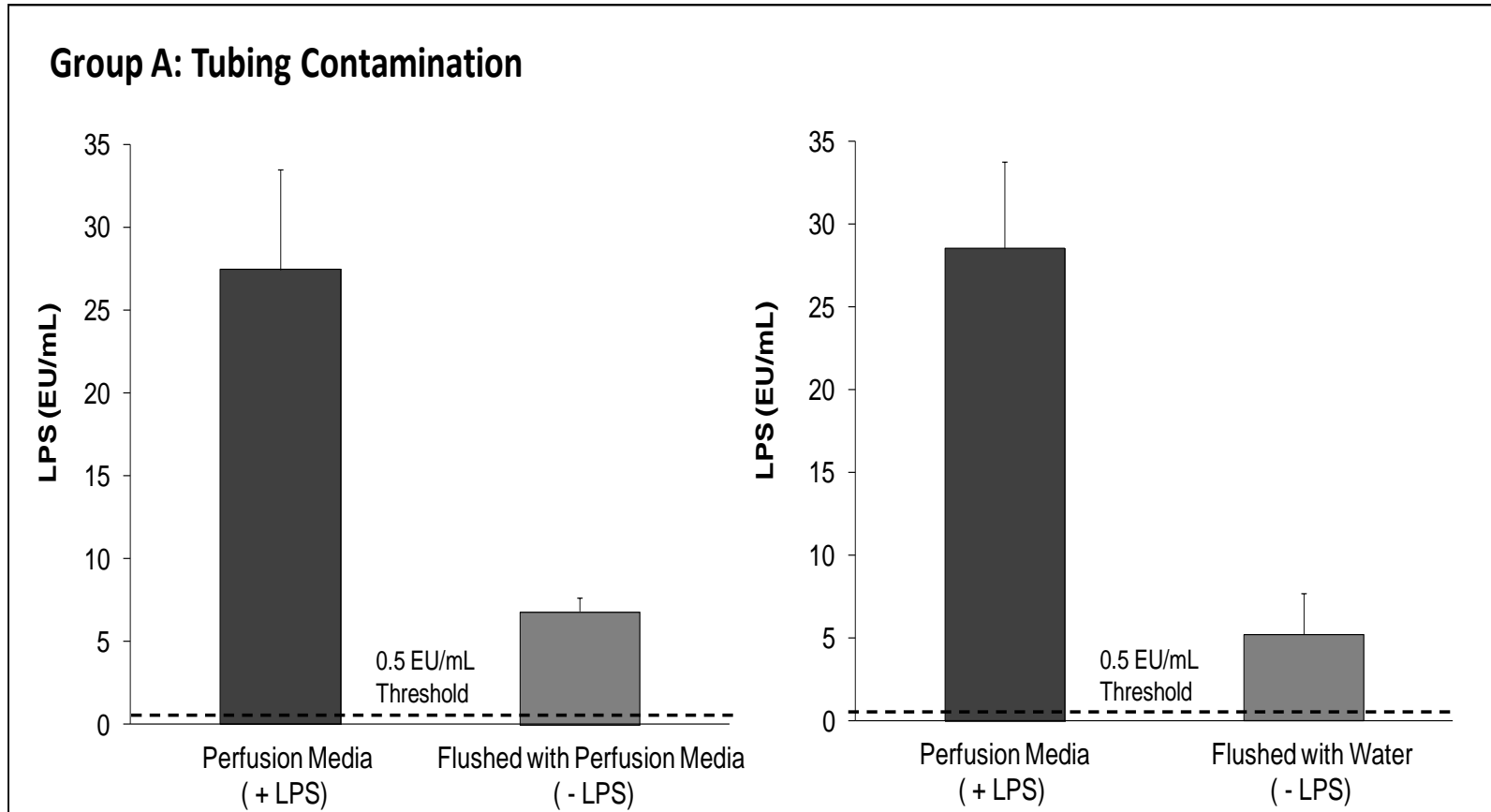
Fig 3.1 LPS levels in the perfusion system when flushed with endotoxin-free perfusion media (n=3) and Sterile Water (n=3)
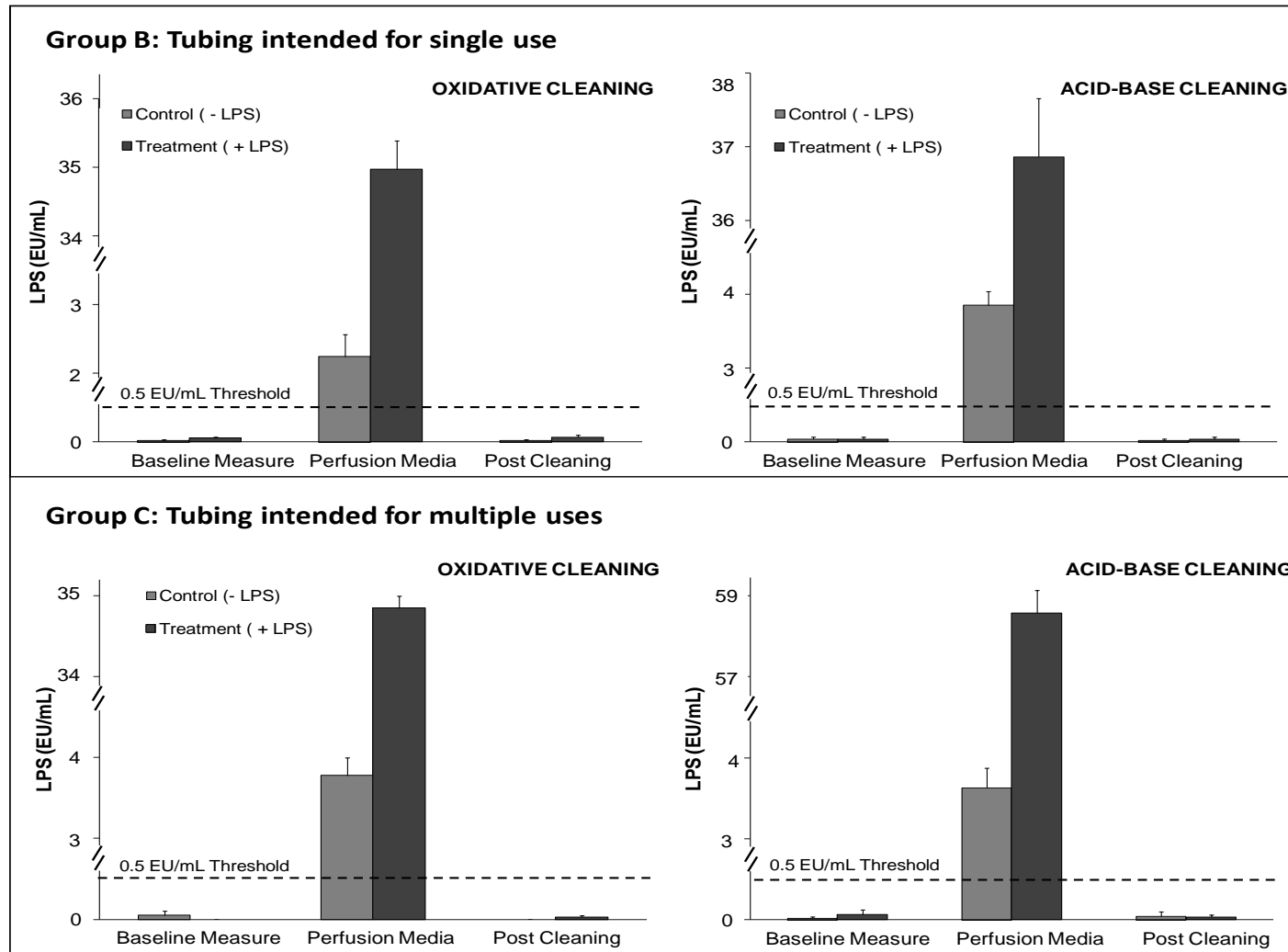
Fig 3.2 LPS levels in the perfusion system following oxidative and acid-base cleaning
in tubing intended for single use (Group B) and multiple uses (Group C)

3. 8 DISCUSSION

Introducing exogenous LPS molecules into the perfusions system leads to LPS-derived contamination if the endotoxin is not effectively removed. Results from Group A demonstrate that without an effective cleaning protocol in place or flushing the system with Sterile Water cannot effectively remove the presence of exogenous LPS molecules.

Tubing intended for single use (Group B) had minimal (< 0.11 EU/mL) to no LPS following acid-base and oxidative cleaning in both control and LPS-treated groups. These findings indicate that both cleaning methods were effective in LPS removal, as LPS-treated groups, were reduced to levels well below the FDA limit of 0.5 EU/mL following cleaning. In addition, following consecutive contamination of the same tubing (Group C), both cleaning methods yielded low levels of LPS that were also below the FDA limit. Thus, these findings demonstrate that both acid-base and oxidative cleaning are capable of maintaining the perfusion system "LPS free" for at least 24 hours following the last cleaning procedure.

Although these findings seem promising, it is still unclear as to whether or not acid-base and/or oxidative cleaning can sustain LPS levels below the 0.5 EU/mL cut off for more than 24 hours. In Chapter 1, I demonstrated that LPS was not adequately removed from the system following acid-base cleaning, however results from Group B and C suggests otherwise. A potential explanation

for the discrepancy between these results may be the ineffectiveness of acid-base cleaning in LPS removal over time. Thus, additional testing is needed to determine if acid-base and/or oxidative cleaning can effectively maintain LPS levels below the 0.5 EU/mL cut off overtime (i.e. ≥ 24 hours) in reused tubing following the last cleaning procedure.

Findings from these experiments also reveal that the endotoxin-free perfusion media is not truly free of LPS. Although the perfusion media was prepared under sterile conditions, traces of LPS were still present in the media. LPS levels in the perfusion media from controls (Group B and C) ranged from 0.45 to 4.93 EU/mL, suggesting that there still may be traces of LPS present in the endotoxin free BSA that needs to be accounted for. Moreover, LPS-treated perfusion media contained LPS levels that either exceeded or fell below the intended LPS dosage of 30 EU/mL. Increased LPS concentration (>30EU/mL) in LPS-treated media may be attributed to an additive effect of the added LPS dose in combination with the traces of LPS already existing in the perfusion media. Furthermore, inaccurate pipetting may also provide a possible explanation to why LPS-treated perfusion media were above or below the intended dosage.

CHAPTER 4:

MATERNO-FETO-PLACENTAL CYOTKINE MAKE-UP

4.1 Introduction

Analysis of perfusates from perfusions without LPS revealed unexpectedly high levels of TNF-α, indicating an activation of the inflammatory pathway that cannot be solely attributed to the added LPS. A potential explanation for this may be that cytokines already present in the placenta are activated upon perfusion. Therefore, the third aim of the thesis was to determine if the placenta was a source for augmenting cytokine expression during placental perfusions, by profiling baseline levels of pro-inflammatory mediators TNF-α, IL-1β, IL-6 and IL-8 in the materno-feto-placental unit.

4.2 Placental Function

Intrauterine fetal growth and development is markedly determined by the placenta – a hemochorial organ that serves as the interface between the mother and fetus (Blackburn, 2007). The placenta functions in fetal respiration, nutrient absorption and waste excretion (Wang, 2010). Additionally, it serves as an immunoprotective barrier by preventing the passage of pathogens into the fetal environment (Blackburn, 2007). Throughout the course of pregnancy, the role of the placenta is to promote an intrauterine environment that is optimal for fetal growth and development. Therefore, alterations to its vascular organization or anatomical structure results in placental dysfunction leading to poor intrauterine fetal development and subsequent postnatal complications (Blackburn, 2007).

4.3 Placental Circulation

Adequate blood flow to the placenta is important in the exchange of nutrients, gases and waste products between the maternal and fetal compartments (Blackburn, 2007). To accommodate the bidirectional transport of substances from the maternal to fetal circulation and *vice versa*, the placenta contains two separate circulations – the uteroplacental (maternal-placental) and fetoplacental (fetal-placental). Equipped with two distinct circulatory systems the placenta thus prevents the mixing of the maternal and fetal blood supply (Wang, 2010).

4.3.1 Uteroplacental Ciruclation*:*

Maternal blood from the systemic circulation enters the placenta via the uterine arteries. From the uterine arteries, blood traverses through the spiral arteries of the basal plate making its way into the intervillous space – the region of the placenta that lacks vascularization (Wang, 2010). Blood from the intervillious space perfuses into the terminal villi – the site of oxygen and nutrient exchange (Blackburn, 2007). Oxygenated nutrient-rich blood from the maternal circulation surrounds the terminal villi where nutrients and oxygen are exchanged with fetal blood (Wang, 2010). Following exchange, deoxygenated blood flows to the intervillious space into the uterine veins where it is returned back to the maternal systemic circulation.

4.3.2 Fetoplacental Circulation:

The umbilical cord is responsible for the fetoplacental circulation, consisting of three vessels – two umbilical arteries that are coiled around a single umbilical vein (Wang, 2010). The vessels of the umbilical cord attach to the chorionic plate of the placenta where it branches radially across the placental surface, entering the terminal villi (Blackburn, 2007). Oxygenated nutrient-rich blood from the placentas is supplied to the fetus via the umbilical vein while deoxygenated nutrient-depleted blood from the fetus is transported to the placenta via the two umbilical arteries (Wang, 2010).

4.4 Cervical ripening

Cervical ripening – the softening of the cervical tissue in pregnant women – is a process that commences approximately 4 weeks before parturition (Blackburn, 2007). Cervical ripening is an inflammatory process orchestrated by the production of pro-inflammatory cytokines. Of particular interest are IL-1, IL-6, IL-8 and TNF-α, all of which have been identified to play a role in cervical ripening (Blackburn, 2007; Keelan, 2003). In brief, IL-1 and TNF-α participate in the changes made to the vascular epithelium; IL-6 has been shown to induce the production of prostaglandin (E2), which promotes dilation of the cervical blood vessels; IL-1 and IL-8 augment the production of matrix metalloproteinase (MMP)-8 – a degradative enzyme that promotes cervical ripening (Blackburn, 2007). While the inflammatory state induced during cervical ripening is predominantly from local cytokine production, the placenta may also exert effects in preparation for parturition.

IL-1, IL-6, IL-8 and TNF-α have all been detected in non-laboring placental tissue during the third trimester (Bowen, 2002). Moreover, mRNA expression of the above four mentioned cytokines has also been identified in trophoblast and decidual tissues (Bowen, 2002). Thus, concentrations of IL-1, IL-6, IL-8 and TNF-α in the placental tissue, following birth, may already be elevated prior to placental perfusion. Therefore, it is important to characterize placental inflammatory responses, prior to experimentation.

To date, placental perfusions have only measured the inflammatory state of the placenta in the presence and absence of an external stimulus. The inflammatory state of the placental unit prior to perfusion (i.e. following birth), however, has yet to be described. Given that the placenta does not function as a single unit and is equipped with two circulatory systems of fetal and maternal origin, assessing pro-inflammatory mediators in the placental tissue alone would not be sufficient. For that reason, maternal and fetal blood need to be concurrently examined with placental tissue – collectively coined as the materno-feto-placental unit. This will allow for a more-holistic understanding of the organ with respect to its cytokine makeup.

4.5 MATERIALS AND METHODS

To comparatively analyze cytokine levels within the materno-feto-placental unit, placentas were obtained from singleton births of pregnant mothers delivering at term (GA≥37) via an elective cesarean section. Patients were recruited to the study from January 2013 to June 2013. All patients were enrolled from McMaster University Children's Hospital, Labor and Delivery Ward (n= 33). Informed written consents were obtained by a health professional. The study was approved by Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board (REB # 11-117-T). Clinical and laboratory health records of each patient were additionally extracted using Sovera Health Information Management system. Extracted maternal parameters include: age (yrs), pre-pregnancy body mass index (BMI) (kg/m^2), systolic blood pressure (mmHg), diastolic blood pressure (mmHg), diabetic status, smoking status and white blood cell count. Fetal birth weight (g), gestational age (wks) and the gender of the infant were parameters that were also extracted from health records.

4.5.1 Maternal Plasma Samples

Routine CBC samples (~1-2 mL) drawn from mothers prior to delivery were collected in K_2 EDTA coated vacutainers (REF # 367842; BD) and stored at 4°C at McMaster University's Hamilton Regional Lab Medicine Program (HRLMP) Facility. Leftover whole blood samples not used for CBC analysis were transported to the research lab within 24 hours of the initial blood draw and centrifuged at 4°C for 10 min at 1207 *g* (Beckman GPR Centrifuge, USA)

Aliquots of the separated plasma (~1.5 mL) were stored at -80°C in polypropylene tubes for analysis.

4.5.2 Umbilical Serum and Placental Tissue Samples:

To establish cytokine levels in the fetal circulation, 4 – 8 mL of blood was drawn from the umbilical vein of the placenta immediately following birth. Blood samples were collected in silicone coated vacutainers (REF # 366430; BD) and centrifuged at 4°C for 10 min at 1207 *g* (Beckman GPR Centrifuge, USA). Aliquots (~1.5 mL) of the separated serum were stored at -80°C in polypropylene tubes for analysis.

From the same placenta, an intact peripheral cotyledon was identified following careful inspection of the chorionic (fetal) and corresponding decidual (maternal) surface. Using tissue forceps and a pair of micro dissecting scissors, tissue segments weighing approximately 1 - 2 grams were extracted from both the maternal and fetal surfaces. Tissue segments were first dissected to remove any connective tissue, vessels and/or calcium deposits prior to sampling. To minimize sampling both the chorionic and decidual tissue together, tissue segments were dissected transversely. All tissue specimens were placed in 1.5 mL polypropylene vials, immediately snap frozen in liquid nitrogen and subsequently transferred to a -80°C freezer.

4.5.3 Tissue Homogenization:

Frozen tissue segments were weighed and homogenized in a ratio of 1:10 in previously cooled phosphate buffered saline (pH 7.4) using a Brinkmann

Polytron homogenizer (Type PT 10-20-3500). Tissue homogenates were centrifuged at 2°C for 30 min at 12,000 g (Eppendorf Centrifuge 5415R). Aliquots (2 mL) of the separated supernatant were stored at -80°C in polypropylene tubes for analysis.

4.5.4 Sample Analysis:

To analyze IL-1β, IL-6, IL-8 and TNF-α expression in serum, plasma and supernatant samples, a ProcartaPlex Multiplex Immunoassay (affymetrix, e-Bioscience) was performed using Luminex-based technology. All samples were measured simultaneously; tissue samples were loaded on one plate and serum and plasma samples were loaded on a second plate. The immunoassay was performed by a trained professional (Pers. Comm., L. Wiltshire 2013). The assay was performed according to protocol, except that 50 µL of the maternal plasma and umbilical serum were diluted in sample diluents at a ratio of 1:4. Tissue samples were not diluted. The standard curve ranges and sensitivity for each of the 4 analytes are described in Table 2.

Table 2: Standard curve range and sensitivity of each analyte

	TNF-α	IL-1β	IL-6	IL-8
Standard Curve Range (pg/mL)	8.54-35,000	2.44-10,000	9.77- 40,000	2.44-10,000
Sensitivity (pg/mL)	0.4	0.2	0.4	1.2

4.5. 5 Total Protein in Placental Tissue

To quantitatively measure total protein concentration in tissue supernatants a BCA (bicinchoninic acid) protein assay was additionally performed (Thermo Scientific). The assay was performed to protocol. Tissue samples were diluted at a ratio of 1:8. Absorbance was read at 563 nm via a plate reader. The standard curve range was 0 to 2000 µg/mL.

4 5.6 Statistical Analysis

Sample size calculation for descriptive analysis of a single study ($N = 4\sigma^2 (Z_{crit})^2/D^2$) was used to determine the number of participants needed for the study using α of 0.05 (Eng, 2003). Standard deviation (SD) was assumed using cord blood IL-6 levels based on the findings reported by Takahashi *et al.* (2010). Based on sample size calculation a minimum of 30 participants were needed to yield significant differences between variables. Cytokine levels below the lower limit of detection were excluded from statistical analysis. Data within the detectable limit underwent a logarithmic transformation given the distributions of the data were not parametric. Continuous and categorical data extracted from participants were represented as means ± SD and percentages, respectively. A student t-test was performed to determine if IL-1β, IL-6, IL-8 and TNF-α were statistically different between maternal plasma and umbilical venous serum. A paired t-test was performed to determine if IL-1β, IL-6, IL-8 and TNF-α were statistically different between decidual and chorionic placental tissue. Additionally, a Spearman rank correlation test was used to determine the

relationship between each variable. Correlations that were statistically significant were additionally graphed as scatter plots. Maternal and fetal variables that yielded a significant correlation with cytokine levels ($P < 0.1$) were included in a multivariate linear regression model. Results were considered statistically significant when P-values were < 0.05. All statistical analyses were performed using IBM SPSS Statistics Software version 19.

4.6 RESULTS

4.6.1 Demographic Characteristics

Maternal age ranged from 18 to 40 years of age with women having an average age of 31.5 years during pregnancy. Regarding pre-pregnancy BMI, only 8 mothers were of normal weight (18.5 to 24.9 kg/m^2); remainder of the women were overweight (n = 8) or obese (n = 9); pre-pregnancy BMI records were missing from 8 mothers (Institute of Medicine, 2009). Pre-delivery, maternal systolic and diastolic pressure ranged from 99 to 154 mmHg and 62 to 100 mmHg, respectively. Maternal diabetes (type I, type II and gestational diabetes mellitus) was reported in 15% of the mothers during pregnancy. Of the mothers enrolled in the study, 88% reported never smoking. Birth weight of the infants ranged from 2800 to 4400 g, of which 24% (n = 8) weighed > 4000 g. All infants in the study were born at term (GA ≥ 37 wks). Demographic characteristic of the 33 mothers and infants enrolled in the study are summarized in Table 3.

4.6.2 Maternal Plasma and Umbilical Serum Samples

The logarithmic distribution for each cytokine in plasma and serum samples is shown in Fig 4. IL-6 was detected in only 15% of plasma samples and thus excluded from statistical analysis. IL-1β was detected in 88% of plasma samples; IL-8 and TNF-α were detected in all maternal plasma samples. IL-1β was not detected in umbilical serum samples moreover, IL-6 was measured in only 2 serum samples; both measurements were excluded from statistical analysis. TNF-α levels were significantly higher in maternal plasma samples than

umbilical serum samples (see Table 5). Cytokine levels measured in maternal plasma and umbilical serum are shown in Table 4.

4.6.3 Decidual and Chorionic Tissue Samples

Cytokines levels in decidual and chorionic tissue samples are summarized in Table 5. IL-6 and TNF-α were detected in all decidual tissue samples; 94% of the samples had detectable levels of IL-1β and IL-8. IL-1β, IL-6 and TNF-α were measured in all chorionic tissue samples; 94% of the samples had detectable levels of IL-6. IL-1β, was significantly higher in decidual tissue compared to chorionic tissue (see Table 7). The logarithmic distribution for each cytokine in tissue samples is shown in Fig 4.

4.6.4 Correlation Coefficient

Correlation coefficients between each variable are shown in Table 8. Scatter plots of cytokines that yielded a significant correlation in placental tissue are shown in Figures 4.1 – 4.5. IL-6 and IL-8 in decidual tissue were strongly correlated with each other while IL-8 and TNF-α showed a moderate correlation. IL-6 and IL-1β were strongly correlated in decidual tissue while IL-8 and IL-1β were moderately correlated.   TNF-α, IL-6 and IL-8 were moderately correlated in chorionic tissue. IL-1β and IL-6 and IL-8 were strongly correlated. A strong correlation was also observed between IL-6 and IL-8 in decidual (r = 0.638) tissue; IL-6 and IL-8 in chorionic tissue showed a very strong correlation (r = 0.812).   No correlation was observed between TNF-α and IL-1β in both tissue

samples. Moreover, cytokines in decidual tissue were not correlated to cytokines in chorionic tissue.

A very strong correlation was observed in maternal plasma IL-1β and TNF-α levels (r = 0.813). A negative weak correlation was observed between maternal plasma and chorionic tissue IL-8 levels. Maternal plasma IL-8 and maternal age showed a moderate correlation. Placental tissue and maternal plasma cytokines were not correlated with umbilical serum cytokines. A moderate correlation however, was observed between umbilical serum IL-1β and maternal plasma IL-6 (r = 0.663). Gestational age (GA) and birth weight (BW) showed a weak correlation. Pre-pregnancy BMI, diabetic status (DS) and systolic pressure (SP) were moderately correlated with one another. Moreover, a strong correlation was observed between maternal systolic and diastolic pressure (DP) (r = 0.858).

4.6.5 Regression Analysis

Multivariate regression analysis included maternal and fetal variables that were significantly correlated with cytokine levels in maternal plasma, umbilical serum and tissue samples (P < 0.1). Variables included in the regression analysis are shown in Table 9. Multivariate analysis did not show any significant association between variables.

Table 3*:* Demographic Characteristics of Participants (n = 33)

Maternal Demographics	
Maternal Age (yrs)	31.5 ± 5.5
Pre-Pregnancy Body Mass Index (BMI) (kg/m^2)	* 30.5 ± 8.2
Systolic Blood Pressure (mmHg)	123.5 ± 13.3
Diastolic Blood Pressure (mmHg)	78.2 ± 10.7
Diabetic	5 (15)
Smoked	4 (12)
White Blood Cell (WBC)	10.4 ± 2.4
Infant Demographics	
Birth Weight (g)	3563 ± 509
Gestational Age (wks)	39 ± 0.9
Sex, Male	20 (60.6)

*Data are represented as means ± SDs or n (%); * mean ± SD for BMI were calculated from n = 25*

Table 4: Cytokine Levels in Maternal Plasma and Umbilical Serum (n = 33)

Cytokines	Within Detectable Range, n (%)	Below Detectable Range, n (%)
Maternal Plasma		
TNF-α	33 (100%)	0 (0%)
IL-1β	27 (81.82%)	6 (18.18%)
IL-6	5 (15.15%)	28 (84.85%)
IL-8	33 (100%)	0(0%)
Umbilical Serum		
TNF-α	29 (87.88%)	4 (12.12%)
IL-1β	0 (0%)	33 (100%)
IL-6	2 (6.06%)	31 (93.94%)
IL-8	9 (27.27%)	24 (72.73%)

Table 5: Differences in Cytokine Values Between Plasma and Serum Samples

Cytokines	Maternal Plasma (pg/mL)		Umbilical Serum (pg/mL)		P-value
	n	Maternal Plasma	n	Umbilical Serum	
TNF-α	33	2.93 ± 0.9	29	1.46 ± 0.44	< 0.0001*

Means ± SDs were calculated using logarithmic transformed data from samples that were within the detectable range. P-values were determined via Student t-test (P < 0.05)*

Table 6: Cytokine Levels in Decidual and Chorionic Tissue Samples (n = 33)

Cytokines	Within Detectable Range, n (%)	Below Detectable Range, n (%)
Decidual Tissue		
TNF-α	33 (100%)	0 (0%)
IL-1β	31 (93.94%)	2 (6.06%)
IL-6	33 (100%)	0 (0%)
IL8	31 (93.94%)	2 (6.06%)
Chorionic Tissue		
TNF-α	33 (100%)	0 (0%)
IL-1β	33 (100%)	0 (0%)
IL-6	33 (100%)	0 (0%)
IL-8	31 (93.94%)	2 (6.06%)

Table 7: Differences in Cytokine Levels Between Decidual and Chorionic Tissue

Cytokine	Decidual Tissue (pg/mg)		Chorionic Tissue (pg/mg)		P-value
	n	Mean ± SD	n	Mean ± SD	
TNF-α	33	1.61 ± 0.73	33	1.61 ± 0.48	0.964
IL-1β	31	2.37 ± 1.28	33	1.93 ± 1.01	0.039*
IL-6	33	4.48 ± 0.40	33	4.55 ± 0.43	0.495
IL-8	31	3.62 ± 0.48	31	3.64 ± 0.53	0.814

Mean ± SD were calculated using logarithmic transformed data from samples that were within the detectable range. P-values were determined via Paired t-test (P < 0.05)*
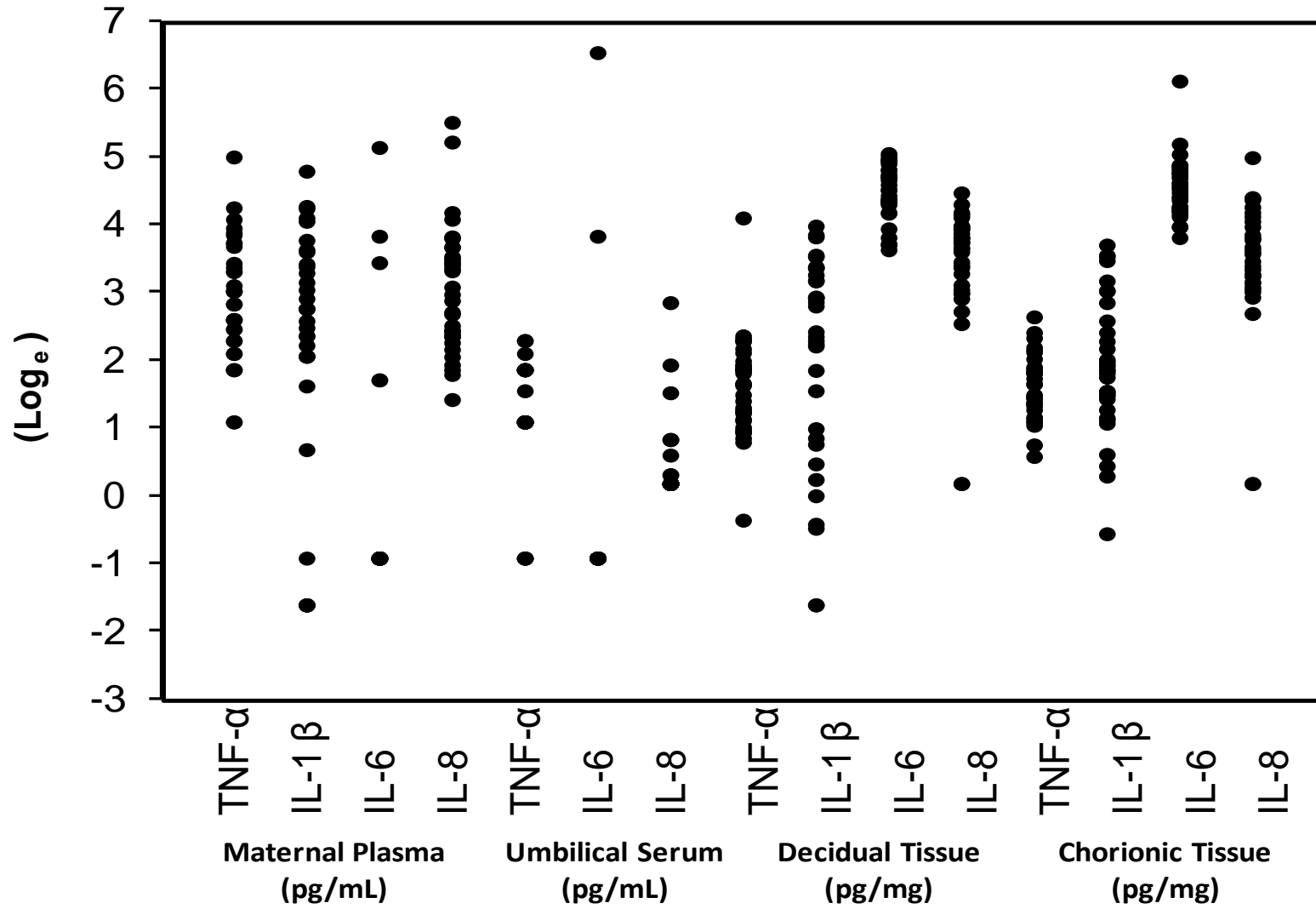
Fig 4. Logarithmic distribution of cytokine levels in maternal plasma umbilical serum and placental tissue

Table 8: Spearman rank correlation coefficient between each variable

		Decidual Tissue				Chorionic Tissue				Maternal Plasma				Umbilical Serum			Infant Variables			Maternal Variables					
		TNF-α	IL1-β	IL-6	IL-8	TNF-α	IL1-β	IL-6	IL-8	TNF-α	IL1-β	IL-6	IL-8	TNF-α	IL1-β	IL-8	SEX	GA	BW	BMI	DS	SS	SP	DP	AGE
Decidual Tissue	TNF-α																								
	IL1-β	0.296																							
	IL-6	.616**	.723**																						
	IL-8	.468**	.507**	.638**																					
Chorionic Tissue	TNF-α	0.125	-0.06	0.05	0.077																				
	IL-1β	0.182	0.012	0.179	0.287	0.329																			
	IL-6	0.231	-0.002	0.135	0.282	.582**	.604**																		
	IL-8	0.047	0.025	0.156	0.256	.596**	.653**	.812**																	
Maternal Plasma	TNF-α	0.174	-0.135	0.046	0.225	0.04	0.127	0.138	-0.081																
	IL1-β	0.194	-0.185	-0.069	0.075	0.114	0.067	0.207	-0.08	.813**															
	IL-6	-0.213	-0.152	-0.163	0.26	0.053	0.19	0.1	0.298	-0.048	-0.026														
	IL-8	-0.083	0.158	0.134	0.004	-0.309	-0.241	-0.334	-.390*	0.301	0.124	-0.123													
Umbilical Serum	TNF-α	0.131	0.065	0.255	0.325	-0.198	0.025	-0.004	0.133	-0.175	-0.216	0.087	-0.014												
	IL1-β	-0.175	-0.088	-0.204	0.284	0.227	0.233	0.284	0.297	-0.072	-0.092	.663**	-0.247	-0.129											
	IL-8	0.149	-0.051	0.145	0.23	0.247	0.078	0.244	0.049	0.189	0.273	0.152	0.098	-0.048	0.206										
Infant Variables	SEX	-0.072	-0.143	-0.098	0.13	0.059	-0.046	-0.059	-0.091	-0.052	-0.101	-0.172	-0.081	0.042	-0.047	0.27									
	GA	-0.004	-0.008	-0.052	-0.082	0.235	0.006	0.208	0.241	-0.071	-0.114	0.198	-0.106	0.265	0.309	0.198	-0.249								
	BW	-0.078	0.112	0.167	0.197	0.133	0.098	-0.029	0.128	-0.041	-0.215	0.167	0.133	0.32	0.026	0.328	0.189	.352*							
Maternal Variables	BMI	-0.046	0.111	0.106	0.087	-0.276	-0.005	0.024	0.004	-0.056	-0.089	0.193	0.111	-0.011	-0.149	0.078	-0.177	-0.148	0.154						
	DS	0.044	0.044	0.124	.355*	-0.178	0.222	-0.018	-0.071	0.098	-0.045	0.299	0.049	-0.215	0.257	0.164	0.168	-0.308	0.115	.510**					
	SS	0.078	0.029	-0.068	0.029	-0.039	-0.176	-0.195	-0.224	0.088	0.015	0.117	-0.132	0.236	0.283	-0.224	-0.081	0.245	-0.117	-0.187	-0.157				
	SP	-0.11	0.136	0.091	0.051	-0.133	0.052	-0.076	-0.043	-0.241	-0.196	.367*	-0.101	-0.298	0.184	0.061	-0.249	-0.11	-0.03	.413*	.530**	-0.294			
	DP	0.053	0.042	0.15	0.108	-0.063	0.301	0.017	0.053	-0.129	-0.122	0.338	-0.148	-0.24	0.246	0.114	-0.234	-0.076	-0.016	0.219	.431*	-0.271	.858**		
	AGE	-0.313	-0.087	-0.153	-0.146	0.03	0.002	-0.104	0.077	-0.04	-0.162	0.118	.402*	0.152	-0.195	-0.212	-0.082	-0.088	0.202	0.246	-0.102	-0.078	-0.089	-0.075	
	WBC	0.031	-.353*	-0.168	-0.006	0.072	0.015	-0.121	-0.035	0.3	0.183	.362*	0.039	-0.149	0.291	0.155	0.033	0.297	0.064	0.053	0.138	0.283	0.007	0.001	-0.181

Bolded values indicate a significant correlation between variables (* p < 0.05; ** p < 0.01); GA = gestational age; BW = birth weight; BMI = pre-pregnancy body mass index; DS = diabetic status; SS = smoking status; SP = systolic pressure; DP = diastolic pressure; WBC = white blood cell
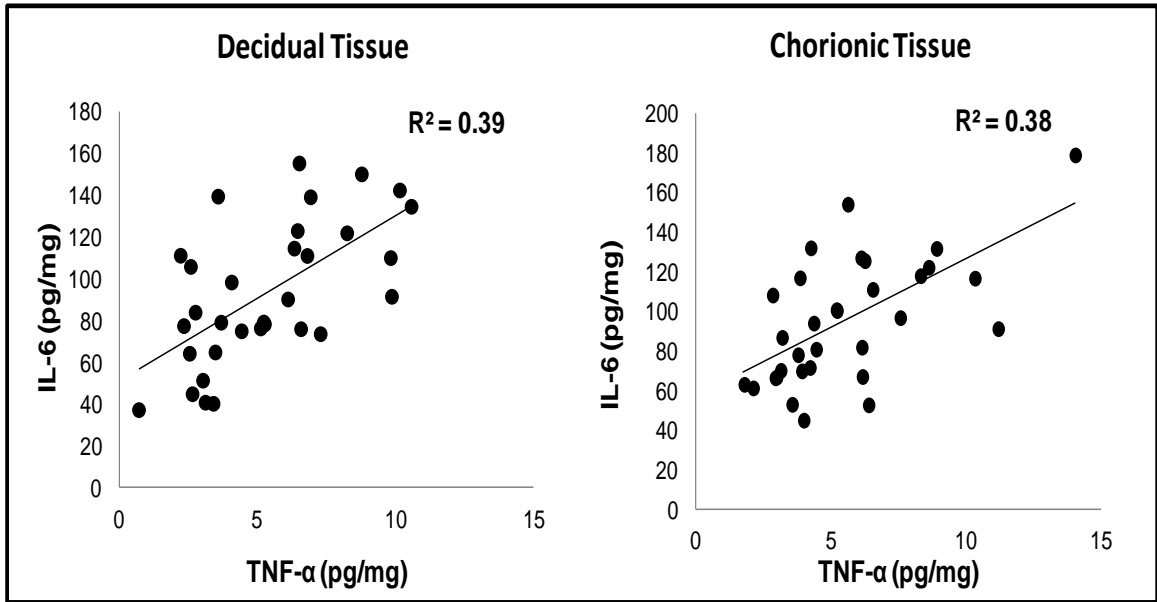
Fig 4.1. Correlation between TNF-α (pg/mg) and IL-6 (pg/mg) in decidual and chorionic tissue
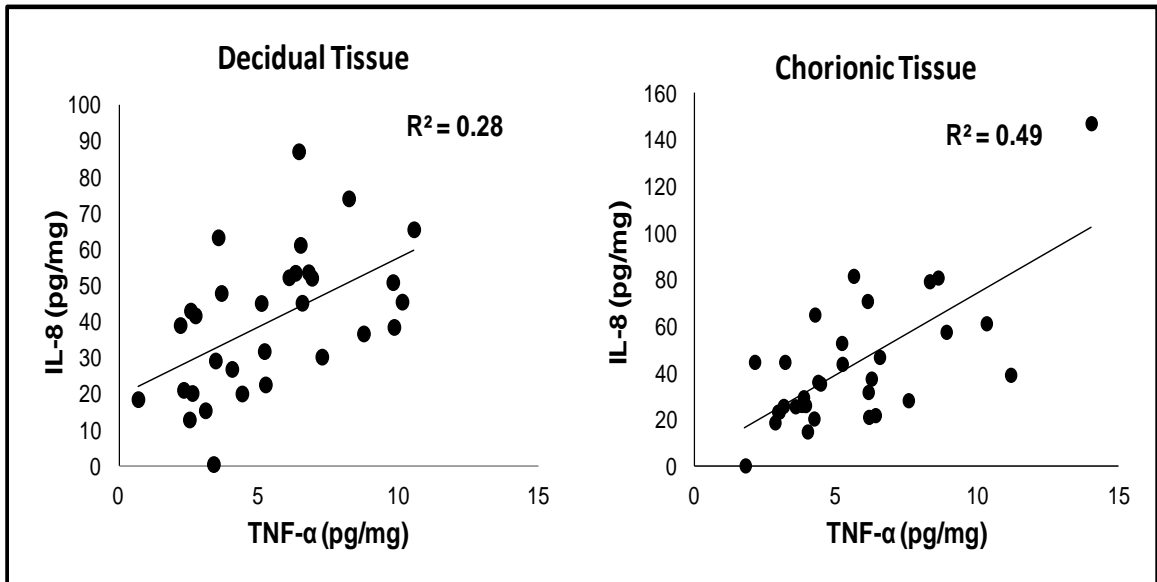

Fig 4.2. Correlation between TNF-α (pg/mg) and IL-8 (pg/mg) in decidual and chorionic tissue
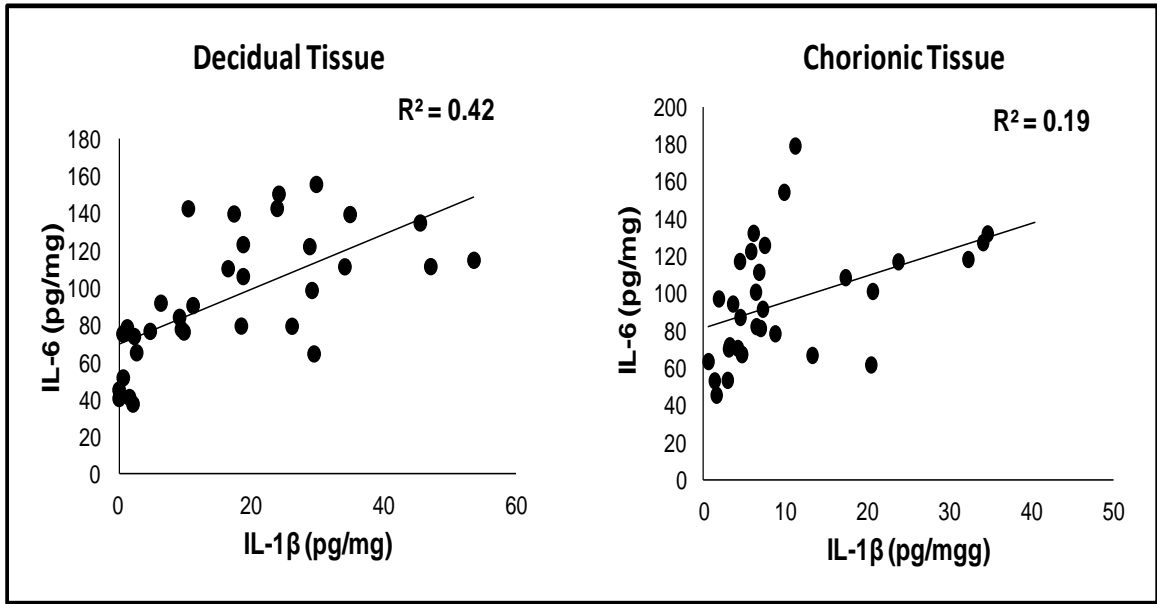
Fig 4.3. Correlation between IL-1β (pg/mg) and IL-6 (pg/mg) in decidual and chorionic tissue
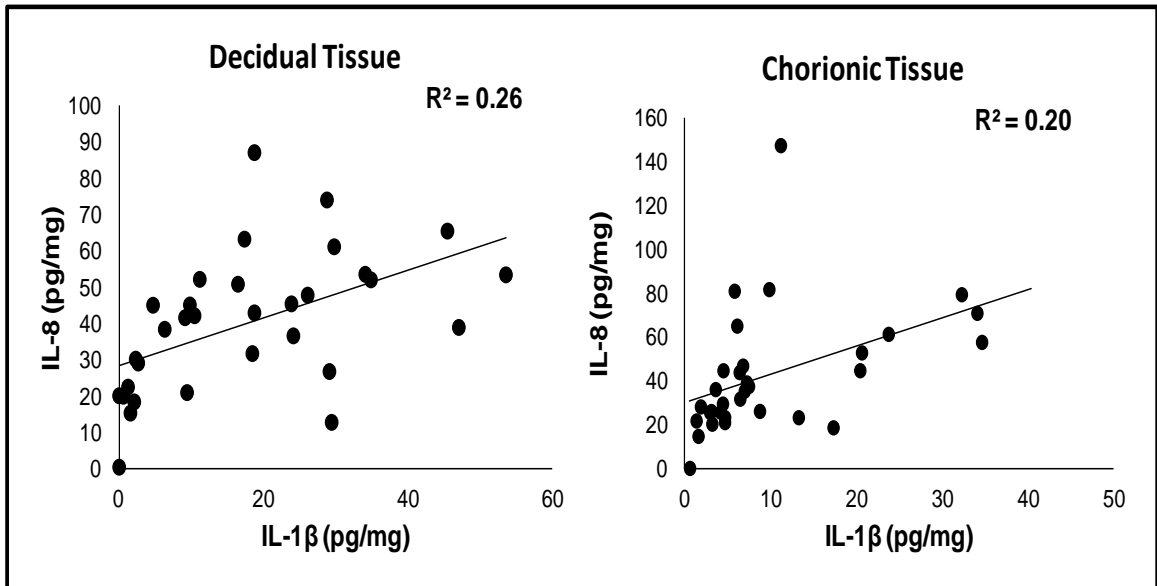
Fig 4.4. Correlation between IL-1β (pg/mg) and IL-8 (pg/mg) in decidual and chorionic tissue
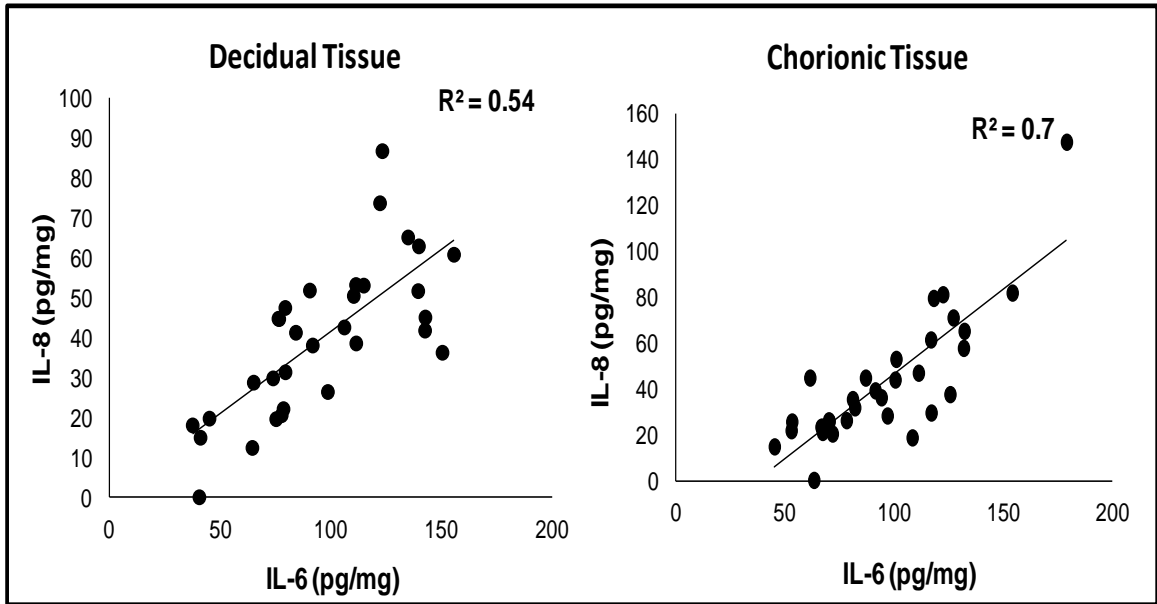
Fig 4.5. Correlation between IL-6 (pg/mg) and IL-8 (pg/mg) in decidual and chorionic tissue

Table 9: Multivariate Linear Regression Analysis

Cytokines	Independent Variables	[a] P-values	[b] P-values
Maternal Plasma			
TNF-α	WBC	0.09	0.05
IL-8	Maternal Age	0.021	0.06
Decidual Tissue			
TNF-α	Maternal Age	0.076	0.149
IL1-β	WBC	0.03	0.919

[a] P-values derived from spearman correlation test

[b] P-values derived from multivariate regression analysis

4.7 DISCUSSION

TNF-α levels in maternal plasma were statistically different from that of umbilical serum, with maternal plasma having higher levels of the cytokine. Moreover, levels of IL-1β in decidual (maternal) tissue were statistically different from chorionic (fetal) tissue with decidual tissue having higher levels of the cytokine. Under normal pregnancy, transplacental transfer of cytokines from maternal to fetal circulation is considered to be minimal (Desoye, 2007). Given that cytokine expression in the fetal environment is tightly regulated by the placenta, expression of pro-inflammatory cytokines TNF-α and IL-1β are assumed to be lower in the fetal circulation, relative to the maternal environment (Desoye, 2007). Thus, differences in TNF-α and IL-1β expression between maternal and fetal samples, with higher levels of the cytokine found in maternal samples, may be explained by the protective role of the placenta in preventing maternally derived cytokines from fetal exposure.

IL-6 and IL-8 showed strong correlation with each other and with TNF-α and IL-1β in placental tissue suggesting that IL-6 and IL-8 expression are dependent on each other and the presence of TNF-α and IL-1β. Given that IL-6 and IL-8 are secondary cytokines, their expression can be enhanced by the presence of TNF-α and IL-1β, which is consistent with the above findings (Malerich, 2006). TNF-α and IL-1β, however, showed no correlation in decidual or chorionic tissue. An explanation for this might arise from the fact that both

TNF-α and IL-1β are primary cytokines, thus it is logical that their expression are independent of each other (Malerich, 2006).

Although cytokines were moderately to strongly correlated in placental tissue samples it is interesting to note that no correlation was found between the two tissue types. The lack of correlation between cytokine make up in decidual and chorionic tissue suggests cytokine expression between the fetal and maternal compartment might be independent of each other. Placental macrophages are present throughout pregnancy aiding in placental development and host defense (Pinhal-Enfield, 2012). Although placental macrophages are localized in both the chorionic villi (fetal surface) and uterine deciduas (maternal surface), they originate from different cells. Placental macrophages found in the fetal chorionic villi are known as Hofbauer cells and are derived from the fetal yolk sac while macrophages on the maternal side are derived from stem cells and are referred to as decidual macrophages (Pinhal-Enfield, 2012). Given that different macrophage cells occupy the maternal and fetal compartments, it is reasonable to think that cytokine expression in the chorionic and decidual tissue might be regulated independently of each other. This may explain why no correlation in cytokine expression was observed between the two tissue types.

Multivariate regression analysis indicated maternal and fetal demographics to not be predictors of cytokine levels in the materno-feto-placental unit. These findings are not consistent with those reported in literature which has shown maternal adiposity, diabetic status and maternal smoking to alter cytokine

expression (Atégbo, 2006; Marseille-Tremblay, 2007; Dowling, 2007). Discrepancies between our findings and those reported in literature may be attributable to insufficient sample size for the above demographics and that our study was not powered for these aspects.

Lastly, the above findings do not implicate the materno-feto-placental unit as a confounding factor for driving high background TNF-α expression. TNF-α levels in maternal plasma and umbilical serum were within physiological range (<10 pg/mL) therefore, cannot be confounding factors for high TNF-α release in the placental perfusion model. Although a mild TNF-α release was observed in tissue specimens the magnitude of release was much lower than that of the perfusates that were initially measured. Thus, although the placenta is the only source for cytokine production in the perfusion model, placentas obtained via c-section (without pathology) were not responsible for driving high background TNF-α expression in maternal and fetal perfusates.

CHAPTER 5:

SUMMARY OF FINDINGS

5.1 SUMMARY AND STRENGTHS

Elevated levels of TNF-α in previously conducted placental perfusions were attributed to (1) LPS contaminated perfusion media and (2) LPS build up in the perfusion system. Using TNF-α expression as a surrogate marker of contamination we found that the use of commercially prepared BSA in perfusion media and inadequate removal of LPS molecules from the perfusion system greatly augmented placental TNF-α expression in the absence of added LPS.

These findings implicate commercially prepared BSA - the chief component of the perfusion media used to perfuse placental lobules - as a source of LPS contamination. As endotoxins, LPS molecules are capable of eliciting immune responses. Traces of LPS found in BSA led to basal contamination of the perfusion media which subsequently resulted in the induction of TNF-α. Presence of LPS in the perfusion media is problematic as controls now contain traces of the same stimulus used in treatment groups. Basal contamination of the perfusion media not only augments cytokine expression in controls but can further enhance cytokine levels in treatment groups. Although we did not observe any difference in TNF-α expression between control and LPS treated groups, the possibility that cytokine expression can be further augmented following LPS challenge exists. In such cases, cytokine expression is largely misinterpreted as it is unclear as to how much of the response is attributable to exogenous LPS vs. basal contamination or the combined effects of both factors. Given that BSA is a common component of the perfusion media, researchers need to be cautious of

its use. Thus, in order to circumvent BSA-derived LPS contamination, the quality of the BSA must be assessed prior to its use. LPS content of BSA is highly dependent on how it is manufactured, thus BSA purchased for the use of placental perfusions, must be certified as endotoxin-free. Although the issue of LPS contamination in BSA has been addressed for decades, the issues of its effects in placental perfusions have not. This is the first study to demonstrate the negative effects between LPS contaminated BSA and augmented placental response.

Inadequate removal of LPS from the perfusion system led to further contamination of the perfusion media. Thus, to overcome LPS contamination of the perfusion system, a cleaning protocol effective in decontaminating the system is of great importance. Oxidative and acid-base depyrogenation techniques effectively reduced the buildup of LPS molecules to endotoxin levels that were in accordance with FDA guidelines for medical equipment (< 0.5 EU/mL). Moreover, both depyrogenation techniques were capable of maintaining the perfusion system "LPS-free" for at least 24 hours following the last cleaning procedure. Additionally, oxidative and acid-base depyrogenation effectively removed LPS molecules without the use of high temperatures – this is of great importance as the perfusion system consists mostly of materials and equipment that cannot tolerate extreme conditions of heat. Both techniques were also cost-effective and not labor intensive. While the effectiveness of both depyrogenation techniques overtime (>24hrs) is yet to be validated in the perfusion system, the above

findings provide a temporary solution for maintaining a LPS-free system. Although the technique of how to dually perfuse a placental lobule has been extensively described in literature, a cleaning protocol for the model has not. To our knowledge, this is the first study that has investigated and validated a cleaning protocol specifically designed for the placenta perfusion model.

By profiling cytokine makeup in the materno-feto-placental unit we were able to determine that the placenta was not driving TNF-α expression in the perfusion system in the absence of exogenous LPS.   Moreover, by profiling baseline levels of TNF-α, IL-1β, IL-6 and IL-8 we have compiled reference values for each cytokine in healthy placentas.   Although other studies have profiled cytokine levels in umbilical serum, maternal plasma and placental tissue independently, to our knowledge this is the first study to have simultaneously analyzed TNF-α, IL-1β, IL-6 and IL-8 expression in the materno-feto-placental unit. Given that the above 4 cytokines play a key role in the pathogenesis of chorioamnionitis these values can serve as reference values for future studies when comparing cytokine levels between healthy pregnancies and those complicated by chorioamnionitis.

5.2 LIMITATIONS

A major limitation to the study was participant recruitment as our study was not the only study actively recruiting mothers from Labor and Delivery at McMaster University Hospital. Mothers that consented for another study could

not be recruited for the purpose our study as other studies needed cord blood samples. Once blood has been completely drained from the placenta it is no longer viable for further use. Thus, participant recruitment does not reflect the number of mothers that were eligible for the study.

Another limitation was sample collection. Placentas that were torn or presented with tissue injury could not be used for tissue analysis. Moreover, placentas that were obtained with an unclamped umbilical cord could not be used to draw cord blood samples (umbilical cords that are not clamped results in blood loss and thus cannot be used for sample collection). Placental sample collection is also time sensitive given that the vessels of the placenta begin to constrict as the temperature drops. Constriction of the vessels restricts blood flow and ultimately results in blood clotting. Occasionally, the core lab (HRLMP) sometimes needed the entire blood sample collected from the participants for additional CBC testing, thus maternal blood samples, at times, were also difficult to obtain. Since we wanted to comparatively assess cytokine levels within the materno-feto-placental unit, any one sample that was missing from a participant (i.e. placental tissue, umbilical cord blood or maternal blood samples) were excluded from the study. Thus, the number samples used for analyses does not reflect the number of participants that were initially recruited for the study.

5.3 FUTURE DIRECTIONS

Having identified the factors contributing to high background TNF-α expression and subsequently minimizing their effects in the system the initial research question - *How does bacterial chorioamnionitis elicit fetal inflammation?* – can now be addressed. In order to circumvent LPS-derived contamination placentas should be perfused with media that contains endotoxin-free BSA. As a quality control, it is also advised that BSA intended for perfusion be additionally tested for traces of LPS using a LAL assay. Moreover, the perfusion system should be cleaned within 24 hours prior to its use using either the acid-base or oxidative protocol outlined in Chapter 3. Given that acid-base and oxidative depyrogenation methods have not been validated for long term use, it is suggested that tubing from the system be periodically exchanged with new tubing. Furthermore, although maternal and fetal demographics did not alter cytokine expression in our findings, maternal and fetal variables that have been reported to alter cytokine expression (i.e. maternal adiposity, maternal diabetes, maternal smoking etc.) should be controlled for when recruiting participants for future placental perfusion studies.

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