11-27-2015 12:00 AM

Brain Injury and Inflammation and Placental Inflammation in Response to Repetitive Umbilical Cord Occlusions in the Near Term Ovine Fetus

Alex Xu, *The University of Western Ontario*

Supervisor: Bryan Richardson, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology

BRAIN INJURY AND INFLAMMATION, AND PLACENTAL INFLAMMATION IN RESPONSE TO REPETITIVE UMBILICAL CORD OCCLUSIONS IN THE NEAR TERM OVINE FETUS

Integrated Article

by

Alex Xu

Graduate Program in Physiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

We hypothesized that repetitive umbilical cord occlusions (UCOs) leading to severe acidemia will stimulate a placental and fetal inflammatory response associated with brain injury, which will be exacerbated by chronic hypoxemia and low-grade infection. Chronically instrumented fetal sheep served as controls or underwent repetitive UCOs for up to 4 hours or until fetal arterial pH was <7.00. Normoxic-UCO and hypoxic-UCO fetuses had arterial O_2 saturation pre-UCOs of >55% and <55%, respectively, while lipopolysaccharide (LPS)-UCO fetuses received LPS intra-amniotically (2mg/h) starting 1 hour pre-UCOs. Fetal plasma and amniotic fluid were sampled for cytokines and after 48 hours of recovery placental and brain tissue were processed for measurement of inflammation and brain injury. Repetitive UCOs resulted in severe fetal acidemia with pH approaching 7.00 for all 3 UCO groups. All UCO groups had increased neutrophils in the placental zona-intima. LPS-UCO animals had elevated IL-6 in fetal plasma at 1-hour recovery, which was highly correlated with nadir pH attained (r =.97). Repetitive UCOs with severe acidemia can induce a placental inflammatory response, more so with low-grade inflammation and likely contributing to cytokine release into umbilical circulation. Despite up to 4 hours of repetitive UCOs and severe acidemia in most of these animals, there was no significant effect on measures of brain inflammation or injury, except in the LPS-UCO animals where TUNEL+ cells were increased in the hippocampus. As such, the near-term ovine fetal brain shows remarkable tolerance for these cord occlusion insults and likely involving protective metabolic mechanisms, despite the severe acidemia noted.

Keywords

Fetal hypoxia; placental inflammation; LPS; microglia; brain necrosis; brain apoptosis; neutrophils; macrophages; interleukin 6; interleukin 1β

Abbreviations

FHR, fetal heart rate; IUGR, intrauterine growth restriction; HIE, Hypoxic ischemic encephalopathy; LPS, Lipopolysaccharide; UCO, Umbilical Cord Occlusion

Acknowledgments

I would like to express my sincere gratitude to my Supervisor Dr. Bryan Richardson for his support of my MSc research, and for his invaluable guidance and patience. I would like to thank my advisory committee, Dr. Stan Leung, Dr. Kelly Summers, and Dr. Doug Fraser for their comments and critiques, as well as Dr. Martin Frasch and Dr. Rob Hammond for their continued guidance.

I would also like to express my thanks to Brad Matushewski for his role in animal care and data collection, Dr. Mingju Cao for his assistance in performing the ELISA assays, and Karen Nygard for her technical expertise and assistance with immunohistochemical staining, all of whom were invaluable to this project.

List of Tables

List of Figures

Table of Contents

Chapter 1

1.1 Inflammation and Fetal Brain Injury

1.1.1 Intrauterine Infection and Fetal Brain Injury

Exposure of the fetus to inflammation in utero is a known risk factor for the development of neurobehavioral disorders later in life, including cerebral palsy, autism and schizophrenia.[1-5] Although inflammation is a natural aspect of pregnancy and labour, various adverse events such as intrauterine infection, perinatal hypoxia, or labour complications can disrupt the balance of pro- and anti-inflammatory cytokines resulting in adverse neonatal outcomes.

Between 25% and 40% of preterm births[6], and between 8.8% and 23.5% of term births[7], are complicated by intrauterine inflammation. Chorioamnionitis is the inflammation of the fetal membranes resulting from ascending bacterial infection of the uterus, and is a confirmed risk factor for the development of cerebral palsy.[5] Yoon *et al* found that among 123 preterm children, those with elevated amniotic IL-6 or IL-8 concentrations at the time of birth were significantly more likely to have developed cerebral palsy by the age of 3.[8] Likewise, Shalak *et al* found that infants born to mothers with chorioamnionitis had elevated levels of IL-6 and IL-8 in umbilical cord blood, and those infants with the highest cytokine levels were more likely to develop hypoxic-ischemic encephalopathy (HIE).[9] Additionally, Viscardi *et al* found that in preterm infants, elevated cerebrospinal fluid IL-6 and TNFα was associated with cranial ultrasound abnormalities.[10] These clinical and epidemiological studies suggest that the release of pro-inflammatory cytokines due to infection during pregnancy is a significant mediator of neonatal brain injury, and could have long term implications on brain development in the infant.

Animal based studies with induced perinatal infection/inflammation further support the contributory role of cytokines and inflammatory cells in perinatal brain injury. Lipopolysaccharide (LPS) is a membrane component of gram-negative bacteria that bind toll-like receptors expressed on a variety of immune and non-immune cell types to stimulate an acute inflammatory response, and is a commonly used tool in the study of infection/inflammation in relation to neonatal brain injury. Kallapur *et al*[11] were the first to demonstrate in preterm fetal sheep that intraamniotic exposure to LPS (~20 mg) resulted in increased expression of IL-6, IL-8, and IL-1β in the chorion and amnion, as seen in

chorioamnionitis. Nitsos *et al*[12] exposed preterm fetal sheep to LPS (1.1 mg/d for 28 days by amniotic infusion), and found that amniotic fluid IL-6 and IL-8 were both elevated at delivery. Additionally, most LPS exposed fetuses had focal areas of white matter injury, as well as a greater degree of activated microglia compared to control animals. In a similar study where preterm sheep were exposed chronically to intravenous LPS over 5 days, exposed fetuses had elevated plasma TNFα, increased number of activated microglia in the subcortical white matter, and also exhibited signs of white matter injury.[13] This study demonstrated that inflammation in the absence of other major physiologic disturbances such as hypoxemia, hypercapnia, or acidemia is able to cause brain injury. In rabbits, intrauterine infection with *e. coli* administration at 80% gestation resulted in notable apoptotic cell death in fetal periventricular white matter, and in some cases cystic white matter lesions comparable to periventricular leukomalacia (PVL) seen in humans.[14] More recently, maternal infection by *E. coli* has been associated with increased neuronal apoptosis in the hippocampus as well as subsequent impairment of spatial learning and memory abilities in rat pups[15]. These studies together corroborate the notion derived from human studies that infection and subsequent elevation of pro-inflammatory cytokines plays a significant contributory role in the development of neonatal brain injury.

1.1.2 Intrauterine Hypoxia and Brain Injury

Intrauterine growth restriction (IUGR) is another known risk factor for neonatal brain injury which may lead to the development of long term neurological sequelae including cerebral palsy, autism, and schizophrenia,[16-18] and is associated with chronic hypoxia during the latter part of pregnancy.[19,20] Both clinical and experimental studies have linked IUGR with increased production of pro-inflammatory cytokines. McElrath *et al* [21] found that infants with IUGR, had increased risk of elevated pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, and IL-8 14 days after birth. In a study looking at IUGR in guinea pigs, gene expression for 22/113 cytokines and/or receptors were upregulated in chronically hypoxic fetuses; among them were TNFα, IL-1β, and IL-1r1 receptor.[22] These chronically hypoxic fetuses also exhibited decreased neuronal density and increased rates of apoptosis in the hippocampus.[22] These studies indicate that chronic hypoxia in the days or weeks prior to birth may be a significant source of inflammation, and thereby has the potential to sensitize the neonatal brain to injury.

While either infection or hypoxia alone is sufficient to induce neonatal brain injury, there is evidence that infection can also predispose a fetus to hypoxic-ischemic brain injury. Epidemiological evidence indicates that the risk for developing cerebral palsy is greater in fetuses exposed to both infection and hypoxia compared to those exposed to hypoxia alone.[23] Similarly, exposure of 7 day old rat pups to LPS, administered to either the peritoneal cavity or the cerebrospinal fluid, sensitized their brains to a subsequent hypoxic/ischemic insult that would otherwise be inert.[24,25] Other studies have shown that rat pups exposed to LPS prior to a hypoxic insult had increased number of apoptotic cells[26] and blood-brain-barrier damage.[27] Taken together, these studies indicate intrauterine infection can sensitize the fetal brain to hypoxic injury. A possible mechanism by which infection could promote hypoxic injury was suggested by Garnier *et al*[28], whereby changes in cardiovascular function due to bacterial infection would result in altered cerebral blood flow. Indeed, intrauterine infection can alter fetal cardiovascular function resulting in reduced cerebral blood flow and subsequent hypoxic injury.[29] Under normal circumstances, a fetus is able to alter brain metabolism and redistribute blood flow in order to maintain its essential energy requirements and to avoid brain injury in response to a hypoxic insult.[30] However increased systemic pro-inflammatory cytokines due to infection might impair the fetus' ability to adapt to a hypoxic insult, thereby increasing the likelihood of injury.

1.1.3 Labour Events and Inflammation

Inflammation is a normal and essential aspect in the induction, maintenance, and termination of healthy pregnancies.[31,32] Normal term labour is accompanied by increases in pro-inflammatory cytokines IL-6, and IL-8[33], indicating that these cytokines play a role in the normal progression of labour. However adverse events near term, such as compression of the umbilical cord during labour leading to severe hypoxic-acidemia, can result in excessive production of pro-inflammatory cytokines, and in turn increase risk for neonatal morbidity.[34,35] Chiesa *et al*[34] studied term neonates and found that IL-6 was elevated in the umbilical cord blood of neonates that had perinatal asphyxia and subsequently developed HIE (22(1.9-9743) pg/ml; $P < 0.0001$) compared to those without perinatal asphyxia. In a similar study, Silveira *et al*[35] found that IL-6 and TNFα were elevated in the cerebrospinal fluid of neonates with HIE compared to normal controls, at 157.5 (66.8-288) pg/ml ($p < 0.00001$) and 14.7 (13.8-15.3) pg.ml ($p < .00001$), respectively. These studies reinforced the notion that pro-inflammatory

cytokines have a role in the pathogenesis of hypoxic brain injury. Additionally, placental hypoperfusion resulting from compression of the umbilical cord during labour has been shown to stimulate secretion of IL-6 and TNFα by the placenta[36], suggesting that labour itself is a possible trigger for the production of pro-inflammatory cytokines.

1.2 Pro-inflammatory Cytokines and the Fetal Inflammatory Response

1.2.1 Pro-inflammatory Cytokines and Pregnancy

Cytokines are a class of small soluble peptides or glycoproteins produced by a variety of cell types including leukocytes and endothelial cells, among others. They function primarily as a means of intracellular communication, with a plethora of autocrine, paracrine, and endocrine effects, and thus play an integral role in the regulation of the innate immune response.[43] Cytokines can be classified based on the effect they have on their effector cells; some cytokines are pro-inflammatory such as IL-1β, IL-8 and TNFα, some are anti-inflammatory such as IL-10, and some can be pleiotropic like IL-6, having both pro- and anti-inflammatory effects depending on the target-cell type, cytokine concentration, and/or other environmental conditions.[43] *In vivo*, cytokines are always acting among a milieu of other cytokines and messenger molecules, and so the end result of an inflammatory cascade usually depends upon a complex interplay between pro- and anti-inflammatory signals.

Inflammation is a normal aspect of a healthy term pregnancy, with pro-inflammatory cytokines having essential functions at all stages including implantation, maintenance, and parturition. Implantation involves the adhesion of a blastocyst to the endometrium, followed by secretion of proteinases thus allowing for proliferation and invasion of trophoblasts. IL-1β promotes secretion of proteinases by trophoblasts[37,38], while IL-6 increases their bioactivity.[39] In addition, both IL-1β and IL-6 have been shown to promote proliferation of first trimester trophoblasts[40]. Insufficient invasiveness of trophoblast cells leading to placental ischemia later in pregnancy is strongly associated with preeclampsia[41], thus emphasizing the importance of these cytokines in the establishment of pregnancy. After implantation, pro-inflammatory cytokines are also involved in the production and secretion of a variety of hormones throughout the course of pregnancy. Production of human chorionic gonadotropin (hCG), is promoted by

IL-1β, IL-6 and TNFα among others; secretion of both corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) by the placenta is stimulated by IL-1β; and finally, estradiol production by placental cells is increased after exposure to IL-1β or TNFα.[32] Additionally, pro-inflammatory cytokines are involved in processes of parturition, including cervical ripening, rupture of the fetal membranes, and contraction of the uterus during labour.[32] Involvement of pro-inflammatory cytokines in these processes not only highlights their functional importance throughout pregnancy, but also illustrates the many points of vulnerability should an abnormal inflammatory response occur.

1.2.2 The Fetal Inflammatory Response Syndrome

The fetal inflammatory response syndrome (FIRS) was first defined by Gomez *et al*.[42] in fetuses with premature labour or preterm premature rupture of membranes (PPROM) by a cord blood IL-6 concentration of 11 pg/ml or greater, and was determined to be an independent risk factor for severe neonatal morbidity, most notably respiratory distress syndrome and sepsis. Presently, the literature has shown that a fetal inflammatory response may be triggered by both bacterial infection or by signals released from injured tissue, is not limited to fetuses with premature labour or PPROM, and involves many cytokines in addition to IL-6. Accordingly, FIRS is now more broadly defined as the systemic activation of the fetal innate immune system.[43] Gotsch *et al*[43] suggested that the FIRS is a means for a fetus to escape a hostile intrauterine environment, whereby signals for infection/injury trigger the release of pro-inflammatory cytokines by the fetus and placenta eventually leading to the induction of labour, citing the fact that infants who survived the neonatal period were more likely to show signs of an inflammatory response than an infant who died in the perinatal period.[44] Despite its potential survival value, the FIRS has been linked to dysfunction or abnormalities in every major organ system, including the fetal brain.[43] It is well established that a fetal inflammatory response can result from intrauterine infection[12,13,24,25,43], IUGR[21,22], and acute hypoxia/ischemia[34,35]. Therefore, neonatal morbidity associated with neonatal brain injury is likely to result from a culmination of all these factors.

1.3 The Placenta and Inflammation

1.3.1 Neutrophils

Neutrophils are the most abundant type of white blood cell, and are an integral part of the innate immune system. When stained with hematoxylin and eosin (H&E) neutrophils appear light pink with a dark multilobular nucleus and visible granules within its cytoplasm. Normally found in the systemic circulation, neutrophils migrate quickly to sites of injury or infection and are usually among the first immune cells to appear. The extravasation of neutrophils from blood vessels is facilitated by the expression of specific cell adhesion molecules on endothelial cells in response to stimulants including bacterial LPS or cytokines such as IL-1β and TNFα.[45] Once at the site of inflammation neutrophils can phagocytose foreign microbes, release cytoplasmic granules containing a variety of antimicrobial molecules, or form neutrophil extracellular traps (NETs) which are networks of extracellular fibers with antimicrobial properties.

The immune response to infection or injury in the placenta, like other tissues, consists firstly of a prompt arrival of neutrophils followed later by macrophages, T-cells and dendritic cells.[46] Neutrophils first appear in the subchorionic fibrin within 6-12 hours of the inflammatory stimulus, migrating from the maternal blood in the intervillous space.[47] Second, neutrophils migrate from the maternal decidual vessels to the fetal amnion, chorion, and associated connective tissue, thus comprising the condition of histological chorioamnionitis.[47] All neutrophils within the placenta are maternal in origin, with the exception of those found in the chorionic plate vessels, which are of fetal origin.[48] Inflammation of the chorionic plate vessels and umbilical cord, termed chorionic vasculitis and funisitis, respectively, is indicative of a fetal inflammatory response. This thesis will use neutrophil infiltration into the ovine placenta as an indicator of inflammation within the placenta.

1.3.2 Macrophages

Macrophages are a type of white blood cell found in tissues under a state of inflammation. Their general functions consist of, but are not limited to, phagocytosis of pathogens and cellular debris, antigen presentation, as well as modulation of the immune response through secretion of cytokines and chemokines. Macrophages are present in the decidua, as well as in the placenta throughout pregnancy and are believed to have a variety of tissue-specific functions

including: vascular remodeling of the uterine lining during placentation, immunomodulation of the materno-fetal interface, and preparation of the cervix for parturition.[49] Additionally, macrophages in the placenta and decidua can function as activators of the innate immune system in response to bacterial infection through activation of membrane bound pattern recognition receptors, for example Toll-like receptors (TLRs)[49] or CD163[50].

CD163 is a scavenger receptor of the cysteine-rich superfamily (SRCR) expressed exclusively on macrophages and monocytes, including those found in the placenta. It is best characterized as a membrane receptor involved in the clearance of free hemoglobin in the blood resulting from the breakdown of red blood cells, thereby protecting tissue from oxidative damage.[51] Recently CD163 has been shown to bind gram-positive and gram-negative bacteria and trigger the production of pro-inflammatory cytokines[52], suggesting that CD163 may also be involved in the initiation of an innate immune response to bacterial infection.

1.3.3 Mast Cells

It is well established that mast cells play a vital role in reproductive processes, particularly during initiation and maintenance of pregnancy where they aid in tissue remodeling, angiogenesis, placentation, and spiral artery modifications.[53] Additionally, there is evidence that excessive mast cell degranulation can lead to preterm labour, as might be seen in women with systemic mastocytosis[54] or allergy-associated induction of preterm labour.[55-57] Conversely, Menzies *et al*[58] found that mast-cell deficient mice underwent labour at the same time as wild-type controls, suggesting that the normal initiation of labour is not dependent on mast cell activity. In the context of hypoxia and/or ischemia, researchers have found that mast cells are the earliest responders to hypoxia-ischemia in the brain, likely by acutely affecting blood brain barrier permeability.[59] In near term fetal sheep subjected to repetitive umbilical cord occlusion with severe acidemia, mast cells in the choroid plexus were increased two fold compared to controls.[60] However, the role of mast cells in placental inflammation is not well studied. To our knowledge this is the first study looking at mast cells in the ovine placental cotyledon.

1.4 The Brain and Inflammation

1.4.1 Microglia as the Primary Immune Cell Within the Brain

Microglia are the innate immune cell of the central nervous system and their physiologic roles include phagocytosis of cellular debris, and induction of neuronal cell death during normal development. While in their resting state, microglia take on a ramified appearance. However, when exposed to certain stimuli such as inflammation[61], infection[13], neuronal death[62], trauma and ischemia[63], they can become activated and more numerous. Activated microglia secrete a variety of inflammatory mediators including reactive oxygen species[64], nitrous oxide[65], pro-inflammatory cytokines[66,67], and quinolinic acid.[68] Although these mediators are normally beneficial, prolonged activation of microglia can result in elevation of these mediators beyond normal physiologic levels, at which point they can become neurotoxic. Aggregations of microglia have been found in the dentate gyrus of neonates exposed to hypoxia-ischemia.[69] Increased microglia activation has also been shown in the white matter of mid-term ovine fetuses exposed to either hypoxia-ischemia or systemic endotoxemia,[70] as well as in the white matter and hippocampus of near-term ovine fetus exposed to hypoxia-ischemia[60] or chronic systemic endotoxemia.[13] Lehnardt *et al*[71] showed that LPS induced oligodendrocyte death is dependent on toll-like receptor 4 (TLR4) which are expressed solely on microglia within the CNS. Similarly, pharmacological inhibition of microglia proliferation after CNS injury *in vivo* reduced neuronal injury compared to control animals.[72] This evidence strongly implicates the role of activated microglia in the pathogenesis of brain injury.

1.4.2 The Role of Mast Cells

Mast cells are potent effector cells in the innate immune system that function primarily by storing and secreting a variety of biological molecules including histamines, prostaglandins, and cytokines.[59,73] In the brain, there is increasing evidence that mast cell activity in response to hypoxia-ischemia can lead to brain injury. In rat pups subjected to unilateral cerebral hypoxia-ischemia via ligation of the right common carotid artery, mast cell numbers were significantly increased in the ligated side compared to the non-ischemic side, and areas of high mast cell numbers were also those with increased activated microglia and brain injury.[74] Furthermore, inhibition of mast cell degranulation attenuated brain injury due to the hypoxic-ischemic insult.[74] In near term ovine fetuses subjected to repetitive umbilical cord occlusion

with severe acidemia, we found a two-fold increase in mast cell numbers in the choroid plexus compared to control animals.[60]

1.5 Umbilical Cord Occlusions in the Ovine Fetus

Variable fetal heart rate (FHR) decelerations are the most common non-reassuring FHR pattern seen intrapartum, and are caused by compression of the umbilical cord leading to fetal hypoxemia.[75] If umbilical cord occlusions (UCO) during labour are of sufficient duration and severity, fetal acidemia may develop,[76] and consequently the infant will be at greater risk of long term neurological sequelae.[77,78] Itskovitz *et al*[79], using the chronically catheterized ovine fetus, elucidated that the fetal heart rate response to UCOs was dependent on the degree of blood flow reduction in the umbilical cord, as well as on the UCO duration. There have been many studies using the catheterized near-term ovine fetus to investigate the effects of UCOs on brain injury and inflammation, all with varying UCO duration, severity and frequency. Intermittent UCOs without cumulative acidemia, consisting of 90-second UCOs every 30 minutes for 3 to 5 hours, is generally well tolerated by the ovine fetus, with minimal signs of necrotic cell injury[80], and no signs of apoptotic cell injury.[81] However, fetuses exposed to these intermittent UCOs did show widespread changes in brain structural proteins, including vimentin, glial fibrillary acidic protein, and nestin.[82] These studies indicate that while the fetal brain can tolerate limited hypoxic insults without suffering overt damage, more subtle changes can occur which may impact long term brain development. Prout *et al*[60] subjected near-term ovine fetuses to a series of repetitive UCOs consisting of 1-minute occlusion every 5 minutes for 1 hour, 1-minute occlusion every 3 minutes for 1 hour, followed by 1-minute occlusions every 2 minutes until the fetus attained an arterial pH of < 7.0. This study found that repetitive UCOs with severe acidemia resulted in a ~2-fold increase in IL-1β in the fetal circulation, a ~2 fold increase in microglia numbers in the white matter and hippocampus, and a ~2-fold increase in mast cells within the thalamus and choroid plexus .[60] This study demonstrated that repetitive UCOs with severe fetal acidemia can result in a fetal inflammatory response, both systemically and locally within the brain, which might then contribute to subsequent brain injury.

1.6 Rationale, Hypothesis, and Research Objectives

1.6.1 Rationale

Variable fetal heart rate (FHR) decelerations are the most common non-reassuring FHR pattern observed intrapartum, and are indicative of acute fetal hypoxia due to repetitive umbilical cord occlusion (UCO) with uterine contractions during labour.[75] Repetitive UCOs of sufficient duration and frequency will result in worsening fetal acidemia, which can become severe defined as fetal arterial pH < 7.0.[83] These infants are at risk of developing hypoxic-ischemic encephalopathy (HIE) and long term neurological sequelae, including cerebral palsy.[78] However, the majority of these infants will be without long term complications, indicating that other factors may be involved in the development of brain injury in addition to the degree of hypoxic-acidemia at birth.[77]

Elevated pro-inflammatory cytokines are well associated with increased risk for fetal-neonatal brain injury.[12,13, 24] The release of pro-inflammatory cytokines into the fetal circulation can be secondary to a variety of conditions, including infection both antenatal and intrapartum[43], chronic hypoxemia with intra-uterine growth restriction during the antenatal period[22], and acute hypoxia occurring during labour and leading to hypoxic-acidemia at birth.[34,35, 43, 84] Since these insults can each on their own initiate an inflammatory response, there is the potential for them to act concomitantly contributing to brain injury. As such, this thesis will study the additive effects of low-grade bacterial infection as might be seen in patients with histologic chorioamnionitis at the time of delivery, as well as chronic hypoxemia as seen with fetal growth restriction, on the fetal-placental inflammatory response to repetitive UCOs.

The placenta is an important source of pro-inflammatory cytokines that under normal physiological conditions play an important role in the initiation, maintenance, and completion of healthy pregnancies.[31] Cells in the placenta shown to produce pro-inflammatory cytokines include trophoblasts, stromal cells, vascular endothelial cells and macrophages.[32] However studies have shown that placental hypoxia or hypoperfusion can also trigger the production of pro-inflammatory cytokines by the placenta with potential for release into the fetal circulation[32,36], supporting the contention that reduced uterine or umbilical blood flow during labour with contractions can cause an increase in circulating pro-inflammatory cytokines in the fetus, thereby predisposing to hypoxic brain injury. This thesis will examine fetal plasma

cytokines as marker for systemic inflammation and amniotic fluid cytokines as marker of placental inflammation in the near term ovine fetus in response to repetitive UCOs.

In the placenta neutrophils are recruited from the maternal blood through expression of cell adhesion molecules on endothelial cells in response to injury or infection.[85] The presence of neutrophils in the amnion and chorion is considered to be the hallmark of placental inflammation with histological chorioamnionitis.[47] Macrophages can be found within the placenta throughout pregnancy where they have a variety of functions.[49] Notably, macrophages within the placenta can function as activators of the innate immune system in response to bacterial infection through activation of their membrane bound receptors such as toll-like receptors (TLRs) or CD163.[49,50] Upon activation, macrophages can secrete reactive oxygen species, nitric oxide, and pro-inflammatory cytokines.[49] Little is known of mast cells in the placenta and their role in the placental inflammatory response. However they are widely recognized as key cellular components of inflammatory processes through the secretion of biologic mediators, including cytokines, histamine, and prostaglandins, among others. This thesis will examine neutrophils, macrophages, and mast cells within the placental cotyledons of the near term ovine fetus as markers of a placental inflammatory response to repetitive UCOs.

Within the fetal brain, microglia are the primary immune cells, which can become activated in response to injury or infection. Upon activation they can secrete various inflammatory mediators including reactive oxygen species[64], nitrous oxide[65], and pro-inflammatory cytokines[65,66] , all of which can be neurotoxic. Mast cells are another type of immune cell that can be found sparsely within the fetal brain, and have been implicated in the pathogenesis of inflammatory brain injury.[59,60,73] This thesis will examine microglia and mast cells within the brain of the near term ovine fetus as markers of a local inflammatory response to repetitive UCOs. Apoptosis and necrosis will additionally be assessed as markers for brain injury.

1.6.2 Hypotheses

1) Repetitive UCOs leading to severe fetal acidemia as might be seen clinically during labour with non-reassuring FHR decelerations, will initiate a placental inflammatory response with an increase in neutrophils, macrophages and mast cells in the placenta causing an increase in pro-inflammatory cytokines in the fetal circulation and amniotic fluid. Pre-existing chronic

hypoxia or low-grade inflammation, as might be seen with fetal growth restriction or histologic chorioamnionitis, will exacerbate the fetal-placental inflammatory response to repetitive UCOs.

2) Repetitive UCOs leading to severe fetal acidemia and systemic inflammation, will initiate a brain inflammatory response with increased activated microglia and mast cells, and thereby an increase in necrotic and apoptotic cell death in the fetal brain. Pre-existing chronic hypoxia or low-grade inflammation, as might be seen with fetal growth restriction or histologic chorioamnionitis, will exacerbate the fetal brain inflammatory and injury response to repetitive UCOs.

1.6.3 Objectives

1) To determine the change in neutrophil, macrophage, and mast cell numbers in the placental cotyledons, and in pro-inflammatory cytokines in the fetal circulation and amniotic fluid in response to repetitive UCOs with severe acidemia in the near-term ovine fetus, with and without pre-existing chronic hypoxia or low grade inflammation.

2) To determine the change in microglia and mast cell numbers, and in necrotic and apoptotic cell death in the brain in response to repetitive UCOs with severe acidemia in the near-term ovine fetus, with and without pre-existing chronic hypoxia or low grade inflammation.

1.7 References

1. Brown AS. Epidemiologic studies of exposure to prenatal infection and risk of schizophrenia and autism. *Dev Neurobiol*. 2012;72(10):1272–1276.

2. Canetta SE, Brown AS. Prenatal infection, maternal immune activations, and risk for schizophrenia. *Transl Neurosci*. 2012;3(4):320–327.

3. Himmelmann K, Ahlin K, Jacobsson B, Cans C, Thorsen P. Risk factors for cerebral palsy in children born at term. *Acta Obstet Gynecol Scand*. 2011;90(10):1070–1081.

4. Nelson KB, Grether JK. Potentially asphyxiating conditions and spastic cerebral palsy in infants of normal birth weight. *Am J Obstet Gynecol*. 1998;179(2):507–513.

5. Wu YW, Colford JM. Chorioamnionitis as a risk factor for cerebral palsy: A meta-analysis. *JAMA*. 2000;284(11):1417–1424.

6. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet*. 2008;371(9606):75–84.

7. Becroft DMO, Thompson JMD, Mitchell EA. Placental Chorioamnionitis at Term: Epidemiology and Follow-Up in Childhood. *Pediatr Dev Pathol*. 2010;13(4):282–290.

8. Yoon BH, Romero R, Park JS, Kim CJ, Kim SH, Choi JH, Han TR. Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of three years. *Am J Obstet Gynecol*. 2000;182(3):675–681.

9. Shalak LF, Laptook AR, Jafri HS, Ramilo O, Perlman JM. Clinical chorioamnionitis, elevated cytokines, and brain injury in term infants. *Pediatrics*. 2002;110(4):673–680.

10. Viscardi RM, Muhumuza CK, Rodriguez A, Fairchild KD, Sun C-CJ, Gross GW, Campbell AB, Wilson PD, Hester L, Hasday JD. Inflammatory markers in intrauterine and fetal blood and cerebrospinal fluid compartments are associated with adverse pulmonary and neurologic outcomes in preterm infants. *Pediatr Res*. 2004;55(6):1009–1017.

11. Kallapur SG, Willet KE, Jobe AH, Ikegami M, Bachurski CJ. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol*. 2001;280(3):L527–L536.

12. Nitsos I, Rees SM, Duncan J, Kramer BW, Harding R, Newnham JP, Moss TJ. Chronic exposure to intra-amniotic lipopolysaccharide affects the ovine fetal brain. *J Soc Gynecol Investig*. 2006;13(4):1–9.

13. Duncan J, Cock M, Suzuki K, Scheerlinck JP, Harding R, Rees S. Chronic Endotoxin Exposure Causes Brain Injury in the Ovine Fetus in the Absence of Hypoxemia. *J Soc Gynecol Investig*. 2006;13(2):87–96.

14. Debillon T, Gras-Leguen C, Vérielle V, Winer N, Caillon J, Rozé JC, Gressens P. Intrauterine infection induces programmed cell death in rabbit periventricular white matter. *Pediatr Res*. 2000;47(6):736–742.

15. Jiang PF, Zhu T, Gao JD, Gao F, Mao SS, Zhao WT, Gu WZ, Yu HM. The effect of maternal infection on cognitive development and hippocampus neuronal apoptosis, proliferation and differentiation in the neonatal rats. *Neuroscience*. 2013;246:422–434.

16. Nelson KB, Grether JK, Dambrosia JM, Walsh E, Kohler S, Satyanarayana G, Nelson PG, Dickens BF, Phillips TM. Neonatal cytokines and cerebral palsy in very preterm infants. *Pediatr Res*. 2003;53(4):600–607.

17. Cannon M, Jones PB. Obstetric complications and schizophrenia: historical and meta-analytic review. *Am J Psychiatry.* 2002 Jul;159(7):1080-92.

18. Dalman C, Thomas HV, David AS, Gentz J, Lewis G, Allebeck P. Signs of asphyxia at birth and risk of schizophrenia. Population-based case-control study. *Br J Psychiatry*. 2001;179:403–408.

19. Soothill PW, Nicolaides KH, Campbell S. Prenatal asphyxia, hyperlacticaemia, hypoglycaemia, and erythroblastosis in growth retarded fetuses. *Br Med J (Clin Res Ed).* 1987;294(6579):1051–1053.

20. Cox WL, Daffos F, Forestier F, Descombey D, Aufrant C, Auger MC, Gaschard JC. Physiology and management of intrauterine growth retardation: a biologic approach with fetal blood sampling. *Am J Obstet Gynecol*. 1988;159(1):36–41.

21. McElrath T, Allred E, Van Marter L, Fichorova R, Leviton A, ELGAN Study Investigators. Perinatal systemic inflammatory responses of growth-restricted preterm newborns. *Acta Paediatr.* 2013 Oct.;102(10):e439-42.

22. Guo R, Hou W, Dong Y, Yu Z, Stites J, Weiner CP. Brain Injury Caused by Chronic Fetal Hypoxemia Is Mediated by Inflammatory Cascade Activation. *Reprod Sci*. 2010;17(6):540–548.

23. Ugwumadu A. Infection and fetal neurologic injury. *Curr. Opin. Obstet. Gynecol.* 2006;18(2):106–111.

24. Eklind S, Mallard C, Leverin AL, Gilland E, Blomgren K, Mattsby-Baltzer I, Hagberg H. Bacterial endotoxin sensitizes the immature brain to hypoxic--ischaemic injury. *Eur J Neurosci*. 2001;13(6):1101–1106.

25. Coumans ABC, Middelanis J, Garnier Y, Vaihinger H-M, Leib SL, Duering Von MU, Hasaart THM, Jensen A, Berger R. Intracisternal Application of Endotoxin Enhances the Susceptibility to Subsequent Hypoxic-Ischemic Brain Damage in Neonatal Rats. *Pediatr Res*. 2003;53(5):770–775.

26. Wang L-W, Chang Y-C, Lin C-Y, Hong J-S, Huang C-C. Low-dose lipopolysaccharide selectively sensitizes hypoxic ischemia-induced white matter injury in the immature brain. *Pediatr Res*. 2010;68(1):41–47.

27. Yang D, Sun Y-Y, Nemkul N, Baumann JM, Shereen A, Dunn RS, Wills-Karp M, Lawrence DA, Lindquist DM, Kuan C-Y. Plasminogen activator inhibitor-1 mitigates brain injury in a rat model of infection-sensitized neonatal hypoxia-ischemia. *Cereb. Cortex*. 2013;23(5):1218–1229.

28. Garnier Y, Coumans ABC, Jensen A, Hasaart THM, Berger R. Infection-related perinatal brain injury: the pathogenic role of impaired fetal cardiovascular control. *J Soc Gynecol Investig*. 2003;10(8):450–459.

29. Fukuda S, Yokoi K, Kitajima K, Tsunoda Y, Hayashi N, Shimizu S, Yoshida T, Hamajima N, Watanabe I, Goto H. Influence of premature rupture of membrane on the cerebral blood flow in low-birth-weight infant after the delivery. *Brain Dev.* 2010;32(8):631–635.

30. Richardson BS. The fetal brain: metabolic and circulatory responses to asphyxia. *Clin Invest Med*. 1993;16(2):103–114.

31. Romero R, Gotsch F, Pineles B, Kusanovic JP. Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury. *Nutr Rev*. 2007;65:S194–S202.

32. Bowen JM, Chamley L, Keelan JA, Mitchell MD. Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. *Placenta*. 2002;23(4):257–273.

33. Chan CJ, Summers KL, Chan NG, Hardy DB, Richardson BS. Cytokines in umbilical cord blood and the impact of labor events in low-risk term pregnancies. *Early Hum Dev*. 2013;89(12):1005–1010.

34. Chiesa C, Pellegrini G, Panero A, De Luca T, Assumma M, Signore F, Pacifico L. Umbilical cord interleukin-6 levels are elevated in term neonates with perinatal asphyxia. *Eur. J. Clin. Invest.* 2003;33(4):352–358.

35. Silveira RC, Procianoy RS. Interleukin-6 and tumor necrosis factor-α levels in plasma and cerebrospinal fluid of term newborn infants with hypoxic–ischemic encephalopathy. *J Pediatr*. 2003;143(5):625–629.

36. Pierce B. Hypoperfusion causes increased production of interleukin 6 and tumor necrosis factor α in the isolated, dually perfused placental cotyledon. *Am J Obstet Gynecol*. 2000;183(4):863–867.

37. Librach CL, Feigenbaum SL, Bass KE, Cui TY, Verastas N, Sadovsky Y, Quigley JP, French DL, Fisher SJ. Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. *J. Biol. Chem.* 1994;269(25):17125–17131.

38. Shimonovitz S, Hurwitz A, Barak V, Dushnik M, Adashi EY, Anteby E, Yagel S. Cytokine-mediated regulation of type IV collagenase expression and production in human trophoblast cells. *J. Clin. Endocrinol. Metab.* 1996;81(8):3091–3096.

39. Meisser A, Cameo P, Islami D, Campana A, Bischof P. Effects of interleukin-6 (IL-6) on cytotrophoblastic cells. *Mol. Hum. Reprod.* 1999;5(11):1055–1058.

40. Bowen JM, Chamley L, Mitchell MD, Keelan JA. Cytokines of the Placenta and Extra-placental Membranes: Biosynthesis, Secretion and Roles in Establishment of Pregnancy in Women. *Placenta*. 2002;23(4):239–256.

41. Raghupathy R. Cytokines as Key Players in the Pathophysiology of Preeclampsia. *Med Princ Pract*. 2013.;22 Suppl 1:8-19

42. Gomez R, Romero R, Ghezzi F, Yoon BH, Mazor M, Berry SM. The fetal inflammatory response syndrome. *Am J Obstet Gynecol*. 1998;179(1):194–202.

43. Gotsch F, Romero R, Kusanovic JP, Mazaki-Tovi S, Pineles BL, Erez O, Espinoza J, Hassan SS. The fetal inflammatory response syndrome. *Clin Obstet Gynecol*. 2007;50(3):652–683.

44. Madan E, Meyer MP, Amortequi A. Chorioamnionitis: a study of organisms isolated in perinatal autopsies. *Ann. Clin. Lab. Sci.* 1988;18(1):39–45.

45. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. 2010;33(5):657–670.

46. Menon R, Taylor RN, Fortunato SJ. Chorioamnionitis--a complex pathophysiologic syndrome. *Placenta*. 2010;31(2):113–120.

47. Redline RW. Inflammatory responses in the placenta and umbilical cord. *Semin Fetal Neonatal Med*. 2006; (11): 296-301.

48. Lee SD, Kim MR, Hwang PG, Shim S-S, Yoon BH, Kim CJ. Chorionic plate vessels as an origin of amniotic fluid neutrophils. *Pathol. Int.* 2004;54(7):516–522.

49. Nagamatsu T, Schust DJ. Review: The Immunomodulatory Roles of Macrophages at the Maternal--Fetal Interface. *Reprod Sci*. 2010;17(3):209–218.

50. Kacerovsky M, Cobo T, Hornychova H, Andrys C, Ryska A, Musilova I, Kriz JT, Kostal M, Jacobsson B. Scavenger receptor for hemoglobin in preterm prelabor rupture of membranes pregnancies complicated by histological chorioamnionitis. *J Matern Fetal Neonatal Med*. 2012;25(11):2291–2297.

51. Fabriek BO, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor CD163. *Immunobiology*. 2005;210(2-4):153–160.

52. Fabriek BO, van Bruggen R, Deng DM, Ligtenberg AJM, Nazmi K, Schornagel K, Vloet RPM, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor

CD163 functions as an innate immune sensor for bacteria. *Blood*. 2009;113(4):887–892.

53. Woidacki K, Jensen F, Zenclussen AC. Mast cells as novel mediators of reproductive processes. *Front Immunol*. 2013;4:29.

54. Metcalfe DD, Akin C. Mastocytosis: molecular mechanisms and clinical disease heterogeneity. *Leuk. Res.* 2001;25(7):577–582.

55. Romero R, Kusanovic JP, Muñoz H, Gomez R, Lamont RF, Yeo L. Allergy-induced preterm labor after the ingestion of shellfish. *J Matern Fetal Neonatal Med*. 2010;23(4):351–359.

56. Habek D, Cerkez-Habek J, Jalsovec D. Anaphylactic shock in response to wasp sting in pregnancy. *Zentralbl Gynakol*. 2000;122(7):393–394.

57. Bytautiene E, Romero R, Vedernikov YP. Induction of premature labor and delivery by allergic reaction and prevention by histamine H 1 receptor antagonist. Am J Obstet Gynecol. 2004 Oct;191(4):1356-61.

58. Menzies FM, Higgins CA, Shepherd MC, Nibbs RJB, Nelson SM. Mast cells reside in myometrium and cervix, but are dispensable in mice for successful pregnancy and labor. *Immunol. Cell Biol.* 2012;90(3):321–329.

59. Lindsberg PJ, Strbian D, Karjalainen-Lindsberg M-L. Mast cells as early responders in the regulation of acute blood–brain barrier changes after cerebral ischemia and hemorrhage. *J Cereb Blood Flow Metab*. 2010;30(4):689–702.

60. Prout AP, Frasch MG, Veldhuizen RAW, Hammond R, Ross MG, Richardson BS. Systemic and cerebral inflammatory response to umbilical cord occlusions with worsening acidosis in the ovine fetus. *Am J Obstet Gynecol*. 2010;202(1):82.e1–9.

61. Kuno R, Wang J, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. Autocrine activation of microglia by tumor necrosis factor-alpha. *J. Neuroimmunol.* 2005;162(1-2):89–96.

62. Dheen ST, Kaur C, Ling E-A. Microglial activation and its implications in the brain diseases. *Curr. Med. Chem.* 2007;14(11):1189–1197.

63. Lai AY, Todd KG. Microglia in cerebral ischemia: molecular actions and interactions. *Can. J. Physiol. Pharmacol.* 2006;84(1):49–59.

64. Yoshida T, Tanaka M, Sotomatsu A, Hirai S. Activated microglia cause superoxide-mediated release of iron from ferritin. *Neuroscience Letters*. 1995;190(1):21–24.

65. Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol.* 1992 Oct.;149(8)2736-2741.

66. Giulian D, Baker TJ, Shih LC, Lachman LB. Interleukin 1 of the central nervous system is produced by ameboid microglia. *J. Exp. Med.* 1986;164(2):594–604.

67. Sawada M, Kondo N, Suzumura A, Marunouchi T. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. *Brain Research*. 1989;491(2):394–397.

68. Heyes MP, Achim CL, Wiley CA, Major EO, Saito K, Markey SP. Human microglia convert l-tryptophan into the neurotoxin quinolinic acid. *Biochem. J.* 1996;320 (Pt2):595–597.

69. Bigio MRD, Beckery LE. Microglial aggregation in the dentate gyrus: a marker of mild hypoxic-ischaemic brain insult in human infants. *Neuropathology and Applied Neurobiology*. 1994;20(2):144–151.

70. Mallard C, Welin A-K, Peebles D, Hagberg H, Kjellmer I. White matter injury following systemic endotoxemia or asphyxia in the fetal sheep. *Neurochem. Res.* 2003;28(2):215–223.

71. Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J. Neurosci.* 2002;22(7):2478–2486.

72. Giulian D, Robertson C. Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord. *Ann Neurol.* 1990;27(1):33–42.

73. Skaper SD, Giusti P, Facci L. Microglia and mast cells: two tracks on the road to neuroinflammation. *The FASEB Journal*. 2012;26(8):3103–3117.

74. Jin Y, Silverman A-J, Vannucci SJ. Mast Cell Stabilization Limits Hypoxic-Ischemic Brain Damage in the Immature Rat. *Dev Neurosci*. 2007;29(4-5):373–384.

75. American College of Obstetricians and Gynecologists. Fetal heart rate patterns: monitoring, interpretation, and management. *Technical bulletin*. 1995;207.

76. Ball RH, Parer JT. The physiologic mechanisms of variable decelerations. *Am J Obstet Gynecol*. 1992;166(6 Pt 1):1683–8– discussion 1688–9.

77. Winkler CL, Hauth JC, Tucker JM, Owen J, Brumfield CG. Neonatal complications at term as related to the degree of umbilical artery acidemia. *Am J Obstet Gynecol*. 1991;164(2):637–641.

78. Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. *Am J Obstet Gynecol*. 2008;199(6):587–595.

79. Itskovitz J, LaGamma EF, Rudolph AM. Heart rate and blood pressure responses to umbilical cord compression in fetal lambs with special reference to the mechanism of variable deceleration. *Am J Obstet Gynecol*. 1983;147(4):451–457.

80. Rocha E, Hammond R, Richardson B. Necrotic cell injury in the preterm and near-term ovine fetal brain after intermittent umbilical cord occlusion. *Am J Obstet Gynecol*. 2004;191(2):488–496.

81. Falkowski A, Hammond R, Han V, Richardson BS. Apoptosis in the preterm and near term ovine fetal brain and the effect of intermittent umbilical cord occlusion. *Brain Res. Dev. Brain Res.* 2002;136(2):165–173.

82. Rocha E, Totten S, Hammond R, Han V, Richardson B. Structural proteins during brain development in the preterm and near-term ovine fetus and the effect of intermittent umbilical cord occlusion. *Am J Obstet Gynecol*. 2004;191(2):497–506.

83. Goldaber KG, Gilstrap LC, Leveno KJ, Dax JS, McIntire DD. Pathologic fetal acidemia. *Obstet Gynecol.* 1991 Dec.;78(6):1103–1107.

84. Hagberg H, Gilland E, Bona E, Hanson LA, Hahin-Zoric M, Blennow M, Holst M, McRae A, and Söde O. Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatr R*es. 1996;40(4): 603-609.

85. Borregaard N. Neutrophils, from marrow to microbes. *Immunity.* 2010 Nov. 24;33(5):657–670.

Chapter 2

Repetitive Umbilical Cord Occlusions and Fetal Placental Inflammation

2.1 Introduction

Variable fetal heart rate (FHR) decelerations due to compression of the umbilical cord with resultant fetal hypoxemia are the most common non-reassuring FHR pattern observed intrapartum.[1] Repetitive umbilical cord occlusions (UCOs) during labour of sufficient duration and frequency result in worsening fetal acidemia which if prolonged can become severe, defined as arterial pH < 7.0.[2] Affected infants have an increased risk of developing hypoxic-ischemic encephalopathy (HIE) and long term neurological sequelae, including cerebral palsy.[3] However, most infants with severe acidemia at birth will be without noted complications, suggesting that an infant's susceptibility to hypoxic-asphyxial injury is multifactorial.[4]

Epidemiological and clinical evidence indicates that an increase in pro-inflammatory cytokines during infectious processes, both antenatal and postnatal, contributes to the risk for perinatal brain injury.[5] Subsequent animal-based studies with induced perinatal infection/inflammation further support the contributory role of cytokines and inflammatory cells in perinatal brain injury[5-7], and also indicate a synergistic interaction between inflammation and hypoxia in the pathogenesis of brain injury.[5,8,9] Similarly, both clinical and experimental evidence suggest that the pathogenesis of newborn HIE, in the absence of infection, is mediated by an increase in pro-inflammatory cytokines,[5,10-12] suggesting hypoxia/asphyxia alone can trigger an inflammatory response. Furthermore pro-inflammatory cytokines have also been implicated in the development of brain injury due to chronic intrauterine hypoxia leading to fetal growth restriction.[13] In short, fetal inflammation can be secondary to a variety of conditions, including intrauterine infection, acute asphyxia intrapartum, and chronic hypoxia during the antenatal period, all of which have the potential to act concomitantly contributing to perinatal brain injury.

The placenta is a major source of pro-inflammatory cytokines, which under normal conditions play a pivotal role in the initiation, maintenance and completion of healthy pregnancies.[14] However, placental hypoxia and/or hypoperfusion can trigger the release of cytokines by the placenta,[15,16] supporting the contention that reduced uterine or umbilical blood flow due to

contractions during labour can cause an increase in circulating inflammatory cytokines in the fetus and thus predispose to hypoxic brain injury. We therefore hypothesized that repetitive UCOs leading to severe fetal acidemia will stimulate a placental inflammatory response, along with a systemic inflammatory response within the fetus. Macrophages, neutrophils, and mast cells were counted as measures of inflammation in the placental cotyledons at 48 hours of recovery, while fetal plasma interleukin (IL) 6 was assessed throughout the experiment as a measure of systemic inflammation in response to the repetitive UCOs with worsening acidemia. Amniotic fluid IL-6 and IL-1β were additionally assessed at select time points as further measures of placental and/or membrane inflammation, since any increase in these cytokines is likely attributable to placental/membrane production and release into the amniotic cavity.[16] We also sought to examine the additional impact on these inflammatory responses of lower oxygenation and low-grade bacterial infection as might be seen clinically during labour with fetal growth restriction and placental chorioamnionitis.[17-20]

2.2 Materials and Methods

2.2.1 Surgical Preparation

Thirty mixed breed ewes were surgically instrumented at 124-128 days gestation (term = 147 days) (Figure 2.1). Anesthesia, surgical procedures, and postoperative (postop) care of the animals have been previously described.[21] General anesthesia was induced using 1 g of sodium pentothal (Abbott Laboratories Ltd., Montreal, Quebec, Canada) given intravenously, followed by 1% to 2% isoflurane in O_2 for maintenance. A polyvinyl catheter was placed in the maternal femoral vein for administering fluids and antibiotics and for blood sampling. A midline incision was made in the lower abdominal wall to expose the uterus, which was palpated to determine fetal number and position. Fetuses were partially exteriorized through an incision in the uterine wall. Polyvinyl catheters were placed in the left and right brachiocephalic arteries for blood sampling and pressure recordings, and the right brachiocephalic vein for administering antibiotics. Stainless-steel electrodes were placed biparietally on the dura to monitor electrocortical (ECoG) activity, as well as on the fetal chest to monitor electrocardiographic (ECG) activity. In experimental animals, an inflatable silicone occluder cuff (OC14; In Vivo Metric, Healdsburg, California) was placed around the proximal portion

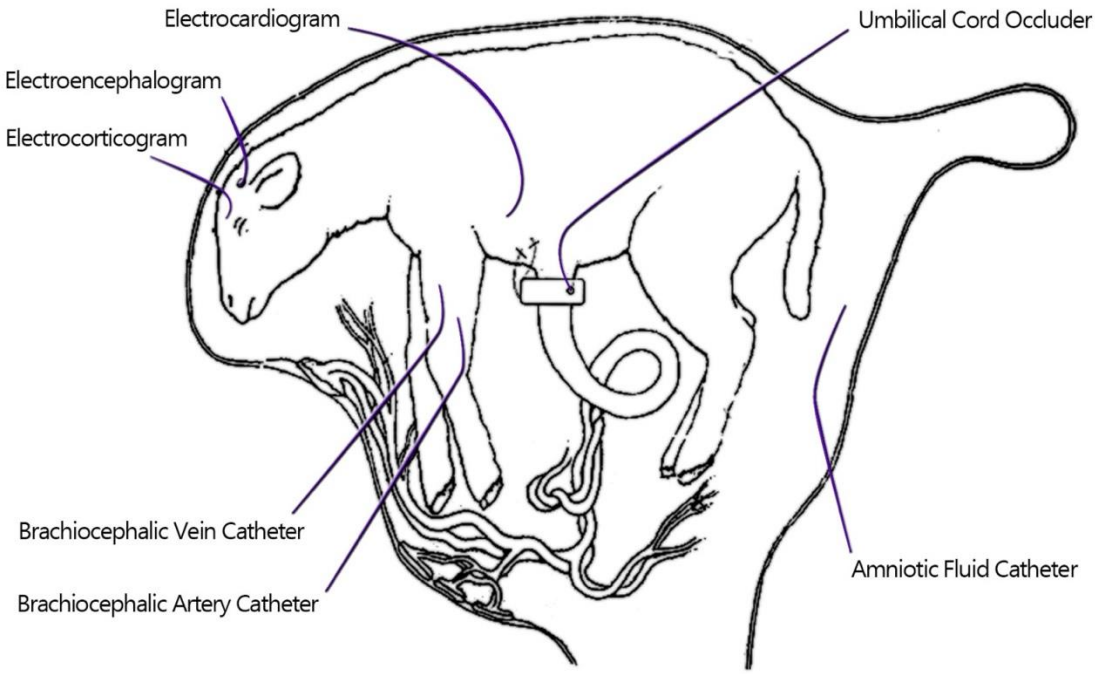

Figure 2.1. Thirty near term ovine fetuses were chronically instrumented at 126 days gestation (term = 147 days), as shown above. Animals were allowed to recover for 4 days prior to experimental study.

of the umbilical cord and secured to the abdominal skin. The fetus was then returned to the uterus, a polyvinyl catheter was placed in the amniotic cavity for amniotic fluid sampling and pressure recordings, and the uterine and abdominal incisions were closed. Antibiotics were administered to the mother (5 cm^3 Tivetrin), fetus (1 cm^3 Penicillin), and amniotic cavity (1 cm^3 Penicillin) at the end of surgery. Animals were allowed to recover for four days, during which daily maternal and fetal blood samples were taken for blood gases and pH to monitor maternal and fetal health. On postop day 2, a fetal arterial plasma sample (3 mL) was also taken for later cytokine analysis. Catheters were flushed daily with saline in order to maintain patency.

2.2.2 Experimental Procedure

Animals were divided retrospectively into 4 groups based on postsurgical, sustained baseline blood oxygen status. Fetal baseline oxygen status was determined by averaging the arterial O_2 saturation of blood samples taken on each of the 4 postop recovery days, as well as at baseline on the day of the UCO experimental study. Fetuses with average baseline arterial O_2 saturation of < 55% comprised a low oxygen or hypoxic-UCO group (n = 5), since this level of oxygenation has been used to denote decreased oxygenation/spontaneous hypoxemia in the near term ovine fetus.[22,23] The remaining fetuses with average baseline arterial O_2 saturation $\geq$ 55% were divided into a normoxic-UCO group (n = 9), LPS-UCO group (n = 6), and control group (n = 10). The LPS (E. Coli 055:B5, Sigma Aldrich, Oakville, Ontario, Canada), a bacterial endotoxin found on the outer membrane of gram-negative bacteria, was administered via hourly intra-amniotic injections of 2 mg/h beginning 1 hour prior to the first UCO to simulate a low-grade bacterial infection in LPS-UCO animals.[24,25] The control group consisted of normoxic fetuses that underwent surgical preparation but were not subjected to UCOs. These animals were used only as tissue controls for histological examination of the placental cotyledons.

Umbilical cord occlusions were induced by inflating occluder cuffs with varying volumes of saline. The severity of occlusion was gauged by observing the magnitude of resultant FHR decelerations, with decelerations of 30, 60, and 90 bpm corresponding to umbilical blood

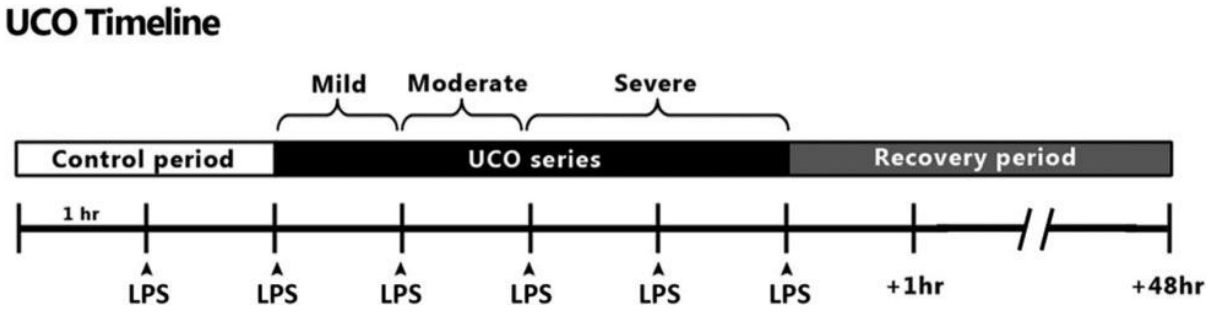

Figure 2.2. Schematic diagram of the experimental timeline. Each division represents a duration of 1 hour. Arrowheads indicate when animals in the LPS-UCO group received intra-amniotic injections of LPS. LPS indicates lipopolysaccharide; UCO, umbilical cord occlusions.

flow reduction of approximately 50%, 75%, and 100%, respectively, as reported by Itskovitz *et al*.[26] These targeted occlusions and associated FHR deceleration of 30, 60, and 90 bpm were deemed, "mild-partial", "moderate-partial", and "severe-complete" UCOs, respectively. All UCOs were 1 minute in duration and occurred every 2.5 minutes.

On the experimental day, a PowerLab system was used for data acquisition and analysis (LabChart 6, Adinstruments, Sydney, Australia) to record fetal arterial blood pressure, amniotic pressure, ECoG, ECG, and heart rate. FHR was triggered and calculated online from arterial pressure systolic peaks. All fetuses were monitored for a 1- to 2-hour baseline control period. Experimental fetuses were then subjected to series of repetitive UCOs consisting of 1 hour of mild partial, 1 hour of moderate partial, and up to 2 hours of severe complete UCOs (Figure 2.2). UCOs were discontinued if fetal arterial pH dropped below 7.00, or after two hours of severe complete UCOs. The ewe and fetus were allowed to recover for 48 hours after the end of the UCOs, at which time they were euthanized with a barbiturate overdose (30 mg sodium pentobarbital, MTC Pharmaceuticals, Cambridge, Ontario, Canada).

Fetal arterial blood samples (1 mL) were taken at baseline, at the end of a representative UCO of each UCO series, every 20 minutes between UCOs, and after 1, 24, and 48 hours of recovery. Blood samples were analyzed for blood gases, pH, glucose, and lactate using an automated blood gas analyzer (ABL 725, Radiometer Medical, Copenhagen, Denmark) with temperature corrected to 39.0°C. Additional blood samples (3 mL) were collected at baseline prior to UCOs, immediately after the final UCO, and at 1 and 48 hours of recovery. These samples were immediately centrifuged at 3400 rpm for 5 minutes at 4° C, with the supernatant (plasma) then transferred to a fresh microfuge tube and frozen at -80° C for later analysis of pro-inflammatory cytokines. Amniotic fluid samples (3 mL) were taken at baseline prior to UCOs, and after 1 and 48 hours of recovery. These samples were also centrifuged at 3400 rpm for 5 minutes at 4° C, with the supernatant then transferred to a fresh microfuge tube and frozen at -80° C for later analysis of pro-inflammatory cytokines. At the end of the experimental day, fetuses were given a transfusion of maternal blood with volume equal to the total volume of blood sampled that day.

2.2.3 Tissue Histochemical Analysis

Placental cotyledons that appeared to be of average size were collected from each fetus at necropsy. Cotyledons were immersed in 4% paraformaldehyde for 24 hours, then in fresh phosphate buffered saline (PBS) for three days, followed by 70% ethanol for 7 days, then blocked and embedded in paraffin for histological analysis.

Macrophages within the cotyledon were visualized by immunohistochemical staining with a mouse anti-CD163 antibody.[27,28] Staining was performed using an avidin/biotin based peroxidase method (Vectastain Elite Kit, Vector Laboratories, Burlingame, California). Tissue sections were deparaffinized with three 5-minute washes in xylene, then rehydrated in a series of ethanol baths (100%, 100%, 90%, 90%, 70%) lasting 2 minutes each. Tissue sections were then rinsed once in pure water for 5 minutes followed by two 5-minute washes in PBS. For antigen retrieval, tissues were boiled in 10 mmol/L citrate buffer for 30 minutes, cooled to room temperature for 30 minutes, then rinsed with two 5-minute washes in PBS. Endogenous peroxidase activity was quenched by incubating tissues in 3% H_2O_2 (Fischer Scientific, Ottawa, Ontario, Canada) for 10 minutes followed by two 5-minute washes in PBS. Endogenous biotin was blocked by incubating tissues in avidin solution followed by biotin solution for 15 minutes each. To reduce non-specific staining, slides were incubated in Background Sniper (Biocare Medical, Concord, California) for 7 minutes at room temperature then rinsed in PBS. Tissue sections were then incubated in mouse anti-CD163 monoclonal antibody (1:1000, Acris, San Diego, California) overnight (~20 hours) at 4° C, rinsed with three 5-minute washes in PBS, incubated with secondary antibody (1:200, biotinylated anti-mouse immunoglobulin G [IgG]; Vector Laboratories) for 30 minutes at room temperature, and again rinsed with three 5-minute washes in PBS. Tissue sections were then incubated with streptavidin-peroxidase solution (Vector Laboratories) for 45 minutes at room temperature, rinsed with three 5-minute washes in PBS, and finally incubated with diaminobenzidine (Biocare Medical) for exactly 2 minutes to develop the stain. Tissue sections were then dehydrated in a series of ethanol baths (70%, 70%, 90%, 100%, 100%) followed by 3 5-minute washes in xylene and finally cover-slipped using Permount (Fischer Scientific). Negative controls were produced using the protocol described above but omitting the primary antibody and confirmed absence of staining.

Neutrophils within the cotyledons were identified by visual examination of hematoxylin and eosin (H&E)-stained slides. Tissue sections were deparaffinized with three 5-minute washes in xylene, then rehydrated in a series of ethanol baths (100%, 100%, 90%, 90%, 70%) lasting 2 minutes each. Tissue sections were then rinsed once in pure water for 5 minutes before being immersed in Haris Modified Haematoxylin stain (Fischer Scientific) for 10 seconds. Stain was differentiated in 1% acid ethanol (2 mL HCl in 198 mL 70% ethanol) for approximately 1 second, and then flushed under running water for 1 minute. Next, sections were stained with eosin (1% eosin Y in 95% ethanol and 0.5% glacial acetic acid, Fischer Scientific) for approximately 1 second. Tissue sections were then dehydrated in a series of ethanol baths (70%, 70%, 90%, 100%, 100%) followed by 3 5-minute washes in xylene and finally cover-slipped using Permount (Fischer Scientific).

Mast cells within the cotyledon were identified by staining with toluidine blue. Tissue sections were deparaffinized with three 5-minute washes in xylene, then rehydrated in a series of ethanol baths (100%, 100%, 90%, 90%, 70%) lasting 2 minutes each. Sections were then rinsed once in pure water for 5 minutes followed by two 5-minute washes in PBS, followed by immersion in toluidine blue solution (5 mg/mL toluidine blue in 0.1M HCl) for 10 minutes. Sections were rinsed under running water for 5 minutes before a quick (10 sec) dip in acid alcohol (0.5 mL of glacial acetic acid in 200 mL of 95% ethanol), then dehydrated in a series of alcohol baths (70%, 90%, 100%) for 30 seconds each, and finally cover-slipped using Permount (Fischer Scientific).

Cotyledon sections were cut vertically through the centre such that each section contained tissue from the entire depth of the cotyledon (Figure 2.3). For analysis, sections were divided into thirds depthwise, with the "fetal zone" consisting of the region most proximal to the fetus, the "zona intima" consisting of the region most proximal to the mother, and the "intermediate zone" consisting of the region in between. Image analysis was performed using a transmitted light microscope (Leica DMRB, Leica-Microsystems, Wetzler, Germany). For each stain, four high-power field (HPF) photomicrographs (400x magnification) were taken per cotyledon region. For the macrophage analysis, endocytosis and breakdown of red blood cells by chorionic epithelial cells was noted to result in nonspecific binding of the CD163 antibody, thus those areas were avoided when imaging. Macrophages were identified by contiguous, dark cytoplasmic staining by DAB and were

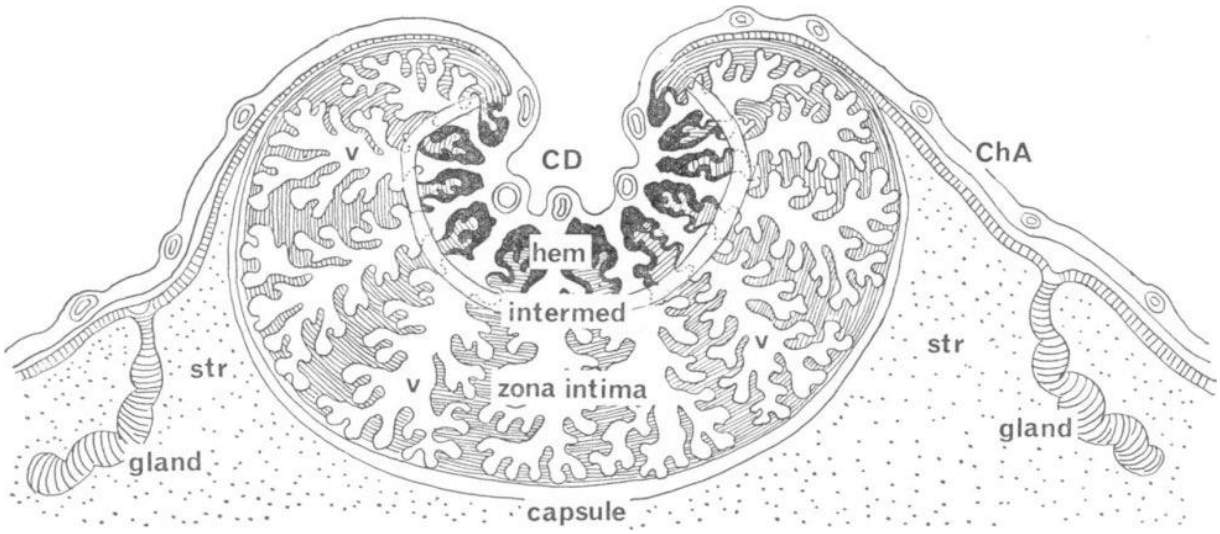

Figure 2.3. Schematic diagram of a vertical section from an ovine cotyledon. Fetal zone images were taken in the chorionic plate above the hemophagous (hem) zone. Intermediate zone images were taken in the middle third of the cotyledon, below the hemophagous zone and above the zona intima. Zona intima images were taken within the zona intima. Figure from Burton *et al* 1976.[29] CD indicates central depression of the cotyledon; ChA, chorioallantois; str, stroma; v, chorionic villi.

counted manually (Figure 2.4A). Neutrophils were identified from H&E stained sections by their round pink cytoplasm and multilobular nucleus (Figure 2.4B), while mast cells were identified by their metachromatic cytoplasmic granules, with these inflammatory cells again counted manually. For all analysis, care was taken to avoid counting cells in areas of extravasated blood or within blood vessels. All slides for each staining procedure were processed on the same day using the same solutions in order to minimize variation in staining. Tissue staining and scoring of cell counts was performed by a single experimenter in a blinded fashion to control and experimental groups.

2.2.4 Enzyme-Linked Immunosorbent Assay Analysis

Pro-inflammatory cytokines in plasma (IL-6) and amniotic Fluid (IL-6 and IL-1β) were analyzed by sheep-specific enzyme-linked immunosorbent assays (ELISA). First, 96-high capacity microtitre well plates were precoated with the capture antibody, using either mouse anti-sheep IL-6 (MCA1659; AbD Serotec, Raleigh, North Carolina) or mouse anti-sheep IL-1β (MCA1658 AbD Serotec) at a concentration of 4 μg/mL overnight at 4° C. Plates were then washed 3 times with PBST (0.05% Tween 20 in PBS, pH 7.4), blocked by incubating with assay buffer (1% BSA in PBST) for 1 hour at room temperature, then washed again 3 times with PBST. Protein standards were prepared by serial dilution of either recombinant ovine IL-6 (Cat No. 968305, Lot No. IL6-911, Protein Express, Cincinnati, Ohio) or recombinant ovine IL-1β (Cat No. 968405, Lot No. IL1β-911, Protein Express) in assay buffer. Amniotic fluid and plasma samples were loaded in duplicate and incubated for 2 hours at room temperature. If necessary, samples were centrifuged then diluted in assay buffer before being loaded, ranging from 1:2 to 1:500. Unbound proteins were removed by washing 3 times with PBST. Plates were then incubated with detection antibody for 30 minutes at room temperature then washed 5 times with washing buffer. Plates were then incubated with detection antibody with either rabbit antisheep IL-6 (1:250 dilution, AHP 424; AbD Serotec) or rabbit antisheep IL-1β (1:250 dilution, AHP 423; AbD Serotec) for 30 minutes at room temperature then washed 5 times with washing buffer. Next, 50 μL of rabbit antisheep IgG-HRP (1:5000, Cat no 111-035-144, Lot no 98733; Jackson ImmunoResearch, West Grove, Pennsylvania) diluted in assay buffer were added to each well, plates were incubated for 30 minutes at room temperature, and then washed 7 times with washing buffer (with 1 minute incubation between washes). Finally, 50 μL of

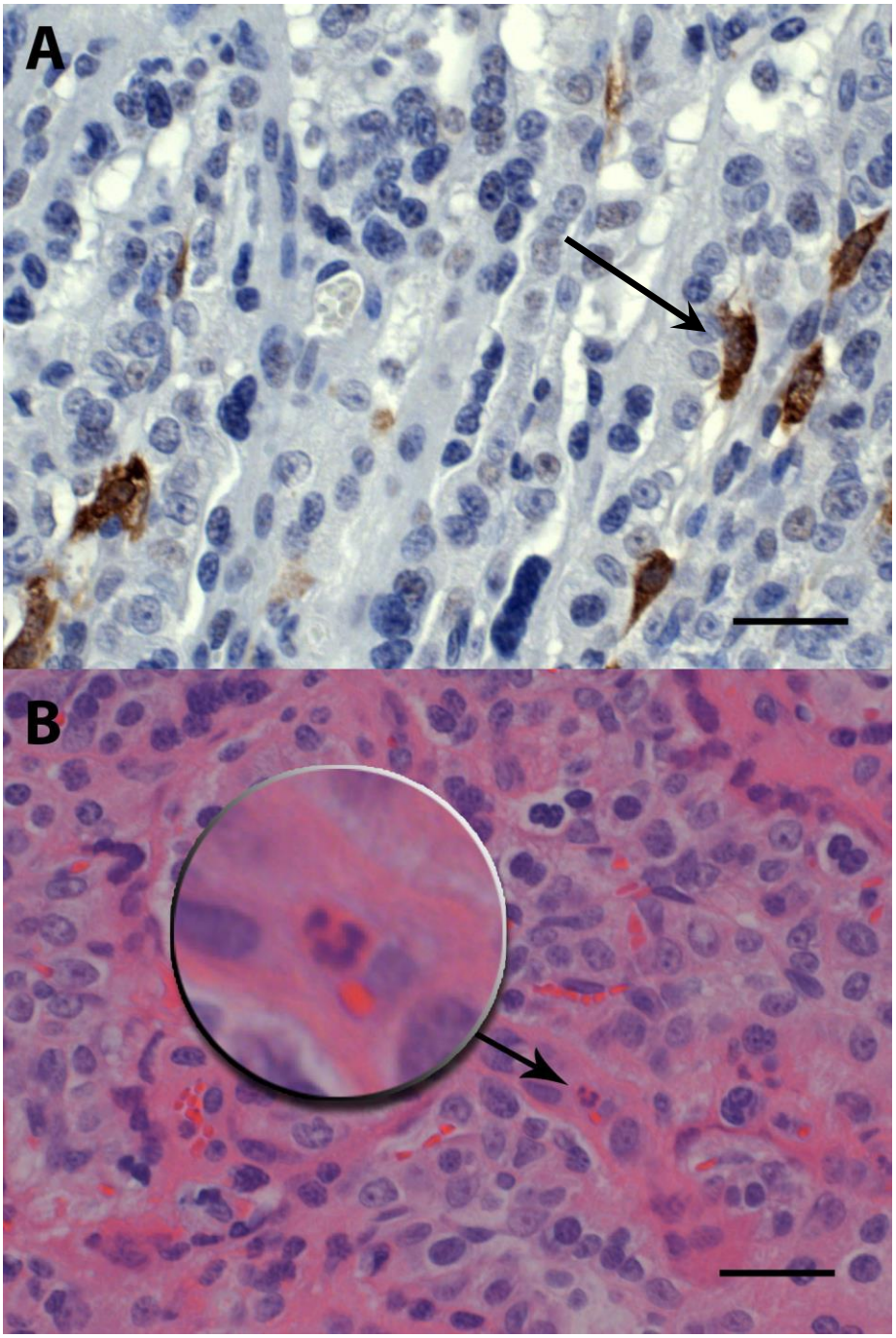

Figure 2.4. Representative photomicrographs (400X) of cotyledon sections from the zona intima, (A) stained with CD163 antibody and counterstained with hematoxylin, with macrophages exhibiting dark, contiguous cytoplasmic staining and (B) stained with hematoxylin and eosin, with neutrophils within the extravascular cotyledon tissue identified manually by their pink cytoplasm and multilobular nucleus (arrow). Scale bar = 20 μm.

3,30,5,50-tetramethylbenzidine (TMB) substrate solution (BD OptEIA TMB substrate Reagent Set, Cat No. 555214; BD Biosciences, Mississauga, Ontario, Canada) was added to each well and incubated for 10 to 15 minutes at room temperature. The reaction was stopped by adding 25 µL of 2 N sulfuric acid to each well. Plates were then read on an ELISA plate reader at 450 nm with 570 nm wavelength correction (EnVision 2104 Multilabel Reader; Perkin Elmer, Waltham, Massachusetts). The sensitivity of the IL-6 ELISA was 16 pg/mL, while the sensitivity of the IL-1β ELISA was 1.12 ng/mL. For all assays, the intraassay and interassay coefficients of variation were <5% and <10%, respectively.

2.2.5 Data Analysis

Differences in arterial blood gases, pH, and metabolites were analyzed using a nonparametric 1-way analysis of variance test (Kruskal-Wallis) with Dunn posttest for comparisons across time points and unpaired T tests for comparisons between groups. Differences in macrophage and neutrophil counts among the 4 experimental groups were analyzed using a nonparametric 1-way analysis of variance test (Kruskal-Wallis) with Dunn posttest for comparing each experimental group to the control group. Differences in plasma and amniotic fluid IL-6 and IL-1 across the various time points were analyzed using a nonparametric 1-way analysis of variance test (Kruskal-Wallis) with Dunn posttest for comparing each time point to baseline levels. All statistical analysis was done using Graphpad Prism (Graphpad Software, La Jolla, California). All values shown are expressed as means ± standard error of the mean. Statistical significance was assumed for $P < .05$. The ECOG, ECG, and arterial blood pressure findings have been reported separately.[30-32]

2.3 Results

2.3.1 Fetal Heart Rate Changes

Normoxic-UCO animals had average FHR of 159 ± 5 bpm at baseline and showed FHR decelerations of 39 ± 8, 59 ± 4, and 99 ± 7 bpm as was intended during the targeted mild partial, moderate partial, and severe complete UCOs, respectively. Hypoxic-UCO animals had average FHR of 168 ± 5 bpm at baseline and showed FHR decelerations of 29 ± 13, 54 ± 17, and 77 ± 15 bpm during the mild, moderate, and severe UCOs, respectively, which did not differ from that of the normoxic-UCO animals. Finally, LPS-UCO animals had average FHR of 160 ± 3 bpm at baseline and showed FHR decelerations of 33 ± 9, 51 ± 10, and 91 ± 12 bpm during the

mild, moderate, and severe UCOs, respectively, which again did not differ from that of the normoxic-UCO animals.

2.3.2 Fetal Arterial Blood Gas, pH, and Metabolites

Oxygen saturation and PaO2 at baseline day 1 of study for the normoxic-UCO and LPS-UCO groups were within the normal physiologic range, averaging 59% ± 5% and 23.0 ± 0.6 mmHg and 57% ± 3% and 23.0 ± 0.6 mmHg, respectively, while that of the hypoxic-UCO group was lower, at 40% ± 7% and 16.1 ± 1.8 mmHg (all $P < .05$). All 3 experimental animal groups had normal PaCO2 and pHa values at baseline, which averaged 50.0 ± 1.1, 53.3 ± 1.7, and 51.4 ± 1.0 mmHg and 7.35 ± 0.01, 7.34 ± 0.01, and 7.35 ± 0.01 for the normoxic-UCO, hypoxic-UCO, and LPS-UCO groups, respectively. A transient decrease in PaO2 was observed during each moderate and severe UCO, at approximately 8 and 13 mmHg, 8 and 12 mmHg, and 8 and 11 mmHg for the normoxic-UCO, hypoxic-UCO, and LPS-UCO animals, respectively (all $P < .05$; Figure 2.5). The LPS-UCO group also showed a transient decrease in PaO2 of 5 mmHg during each mild UCO ($P < .05$). PaO2 recovered rapidly after the release of the occluder cuff and there were no significant changes from respective baseline values as measured at the end of each of the UCO series for any of the 3 experimental groups. PaCO2 changed little during each mild and moderate UCO but increased during the severe UCOs by approximately 14, 17, and 13 mmHg in the normoxic-UCO, hypoxic-UCO, and LPS-UCO groups, respectively (all $P < .05$; Figure 2.5). The increase in PaCO2 values continued to be evident at the end of the severe UCO series for all 3 experimental groups but recovered to baseline by 1 hour of recovery. Fetal arterial pH likewise changed little during each mild and moderate UCO but decreased during the severe UCOs to 7.24 ± 0.01 and 7.10 ± 0.06 in the normoxic-UCO and hypoxic-UCO groups, respectively (both $P < .05$; Figure 2.5). Accordingly, there was a cumulative decrease in pHa with the repetitive UCOs, which became significant for the normoxic-UCO and hypoxic-UCO groups by the end of the moderate UCO series at 7.28 ± 0.01 and 7.19 ± 0.05, respectively (both $P < .05$); and for all 3 experimental groups by the end of the severe UCO series at 7.00 ± 0.03, 7.01 ± 0.03, and 7.07 ± 0.06 for the normoxic-UCO, hypoxic-UCO, and LPS-UCO groups, respectively (all $P < .05$; Figure 2.5). Despite up to 2 hours of severe UCOs, 1 normoxic-UCO animal only reached a nadir pH of 7.19, 1 hypoxic-UCO animal only

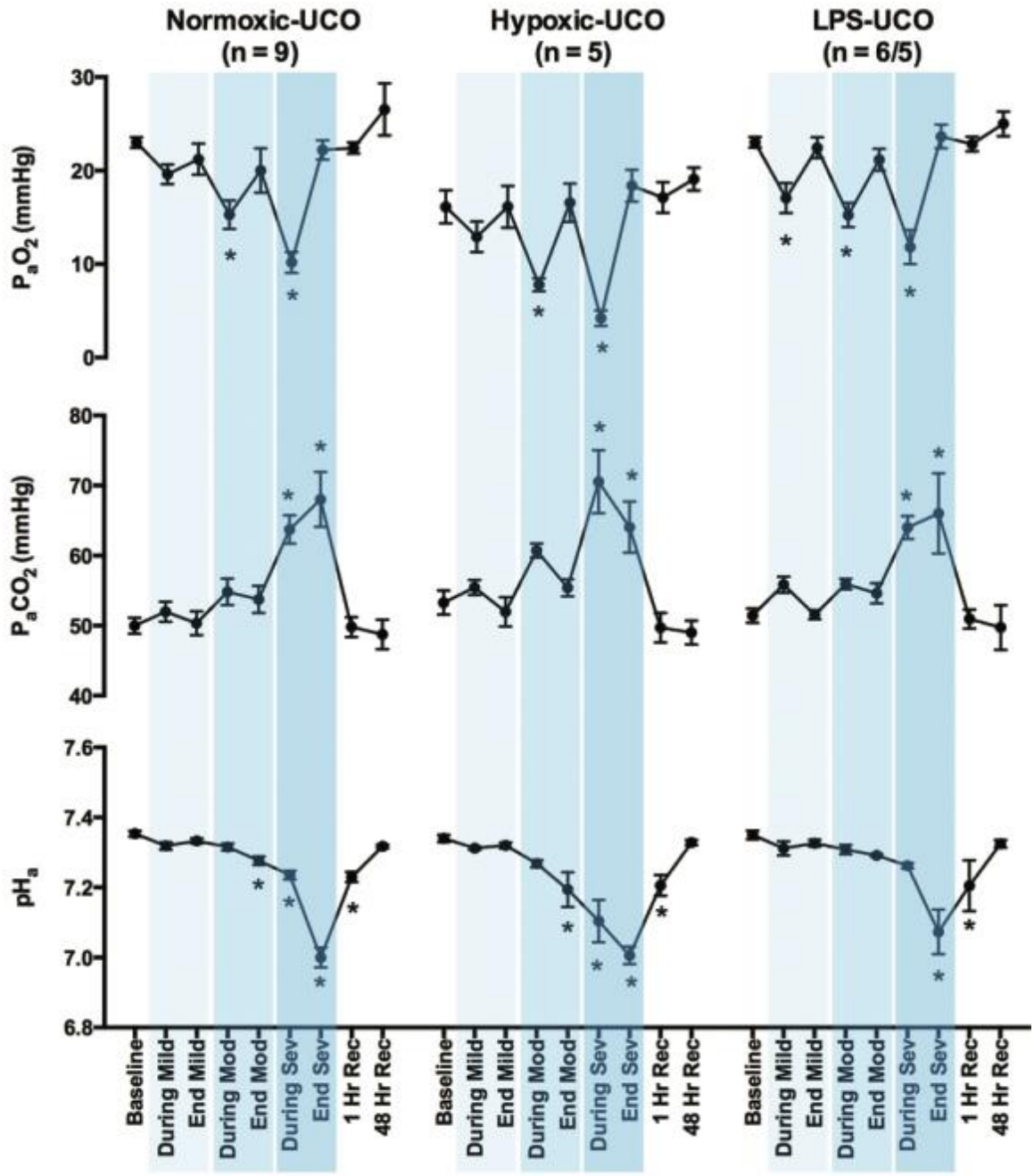

Figure 2.5. Graph of fetal arterial PO_2, PCO_2, and pH (mean ± standard error of the mean [SEM]) throughout the UCO and recovery periods. "During" measurements were taken during the final 10 seconds of a UCO, while ''end'' measurements were taken at the end of each UCO series between UCOs. *P < .05 compared to respective baseline values. UCO indicates umbilical cord occlusion.

reached a nadir pH of 7.06, and 1 LPS-UCO animal only reached a nadir pH of 7.14. Additionally, in 2 of the LPS-UCO animals, there was difficulty attaining complete inflation of the occluder cuff throughout the severe UCO series as indicated by the pressure recording from the occluder, with these animals only reaching nadir pHs of 7.25 and 7.20. The average time between onset of UCOs and reaching the target nadir pH of 7.00 was shorter in the hypoxic-UCO animals at 151 ± 16 minutes compared to the normoxic-UCO animals at 203 ± 14 minutes ($P < .05$). Although pHa values showed improvement when measured at 1 hour of recovery, they continued to be significantly decreased from respective baseline values for all 3 experimental groups (all $P < .05$; Figure 2.5).

Glucose values at baseline were similar for all 3 experimental groups averaging 1.1 ± 0.1 mmol/L and showed no significant change until the end of the severe UCO series when increased to 2.0 ± 0.2, 2.1 ± 0.2, and 1.7 ± 0.2 mmol/L for the normoxic-UCO, hypoxic-UCO and LPS-UCO groups, respectively (all $P < .05$; Figure 2.6). Lactate values at baseline were also similar for the 3 experimental groups averaging 2.0 ± 0.4 mmol/L and again showed no significant changes until the end of the severe UCO series when increased to 10.3 ± 1.7, 10.0 ± 2.0, and 10.5 ± 1.6 mmol/L for the normoxic-UCO, hypoxic-UCO, and LPS-UCO groups, respectively (all $P < .05$; Figure 2.6). Although lactate values were somewhat decreased when again measured at 1 hour of recovery, they continued to be significantly increased from respective baseline values for all 3 experimental groups (all $P < .05$; Figure 2.6). Of note, one of the LPS-UCO animals whose blood gas, pH, and metabolite values appeared to be normalizing when measured at 1 hour of recovery, nonetheless had extremely acidotic values when measured at 24 hours of recovery, pH 6.86, and lactate 17.0 with this animal then immediately sacrificed. Accordingly, while this animal's measurements up until 1 hour of recovery have been included in the LPS-UCO results, they have been excluded thereafter.

Fetal arterial oxygen saturation, PO2, PCO2, pH, glucose, and lactate values for the control group animals, whose tissues were collected as controls for the experimental group animals and studied at the same gestational age, were also within the normal physiologic range averaging 56% ± 3%, 21.0 ± 0.9 mmHg, 52.3 ± 1.2 mmHg, and 7.35 ± 0.01, respectively.

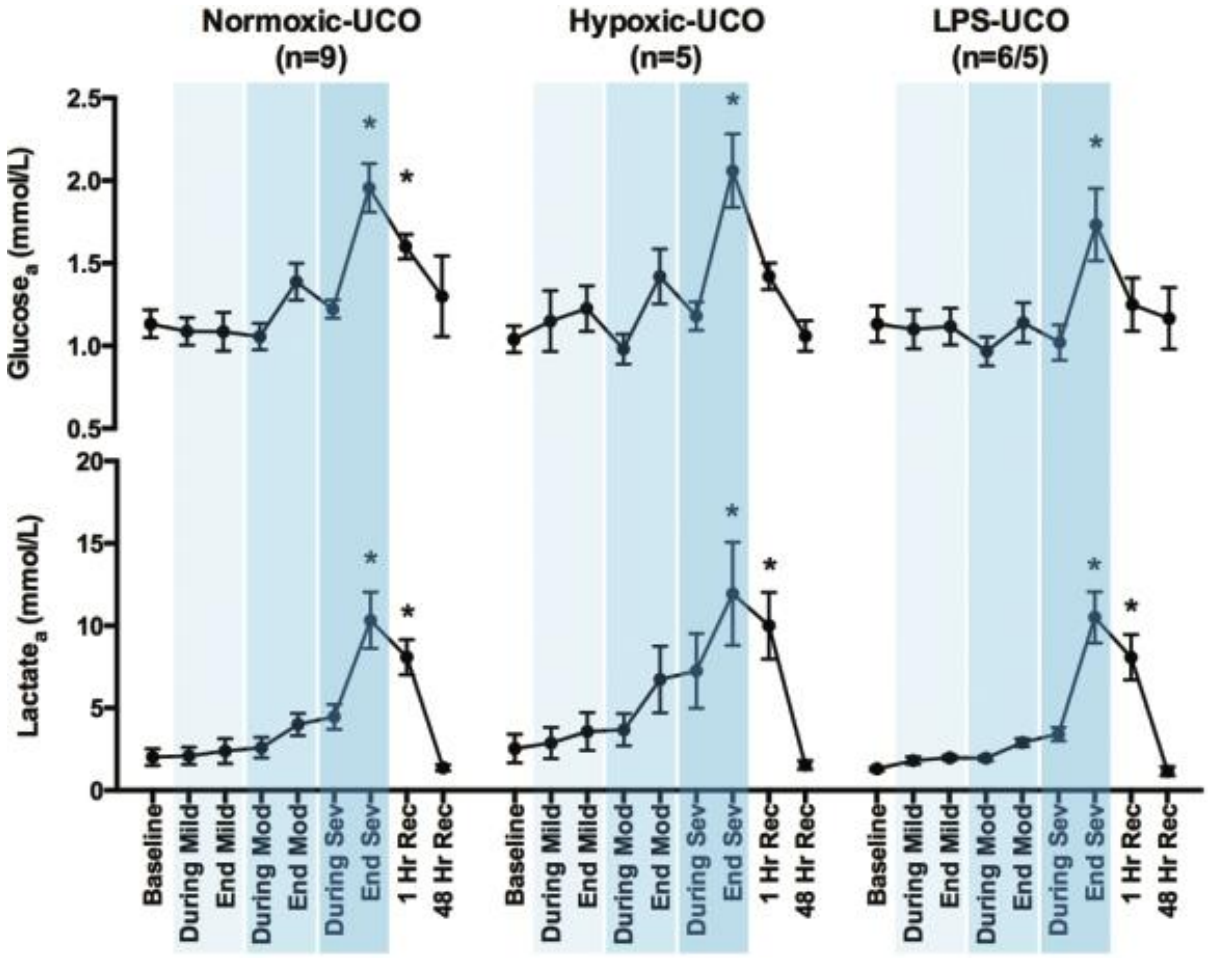

Figure 2.6. Graph of fetal arterial glucose and lactate (mean ± standard error of the mean [SEM]) throughout the UCO and recovery periods. "During" measurements were taken during the final 10 seconds of a UCO, while "end" measurements were taken at the end of each UCO series between UCOs. *P < .05 compared to respective animal group baseline values. UCO indicates umbilical cord occlusion.

2.3.3 Placental Immunohistochemistry

In the control group animals, macrophage cell counts were significantly higher within the fetal zone at 13.7 ± 2.0 cells/HPF than in the intermediate zone and zona intima at 7.9 ± 1.6 and 7.0 ± 0.9 cells/HPF, respectively (Figure 2.7; both $P < .05$). However, the 3 experimental UCO groups showed similar macrophage cell counts to that of the control group for each of the cotyledon zones examined with no significant differences noted (Figure 2.7). In the control group animals, neutrophils were found to be absent within the fetal zone, while significantly higher in the intermediate zone at 0.7 ± 0.3 cells/HPF and the zona intima at 0.6 ± 0.1 cells/HPF (Figure 2.8; both $P < .05$). In the 3 experimental groups, neutrophils were again found to be largely absent within the fetal zone, and cell counts within the intermediate zone were not significantly different from control animals (Figure 2.8). However, in the zona intima, neutrophil cell counts were approximately 2-fold higher than that of the control group at 1.2 ± 0.2, 1.4 ± 0.2, and 1.4 ± 0.3 cells/HPF in the normoxic-UCO, hypoxic-UCO, and LPS-UCO groups, respectively (Figure 2.8; all $P < .05$), although these did not differ from one another. There was additionally no correlation between baseline oxygen status the day of UCO study or averaged postoperatively and zona intima neutrophil cell counts for any of the UCO study groups. There were no mast cells seen in any of the cotyledon zones for either the control or any of the UCO experimental groups of animals.

2.3.4 Plasma and Amniotic Fluid Cytokines

Fetal plasma and amniotic fluid cytokine levels as assessed at select time points throughout the study are shown in Table 1. IL-6 was undetectable (concentration <0.016 ng/mL) in fetal plasma on postop day 2 for all 3 experimental groups, indicating that any increase in this cytokine as a measure of systemic inflammation that might have occurred as a result of the surgical procedure was no longer evident. IL-6 was also undetectable in fetal plasma at baseline for all 3 experimental groups and continued to be undetectable throughout the UCO and recovery periods for the normoxic-UCO and hypoxic-UCO groups. However, plasma IL-6 values began to increase in the LPS-UCO group animals by the end of the severe UCOs and significantly so after 1 hour recovery at 0.22 ± 0.11 ng/mL when compared to respective baseline values ($P < .05$). Of note, this increase in plasma IL-6 values in the LPS-UCO animals at 1 hour recovery was highly correlated with the fetal arterial pH nadir at the end of the UCOs and thereby the degree of fetal acidemia attained (Spearman $r = -.97$). Fetal plasma

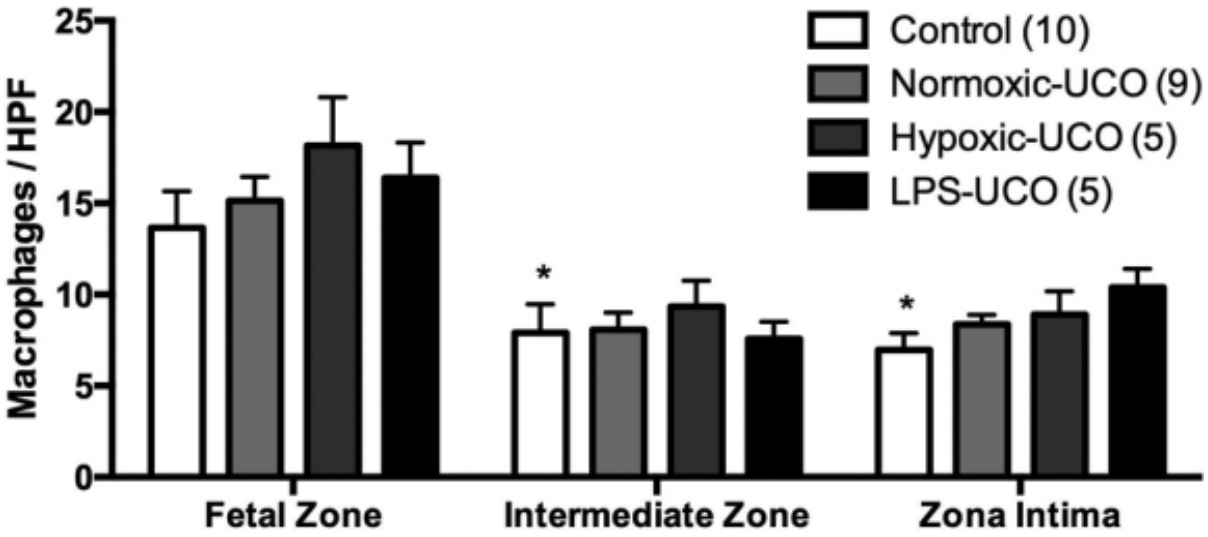

Figure 2.7. Bar graph of CD163-positive macrophages (mean ± standard error of the mean [SEM]) within the cotyledon zones expressed as cells/high-power field (HPF). *P < .05 compared to fetal zone control values. UCO indicates umbilical cord occlusion.

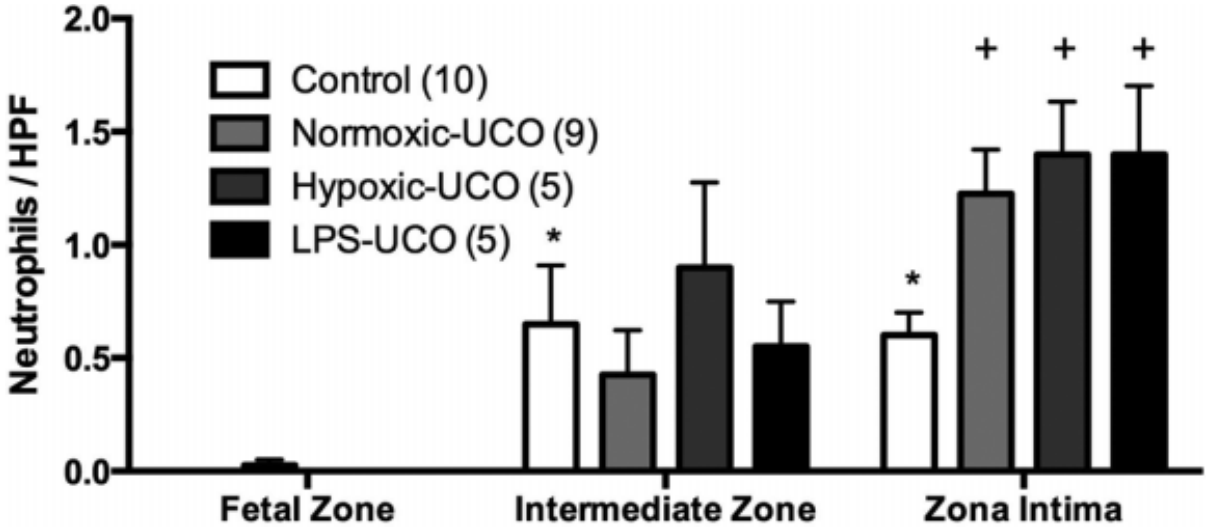

Figure 2.8. Bar graph of neutrophils (mean ± standard error of the mean [SEM]) within the cotyledon zones expressed as cells/high power field (HPF). *P < .05 compared to fetal zone control values; +P < .05 compared to respective control group values. UCO indicates umbilical cord occlusion.

IL-1β was also analyzed at these same time points in the 3 UCO experimental groups. However, results here were extremely variable with values often outside the reference range and possibly due to interference with the primary antibody from the assay buffer used in this analysis. Accordingly, these results have not been reported.

Amniotic fluid IL-6 and IL-1β baseline values were similar for all 3 UCO experimental groups, averaging 0.08 ± 0.03 and 0.69 ± 0.14 ng/mL for IL-6 and IL-1β, respectively (Table 1). Although amniotic fluid IL-6 values in the normoxic-UCO and hypoxic-UCO groups remained consistent at 1 and 48 hours of recovery, LPS-UCO animals showed an ~16-fold increase at these time points, although this was not significant due to the high degree of variance in the response. Likewise, IL-1β values in the normoxic-UCO and hypoxic-UCO groups remained consisten at 1 and 48 hours of recovery, whereas the LPS-UCO animals showed an ~10-fold (nonsignificant) and 50-fold increase ($P < .05$) at these respective time points, although again with considerable variance in this response.

Table 2.1. Plasma and Amniotic Fluid Cytokine Measurements (ng/mL).[a]

	Postop D2	Baseline	End UCO	1 Hour Rec	48 Hours Rec
Plasma IL-6					
Normoxic-UCO	ND	ND	ND	ND	ND
Hypoxic-UCO	ND	ND	ND	ND	ND
LPS-UCO	ND	ND	0.07 ± 0.04	0.22 ± 0.11[b]	0.03 ± 0.01
Amniotic Fluid IL-6					
Normoxic-UCO	–	0.14 ± 0.06	–	0.10 ± 0.09	0.17 ± 0.08
Hypoxic-UCO	–	0.03 ± 0.01	–	0.02 ± 0.01	0.05 ± 0.02
LPS-UCO	–	0.07 ± 0.03	–	1.13 ± 0.82	1.19 ± 0.97
Amniotic Fluid IL-1β					
Normoxic-UCO	–	0.91 ± 0.45	–	0.90 ± 0.48	0.35 ± 0.14
Hypoxic-UCO	–	0.73 ± 0.35	–	0.62 ± 0.49	0.18 ± 0.05
LPS-UCO	–	0.42 ± 0.19	–	4.41 ± 1.16	22.3 ± 12.7[b]

Abbreviations: IL, interleukin; LPS, lipopolysaccharide; ND, not detectable; Postop, postoperative; rec, recovery; SEM, standard error of the mean; UCO, umbilical cord occlusion.

[a]Data are presented as means ± SEMs.

[b]$P < .05$ compared to respective baseline values; not detectable (concentration <0.016 ng/mL).

2.4 Discussion

Variable FHR decelerations due to compression of the umbilical cord during contractions are the most common nonreassuring FHR pattern seen during human labour, and when frequent and/or severe can give rise to concerning fetal acidemia at birth.[33] The relationship between the cardiovascular response and reductions in umbilical blood flow from compression of the umbilical cord has been characterized in the ovine fetus by Itskovitz *et al*[26] who found that the magnitude of FHR decelerations was dependent on the degree of UCO, and that a blood flow reduction of at least 50% was required to elicit a cardiovascular response. In order to induce worsening fetal acidemia with repetitive UCOs, as might occur clinically during human labour, we used a UCO paradigm consisting of 60-second UCOs occurring every 2.5 minutes, with 1 hour of mild-partial cord occlusions, followed by 1 hour of moderate-partial cord occlusions, then up to 2 hours of severe-complete cord occlusion. The degree of occluder cuff inflation for the mild-partial and moderate-partial occlusions was titrated to affect an FHR response associated with a 50% (~25 bpm decelerations) and 75% (~65 bpm decelerations) reduction in umbilical cord blood flow, respectively, whereas severe complete occlusions involved maximal inflation of the occluder cuff and a complete cessation in umbilical blood flow as determined by Itskovitz *et al*.[26] We were able to attain FHR decelerations in the 3 UCO animal groups, similar to those seen by Itskovitz as intended, and thereby indicating ~50%, ~75%, and 100% reductions in umbilical cord blood flow for the mild partial, moderate partial, and severe complete UCOs, respectively. However, since umbilical blood flow was not monitored in the present study, it is possible that the cardiovascular response to induced UCOs differed among the 3 UCO groups and thereby the umbilical blood flow reductions invoked for the mild partial and moderate partial UCO series.

In the normoxic-UCO animals, the mild partial, moderate partial, and severe complete UCOs resulted in transitory fetal hypoxemia and hypercapnia of increasing severity the greater the reduction in umbilical blood flow. This response was overall similar to that reported by Itskovitz *et al*[26] taking into account that their UCOs were of 40-second duration rather than 60 seconds, and that they directly titrated to the decrease in umbilical blood flow. These repetitive UCOs resulted in a cumulative acidosis of a modest degree by the end of the moderate partial UCOs, fetal arterial pH to 7.28, and of a marked degree by the end of the severe complete UCOs, with pH on average to 7.00 and with 8 of the 9 animals reaching the targeted pH. This

worsening acidosis was both respiratory and metabolic in nature with the sustained increase in PCO_2 and lactate values noted. The hypoxic-UCO animals displayed lower PaO_2 values at each of the time points studied and not surprisingly took ~50 minutes less on average than the normoxic-UCO animals to reach the targeted pH with the repetitive UCOs as studied. Although the arterial PO_2 and PCO_2 changes for the LPS-UCO animals were similar to that of the normoxic-UCO animals, it is of note that only 3 of the 6 animals here attained the targeted pH of 7.00. This can be attributed in part to the difficulty in 2 of the animals with complete inflation of the occluder cuff during the severe UCO series as previously noted but may also relate to increased uterine blood flow in these animals with the LPS administration and thereby increased oxygen delivery as we have also speculated in the human fetus where oxygenation is increased with chorioamnionitis.[34]

In all 3 UCO groups, fetal glucose levels increased with repetitive severe complete UCOs to 1.9 mmol/L on average by the end of the UCOs, likely reflecting an increase in fetal glycogenolysis as previously reported in the ovine fetus with sustained hypoxemia and a rise in catecholamine levels.[35] Fetal lactate levels were likewise similarly increased in all 3 UCO groups to 10.3 mmol/L on average by the end of the severe complete UCOs, thereby contributing to the marked acidemia at this time as the principal fixed acid leading to metabolic acidosis in the fetus.[36] This increase in lactate levels presumably reflects the cumulative increase in lactate after each cord occlusion and more so for the severe complete UCOs as we have previously shown.[37]

In the control group animals, no extravascular neutrophils were seen in the chorionic plate or fetal zone of the cotyledon while low levels were seen in the intermediate zone and zona intima encompassing interdigitating fetal and maternal cotyledon tissues. The presence of neutrophils in these areas without apparent inflammatory stimuli is likely due to their close proximity to the high volume flow of maternal blood here. In all 3 UCO groups, no differences in neutrophil counts were observed in the fetal and intermediate zones compared to that of the control animals. However, in the zona intima, neutrophil counts were similarly increased ~2-fold in all 3 UCO groups, with no additive effect from underlying hypoxia or LPS. This indicates that repetitive UCOs leading to severe fetal acidemia can induce a neutrophil inflammatory response within the placenta. Initiating stimuli likely involves placental hypoxia and/or

hypoperfusion resulting from the UCOs, which have been shown to increase inflammatory cytokines within placental tissues from leukocyte infiltration/activation, decidual stromal cells, and placental trophoblast and endothelial cells.[15,16] The finding that this UCO-related increase in neutrophils was confined to the zona intima of the ovine fetal cotyledon would also suggest that this was largely maternal in origin, which is the source of most neutrophils within the placenta.[38] Although it is somewhat surprising that LPS given intraamniotic did not have an apparent effect on neutrophil counts as studied, since infiltration of neutrophils in the fetal membranes is a hallmark of chorioamnionitis[5]. This may be due to the fact that the extraplacental fetal membranes were not examined but rather the fetal zone of the ovine cotyledon. Although this included the chorionic plate membranes overlying the cotyledon depression, the membranes here will be adjacent to fetal tissue and thereby less reflective of a maternal inflammatory response than might be expected in the extraplacental membranes that are adjacent to the maternal decidua. Although the cotyledon tissues were collected 48 hours after the UCOs and LPS administration, and some of the neutrophil inflammatory response from these stimuli might now be lessened or no longer evident, this seems unlikely since similar LPS administration results in persistence of an inflammatory cell infiltrate in the extraplacental membranes for several days.[24,25]

Macrophages are normally present in the placenta and contain membrane receptors that recognize signaling molecules resulting from injury and/or infectious processes thereby triggering inflammatory responses through the release of cytokines including IL-6 and IL-1 β.[39] CD163 is a membrane bound receptor that is highly expressed on tissue macrophages including those within the placenta and is not found on other hematopoietic or nonhematopoietic tissue cell types.[28,39-41] In the control group animals, extravascular CD163-positive macrophages were seen throughout the cotyledon as studied but were ~2-fold higher in the fetal zone when compared to that of the intermediate zone and zona intima. The higher number of CD163+ cells within the fetal zone is consistent with the fact that these cells are primarily of fetal origin.[42] CD163+ macrophage counts were not affected by UCOs and associated acidemia nor by underlying hypoxia and LPS. These findings indicate that no increased infiltration or proliferation of macrophages was evident as measured at 48 hours of recovery but do not rule out increased activation of macrophages already present in the placenta

nor of an earlier macrophage inflammatory response within the placenta to these stimuli, although this again seems unlikely.[41,43]

Mast cells are widely recognized as mediators of inflammatory processes through the secretion of a variety of biological molecules including histamine, prostaglandins, and cytokines.[44] To our knowledge, this is the first study of mast cells in the ovine placental cotyledons, which were absent under control conditions and in response to repetitive UCOs and underlying hypoxia and LPS exposure, although they can be found in the term human placenta.[45] Accordingly, mast cells may play little if any role in the placental inflammatory response to noxious stimuli, which could be protective since the placenta has a high potential for encountering triggering stimuli, and mast cell degranulation and histamine release can have a variety of effects on the fetus,[46] including a trigger for preterm labor.[47]

The proinflammatory cytokines IL-1β and IL-6 have been identified as measures of fetal/placental inflammation because these cytokines play a prominent regulatory role in the inflammatory response and have been shown to increase as part of the fetal/neonatal inflammatory response to infection and HIE.[5,10-12] Fetal plasma IL-6 was undetectable in all animals on postop day 2 indicating that any increase in this cytokine as a result of the surgical procedure was no longer evident. This is to be expected given the short half-life of cytokines and need for repeated stimuli for continued production.[5] Plasma IL-6 continued to be undetectable in the normoxic-UCO and hypoxic-UCO animals throughout the UCO and recovery periods, which is somewhat surprising since IL-6 has been shown to increase in the cord blood of newborn infants with perinatal asphyxia.[10,11] Although this may relate to species-specific differences, it is also likely that additional triggering stimuli are contributory in the human newborns including the impact of labor and in some instances placental infection.[20] The present IL-6 findings are in fact similar to that we have previously reported in the ovine fetus subjected to repeated UCOs with severe acidemia whereby IL-6 levels remained unchanged, but IL-1β levels increased 2-fold at nadir pH.[48] As such, a fetal systemic inflammatory response to the UCOs/severe acidemia is likely, but involving other cytokines than IL-6 or other substances suchas prostaglandins, at least for the time points herein studied. The LPS-UCO animals received injections of LPS at 2 mg/h intraamniotic to simulate a low-grade bacterial infection as often observed in low-risk labouring patients at term with histologic

chorioamnionitis in the absence of clinical chorioamnionitis.[20] This LPS dosing is similar to that of Kramer *et al*[25] and approximately half of that used by Kallapur *et al*[24] with both showing a rapid and sustained leukocytic infiltration in the extraplacental membranes lasting several days with associated increases in cytokine messenger RNA (mRNA) levels, although mainly for the first 24 hours. Plasma IL-6 began to increase in the LPS-UCO animals by the end of the severe UCOs and significantly so after 1 hour recovery and indicating a systemic inflammatory response in these animals. Although the effects of intraamniotic LPS alone were not studied which is a limitation, it is likely that the LPS-and UCO-induced fetal acidemia together contributed to this response since the increase in IL-6 was highly correlated with the degree of fetal acidemia at nadir pH in these animals. Additionally, another study in fetal sheep by Grigsby *et al*[49] failed to show any increase in plasma or amniotic fluid IL-6 after intra-amniotic LPS alone in a dosing similar to that used by Kallapur *et al*. However, in the study of Kramer *et al*[25] with LPS dosing similar to the present study, cord plasma IL-6 levels at delivery were increased approximately 2-fold 5 hours post-LPS, although animals were 2 weeks younger and amniotic fluid IL-6 mRNA levels remained unchanged. This suggests interaction between the degree of fetal maturation and severity of endotoxin exposure in eliciting systemic inflammatory responses, which has been well described in the clinical literature relating to inflammation in preterm newborns.[5] The additive effect of simulated low-grade infection is also consistent with our findings in labouring patients at term where umbilical cord levels for several cytokines including IL-6 are substantially increased in those patients with histologic chorioamnionitis.[20] Although the cellular origin for the increase in plasma IL-6 seen remains unknown, it is likely that the extraplacental membranes are involved given the findings of Kramer *et al*[25] and Kallapur *et al*,[24] as well as the placental cotyledons given the greater impact here of UCO-induced changes in tissue perfusion and oxygenation.

Amniotic fluid IL-1β and IL-6 in the normoxic-UCO and hypoxic-UCO animals were unchanged at 1 and 48 hours recovery from the UCOs/severe acidemia and indicating the absence of a placental and/or membrane inflammatory response for these cytokines or associated release into the amniotic cavity. As such, the neutrophil inflammatory response seen in the cotyledons of these animals was unlikely to involve secretion of cytokines to the amniotic cavity, which is not surprising since this response was confined to the zona intima away from the amniotic cavity. To the extent that plasma IL-1β was increased in these animals and

involved placental release as previously discussed, then cellular sources are again more likely to be away from the amniotic cavity. Of note, Nitsos *et al*[50] also found amniotic fluid IL-6 as well as IL-8 to be unchanged in fetal sheep after intermittent UCOs over a 2-day period, although the degree of fetal acidemia was much less than in the present study, and cytokine levels were obtained at delivery 1 week after the UCOs. Amniotic fluid IL-1β and IL-6 in the LPS-UCO animals were increased at 1 and 48 hours recovery and markedly so for IL-1β at 48 hours, albeit with considerable variance that related to the degree of UCO-induced acidemia. This finding indicates a placental and/or membrane inflammatory response in these animals, the likely synergistic effect of LPS together with UCO-induced fetal acidemia in triggering this response, and again supports the placenta and/or membranes as a major contributor to the fetal inflammatory response with the related increase in plasma IL-6 levels. It is also of note that the amniotic fluid cytokine response in these animals was much greater and longer lasting than the plasma cytokine response, which might impact developmental events for organ systems exposed to amniotic fluid as well studied for the lung.[24,25,51] In the study of Kramer *et al*[25] with similar LPS dosing to the present study, amniotic fluid IL-1β mRNA levels were also increased from 24 hours to 7 days and indicating a differential response to that of IL-6, which as noted remained unchanged with LPS alone. Of interest, they also found an increase in inflammatory cells within the amniotic fluid which related to the increase in cytokine mRNA levels post LPS, again supporting the amniotic fluid as an important source of inflammatory stimulus for the fetus.[25]

In this study, we determined that repetitive UCOs with severe acidemia in the ovine fetus will result in increased neutrophil infiltration of the zona intima of the cotyledon placenta, and when combined with intra-amniotic LPS, increased IL-1β and IL-6 within the amniotic fluid and IL-6 within the fetal plasma. As such, repetitive UCOs with severe acidemia can induce a placental and/or membrane inflammatory response and more so with simulated low-grade infection and likely involving multiple cellular sources with the potential for cytokine release to the umbilical circulation. Variable-type FHR decelerations due to umbilical cord compression are the most common nonreassuring FHR pattern observed intrapartum[1] and when frequent and/or severe have been associated with increased incidence of neonatal acidosis and nuchal cord at delivery,[2,4] and in turn neurologic sequelae.[3,52] Additionally, there is now considerable evidence that fetal/neonatal inflammation in response to perinatal infection[5-7] and hypoxic

asphyxia at birth[10-12] plays a causal role in brain injury of the newborn. The present findings in an animal model relevant to human labour with repetitive UCOs, leading to severe acidemia in the presence or absence of simulated infection, adds to this evidence confirming the potential for placental and/or membrane inflammation.

2.5 References

1. American College of Obstetricians and Gynecologists. Fetal heart rate patterns: monitoring, interpretation, and management. *Technical bulletin*. 1995;207.

2. Goldaber KG, Gilstrap LC, Leveno KJ, Dax JS, McIntire DD. Pathologic fetal acidemia. *Obstet Gynecol*. 1991;78(6):1103-1107.

3. Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. *Am J Obstet Gynecol*. 2008;199(6):587-595.

4. Winkler CL, Hauth JC, Tucker JM, Owen J, Brumfield CG. Neonatal complications at term as related to the degree of umbilical artery acidemia. *Am J Obstet Gynecol*. 1991;164(2): 637-641.

5. Gotsch F, Romero R, Kusanovic JP, Mazaki-Tovi S, Pineles BL, Erez O, Espinoza J, and Hassan SS. The fetal inflammatory response syndrome. *Clin Obstet Gynecol*. 2007;50(3):652-683.

6. Nitsos I, Rees SM, Duncan J, Kramer BW, Harding R, Newnham JP, and Moss TJM. Chronic exposure to intraamniotic lipopolysaccharide affects the ovine fetal brain. *J Soc Gynecol Investig*. 2006;13(4):239-247.

7. Duncan J, Cock M, Suzuki K, Scheerlinck JP, Harding R, Rees S. Chronic endotoxin exposure causes brain injury in the ovine fetus in the absence of hypoxemia. *J Soc Gynecol Investig*. 2006;13(2): 87-96.

8. Eklind S, Mallard C, Leverin AL, Gilland E, Blomgren K, Mattsby-Baltzer I, and Hagberg H. Bacterial endotoxin sensitizes the immature brain to hypoxic–ischaemic injury. *Eur J Neurosci*. 2001;13(6):1101-1106.

9. Coumans ABC, Middelanis J, Garnier Y, Vaihinger HM, Leib SL, Von Duering MU, Hasaart THM, Jensen A, and Berger R. Intracisternal application of endotoxin enhances the susceptibility to subsequent hypoxic–ischemic brain damage in neonatal rats. *Pediatr Res*. 2003;53(5):770-775.

10. Silveira RC, Procianoy RS. Interleukin-6 and tumor necrosis factor-α levels in plasma and cerebrospinal fluid of term newborn infants with hypoxic–ischemic encephalopathy. *J Pediatr*. 2003; 143(5):625-629.

11. Chiesa C, Pellegrini G, Panero A, De Luca T, Assumma M, Signore F, and Pacifico L. Umbilical cord interleukin-6 levels are elevated in term neonates with perinatal asphyxia. *Eur J Clin Invest*. 2003;33(4):352-358.

12. Hagberg H, Gilland E, Bona E, Hanson LA, Hahin-Zoric M, Blennow M, Holst M, McRae A, and Söde O. Enhanced expression of interleukin (IL)-1 and IL-6

messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatr R*es. 1996;40(4): 603-609.

13. Guo R, Hou W, Dong Y, Yu Z, Stites J, Weiner CP. Brain injury caused by chronic fetal hypoxemia is mediated by inflammatory cascade activation. *Reprod Sci*. 2010;17(6):540-548.

14. Romero R, Gotsch F, Pineles B, Kusanovic JP. Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury. *Nutr Rev*. 2007;65(12 pt 2): S194-S202.

15. Pierce BT, Pierce LM, Wagner RK, Apodaca CC, Hume RF, Nielsen PE, and Calhoun BC. Hypoperfusion causes increased production of interleukin 6 and tumor necrosis factor a in the isolated, dually perfused placental cotyledon. *Am J Obstet Gynecol*. 2000;183(4):863-867.

16. Bowen JM, Chamley L, Keelan JA, Mitchell MD. Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. *Placenta*. 2002;23(4): 257-273.

17. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet*. 2008;371(9606):75-84.

18. Becroft DMO, Thompson JMD, Mitchell EA. Placental chorioamnionitis at term: epidemiology and follow-up in childhood. *Pediatr Dev Pathol*. 2010;13(4):282-290.

19. Soothill PW, Nicolaides KH, Campbell S. Prenatal asphyxia, hyperlacticaemia, hypoglycaemia, and erythroblastosis in growth retarded fetuses. *Br Med J (Clin Res Ed)*. 1987;294(6579): 1051-1053.

20. Chan CJ, Summers KL, Chan NG, Hardy DB, Richardson BS. Cytokines in umbilical cord blood and the impact of labor events in low-risk term pregnancies. *Early Hum Dev*. 2013;89(12): 1005-1010.

21. Green LR, Homan J, White SE, Richardson BS. Cardiovascular and metabolic responses to intermittent umbilical cord occlusion in the preterm ovine fetus. *J Soc Gynecol Investig*. 1999;6(2): 56-63.

22. Richardson BS, Carmichael L, Homan J, Patrick JE. Electrocortical activity, electroocular activity and breathing movements in fetal sheep with prolonged and graded hypoxemia. *Am J Obstet Gynecol.* 1992;167(2):553-558.

23. Gardner DS, Fletcher AJ, Bloomfield MR, Fowden AL, Giussani DA. Effects of prevailing hypoxaemia, acidaemia or hypoglycaemia upon the cardiovascular, endocrine and metabolic responses to acute hypoxaemia in the ovine fetus. *J Physiol*. 2002;540(pt 1):351-366.

24. Kallapur SG, Willet KE, Jobe AH, Ikegami M, Bachurski CJ. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol*

Lung Cell Mol Physiol. 2001;280(3): L527-L536.

25. Kramer BW, Moss TJ, Willet KE, Newnham JP, Sly PD, Kallapur SG, Ikegami M, and Jobe AH. Dose and time response after intraamniotic endotoxin in preterm lambs. *Am J Respir Crit Care Med*. 2001;164(6):982-988.

26. Itskovitz J, LaGamma EF, Rudolph AM. Heart rate and blood pressure responses to umbilical cord compression in fetal lambs with special reference to the mechanism of variable deceleration. *Am J Obstet Gynecol*. 1983;147(4):451-457.

27. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, HoffmanHJ, Law SKA, and Moestrup SK. Identification of the haemoglobin scavenger receptor. *Nature*. 2001;409(6817): 198-201.

28. Lau SK, Chu PG, Weiss LM. CD163: a specific marker of macrophages in paraffin-embedded tissue samples. *Am J Clin Pathol*. 2004;122(5):794-801.

29. Burton GJ, Samuel CA, Steven DH. Ultrastructural studies of the placenta of the ewe: phagocytosis of erythrocytes by the chorionic epithelium at the central depression of the cotyledon. *Q J Exp Physiol Cogn Med Sci*. 1976;61(4):275-286.

30. Durosier LD, Green G, Batkin I, Seely AJ, Ross MG, Richardson BS, and Frasch MG. Sampling rate of heart rate variability impacts the ability to detect acidemia in ovine fetuses near-term. *Front Pediatr*. 2014;2:38.

31. Rivolta MW, Stampalija T, Casati D, Richardson BS, Ross MG, Frasch MG, Bauer A, Ferrazzi E, and Sassi R. Acceleration and deceleration capacity of fetal heart rate in an in-vivo sheep model. *PLoS One*. 2014;9(8):e104193.

32. Wang X, Durosier LD, Ross MG, Richardson BS, Frasch MG. Online detection of fetal acidemia during labour by testing synchronization of EEG and heart rate: a prospective study in fetal sheep. *PLoS One*. 2014;9(9):e108119.

33. Ball RH, Parer JT. The physiologic mechanisms of variable decelerations. *Am J Obstet Gynecol*. 1992;166(6 pt 1):1683-1688; discussion 1688-1689.

34. Richardson BS, Wakim E, daSilva O, Walton J. Preterm histologic chorioamnionitis: impact on cord gas and pH values and neonatal outcome. *Am J Obstet Gynecol.* 2006;195(5):1357-1365.

35. Gu W, Jones CT, Parer JT. Metabolic and cardiovascular effects on fetal sheep of sustained reduction of uterine blood flow. *J Physiol (Lond).* 1985;368:109-129.

36. Low JA. The role of blood gas and acid–base assessment in the diagnosis of intrapartum fetal asphyxia. *Am J Obstet Gynecol*. 1988;159(5):1235-1240.

37. Richardson BS, Carmichael L, Homan J, Johnston L, Gagnon R. Fetal cerebral, circulatory, and metabolic responses during heart rate decelerations with umbilical cord compression. *Am J Obstet Gynecol*. 1996;175(4):929-936.

38. Lee SD, Kim MR, Hwang PG, Shim SS, Yoon BH, Kim CJ. Chorionic plate vessels as an origin of amniotic fluid neutrophils. *Pathol Int*. 2004;54(7):516-522.

39. Nagamatsu T, Schust DJ. Review: the immunomodulatory roles of macrophages at the maternal–fetal interface. *Reprod Sci.* 2010;17(3):209-218.

40. Fabriek BO, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor CD163. *Immunobiology*. 2005;210(2-4):153-160.

41. Kacerovsky M, Cobo T, Hornychova H, Andrys C, Ryska A, Musilova I, Kriz JT, Kostal M, and Jacobsson B. Scavenger receptor for hemoglobin in preterm prelabor rupture of membranes pregnancies complicated by histological chorioamnionitis. *J Matern Fetal Neonatal Med*. 2012;25(11):2291-2297.

42. Kim J-S, Romero R, Kim MR, Kim YM, Friel L, Espinoza J, and Kim CJ. Involvement of Hofbauer cells and maternal T cells in villitis of unknown aetiology. *Histopathology*. 2008;52(4):457-464.

43. Tang Z, Abrahams VM, Mor G, Guller S. Placental Hofbauer cells and complications of pregnancy. *Ann N Y Acad Sci*. 2011; 1221:103-108.

44. Lindsberg PJ, Strbian D, Karjalainen-Lindsberg ML. Mast cells as early responders in the regulation of acute blood–brain barrier changes after cerebral ischemia and hemorrhage. *J Cereb Blood Flow Metab*. 2010;30(4):689-702.

45. Purcell WM, Hanahoe TH. A novel source of mast cells: the human placenta. *Agents Actions*. 1991;33(1-2):8-12.

46. Lieberman P. The basics of histamine biology. *Ann Allergy Asthma Immunol*. 2011;106(2 suppl):S2-S5.

47. Romero R, Kusanovic JP, Mun˜oz H, Gomez R, Lamont RF, Yeo L. Allergy-induced preterm labor after the ingestion of shellfish. *J Matern Fetal Neonatal Med*. 2010;23(4):351-359.

48. Prout AP, Frasch MG, Veldhuizen RAW, Hammond R, Ross MG, Richardson BS. Systemic and cerebral inflammatory response to umbilical cord occlusions with worsening acidosis in the ovine fetus. *Am J Obstet Gynecol*. 2010;202(1):82.e1-e9.

49. Grigsby PL, Hirst JJ, Scheerlinck J-P, Phillips DJ, Jenkin G. Fetal responses to maternal and intra-amniotic lipopolysaccharide administration in sheep. *Biol Reprod*. 2003;68(5):1695-1702.

50. Nitsos I, Newnham JP, Rees SM, Harding R, Moss TJ. The impact of chronic intrauterine inflammation on the physiologic and neurodevelopmental consequences of intermittent umbilical cord occlusion in fetal sheep. *Reprod Sci*. 2014;21(5):658-670.

51. Newnham JP, Moss TJM, Kramer BW, Nitsos I, Ikegami M, Jobe AH. The fetal

maturational and inflammatory responses to different routes of endotoxin infusion in sheep. *Am J Obstet Gynecol*. 2002;186(5):1062-1068.

52. Nelson KB, Grether JK. Potentially asphyxiating conditions and spastic cerebral palsy in infants of normal birth weight. *Am J Obstet Gynecol*. 1998;179(2):507-513.

Chapter 3

Repetitive Umbilical Cord Occlusions and Brain Inflammation-Injury

3.1 Introduction

Fetal hypoxia with severe acidemia at birth, as indicated by arterial pH of < 7.00[1,2] is associated with an increased risk for hypoxic-ischemic encephalopathy (HIE) and longer term neurologic sequelae, including cerebral palsy (CP).[3] Variable fetal heart rate (FHR) decelerations are the most common non-reassuring FHR pattern observed intrapartum[4] and are indicative of acute fetal hypoxemia due to compression of the umbilical cord during contractions.[4,5] When frequent and/or severe they have been associated with an increased incidence of neonatal acidosis, low Apgar scores, and nuchal cord at the time of delivery,[6,7] and thereby risk for later adverse neurologic outcome. However, most infants with severe acidemia at birth will not have longer term sequelae, suggesting that an infant's susceptibility to hypoxic-acidemic brain injury may be influenced by a variety of factors in addition to the degree of birth asphyxia.[8]

Both epidemiological and clinical studies have suggested that elevated pro-inflammatory cytokines through the course of infection are associated with an increased risk for perinatal brain injury, whether antenatal with chorioamnionitis or postnatal in the neonate.[9] Subsequent animal-based studies with induced perinatal infection/inflammation have further supported the contributory role of pro-inflammatory cytokines and inflammatory cells in perinatal brain injury,[9-11] and have also suggested that hypoxia and inflammation may act synergistically in the pathogenesis of brain injury.[9,12,13] Hypoxia alone can also trigger an inflammatory response in the fetus, as indicated in both clinical and experimental studies showing that newborns with HIE can have elevated pro-inflammatory cytokines in the absence of infection.[9,14-16] Furthermore, pro-inflammatory cytokines have also been implicated in the development of brain injury due to chronic intrauterine hypoxia leading to fetal growth restriction.[17] These data together indicate that intrauterine infection, acute asphyxia intrapartum, and chronic hypoxia during the antenatal period can induce an inflammatory response in the fetus which can act concomitantly and contribute to perinatal brain injury.

Microglia are the resident immune cells within the brain, which can become activated in response to injury or infection, and have been shown to increase in number with fetal/neonatal

hypoxia and inflammation.[17-19] Mast cells are another immune cell normally found sparsely within the developing brain, but are able to cross the blood-brain-barrier and also increase in number in response to hypoxic-ischemic insults.[20,21] We have previously shown in the near-term ovine fetus that repetitive umbilical cord occlusions (UCOs) with worsening acidemia will lead to an inflammatory response within the brain as indicated by elevated numbers of microglia and mast cells at 24 hours of recovery.[22] We therefore hypothesized that repetitive UCOs with severe fetal acidemia and leading to an inflammatory response within the brain, will consequently result in increased brain injury. In the present study we therefore examined brain inflammation as measured by changes in microglia and mast cell number, and brain injury as measured by the presence of cellular necrosis and apoptosis at 48 hours of recovery from repetitive UCOs with severe acidemia in the near-term ovine fetus. We also examined the additional impact on brain inflammation and injury of lower oxygenation and low-grade bacterial infection as might be seen clinically during labour with fetal growth restriction and placental chorioamnionitis.[23-25]

3.2 Materials and Methods

3.2.1 Surgical Preparation and Experimental Procedure

The data obtained in this chapter were from the same animals used in chapter 2. Thirty mixed breed ewes were surgically instrumented at 124-128 days gestation (term = 147 days), then subjected to the repetitive UCO protocol or served as tissue controls for histological examination of the fetal brains. Anesthesia, surgical preparation, post-operative care and experimental procedure have previously been described in detail.[26,27] Briefly, under general anesthesia polyvinyl catheters were placed in the maternal femoral vein for administering fluids and antibiotics and for blood sampling, in the fetal left and right brachiocephalic arteries for blood sampling and pressure recordings, the right brachiocephalic vein for administering antibiotics, and the amniotic cavity for amniotic fluid sampling and pressure recordings. Stainless-steel electrodes were placed biparietally to monitor fetal electrocortical (ECOG) activity and on the chest to monitor fetal electrocardiographic (ECG) activity. In experimental animals an inflatable occluder cuff was placed around the proximal portion of the umbilical cord. Animals were allowed to recover for 4 days during which antibiotics were administered and maternal and fetal blood samples were taken for blood gases and pH each day. Animal

care followed the guidelines of the Canadian Council on Animal Care and was approved by the University of Western Ontario Council on Animal Care.

Animals were divided retrospectively into 4 groups based upon post-surgical fetal blood oxygen status. This was determined by averaging the arterial O_2 saturation of blood samples taken on each of the 4 post-op recovery days, as well as at baseline on the day of the UCO experimental study. Fetuses with average baseline arterial O_2 saturation of < 55% comprised a low oxygen or hypoxic-UCO group (n=5), since this level of oxygenation has been used to denote decreased oxygenation/spontaneous hypoxemia in the near-term ovine fetus.[28,29] The remaining fetuses with average baseline arterial O_2 saturation ≥ 55% were divided into a normoxic-UCO group (n=9), lipopolysaccharide (LPS)-UCO group (n=6), and control group (n=10). LPS was administered via hourly intra-amniotic injections of 2 mg/hr beginning 1 hour prior to the first UCO and continuing through the period of repetitive UCOs to simulate a low-grade bacterial infection in LPS-UCO animals.[30,31] The control group consisted of normoxic fetuses that underwent surgical preparation but were not subjected to UCOs. These animals were used as tissue controls for histological examination of the fetal brains.

UCOs were induced by inflating occluder cuffs with varying volumes of saline, targeting FHR decelerations of 30, 60, and 90 beats per minute (bpm) corresponding to umbilical blood flow reductions of approximately 50%, 75%, and 100%, and thereby "mild-partial", "moderate-partial", and "severe-complete" UCOs, respectively.[32] All UCOs were 1 minute in duration and occurred every 2.5 minutes. A data acquisition and analysis system was used to record fetal arterial blood pressure, heart rate and amniotic pressure, with FHR triggered and calculated online from arterial pressure systolic peaks. After a 1-2 hour baseline control period, experimental fetuses were subjected to repetitive UCO series consisting of 1 hour of mild-partial, 1 hour of moderate-partial, and up to 2 hours of severe-complete UCOs. UCOs were discontinued if fetal arterial pH dropped below 7.00, or after 2 hours of severe-complete UCOs. Animals were then allowed to recover for 48 hours at which time they were euthanized.

Fetal arterial blood samples (1 mL) were taken at baseline, at the end of a representative UCO from each UCO series, every 20 minutes between UCOs, and after 1, 24, and 48 hrs of recovery. These blood samples were analyzed for blood gases, pH, glucose and lactate using an automated blood gas analyzer.[26] Additional blood samples (3 mL) were collected at baseline

prior to UCOs, immediately after the final UCO, and at 1 and 48 hours of recovery, and processed for later analysis of pro-inflammatory cytokines.[26]

3.2.2 Tissue Histochemical Analysis

Fetal brains were perfusion fixed immediately after animals were euthanized. Briefly, the fetal carotid artery and jugular vein were cannulated and the brain was perfused with 500 mL of cold saline followed by 500 mL of 4% paraformaldehyde. The brain was then removed and immersed in 4% paraformaldehyde for 24 hours, then in fresh phosphate-buffered saline (PBS) for 3 days, followed by 70% ethanol for at least 7 days, and subsequently blocked and embedded in paraffin for histological analysis.

Microglia within the brain were visualized using rabbit anti-Iba1 (1:500, Wako Chemicals USA, Richmond, VA) (Cat No. 019-19741, Lot No. lac4357) as the primary antibody along with a Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). Tissue sections were deparaffinized with three 5-minute washes in xylene, then rehydrated in a series of ethanol baths (100%, 100%, 90%, 90%, 70%) lasting 2 minutes each. Tissue sections were then rinsed once in pure water for 5 minutes followed by two 5-minute washes in PBS. For antigen retrieval, tissues were boiled in 10 mM citrate buffer for 30 minutes, cooled to room temperature for 30 minutes, then rinsed with two 5-minute washes in PBS. Endogenous peroxidase activity was quenched by incubating tissues in 3% H_2O_2 (Fischer Scientific, Ottawa, ON) for 10 minutes followed by two 5-minute washes in PBS. Endogenous biotin was blocked by incubating tissues in avidin solution followed by biotin solution for 15 minutes each. To reduce non-specific staining, slides were incubated in Background Sniper (Biocare Medical, Concord, CA) for 7 minutes at room temperature then rinsed in PBS. Tissue sections were then incubated in diluted primary antibody overnight (~20 hours) at 4°C, rinsed with three 5-minute washes in PBS, incubated with secondary antibody (biotinylated goat anti-rabbit IgG) for 30 minutes at room temperature, and again rinsed with three 5-minute washes in PBS. Tissue sections were then incubated with streptavidin-peroxidase solution for 45 minutes at room temperature, rinsed with three 5-minute washes in PBS, and finally incubated with diaminobenzidine (Biocare Medical) for exactly 2 minutes to develop the stain. Tissue sections were then dehydrated in a series of ethanol baths (70%, 70%, 90%, 100%, 100%) followed by 3 5-minute washes in xylene and finally cover-slipped using Permount (Fischer Scientific).

Mast cells within the brain were identified by staining with toluidine blue. Tissue sections were deparaffinized as noted, rinsed once in pure water for 5 minutes followed by two 5-minute washes in PBS, then immersed in toluidine blue solution (5 mg/ml toluidine blue in 0.1M HCl) for 10 minutes. Sections were rinsed under running water for 5 minutes before a quick (10 sec) dip in acid alcohol (0.5 mL of glacial acetic acid in 200 mL of 95% ethanol), then dehydrated, and cover-slipped as noted.

Necrotic cell injury in the brain was studied by staining with H&E. Tissue sections were deparaffinized as noted, then rinsed once in pure water for 5 minutes before being immersed in Harris's Modified Haematoxylin stain (Fisher Scientific) for 10 seconds. Stain was differentiated in 1% acid ethanol (2 mL HCl in 198 mL 70% ethanol) for approximately 1 second, and then flushed under running water for 1 minute. Next, sections were stained with eosin (1% eosin Y in 95% ethanol and 0.5% glacial acetic acid, Fisher Scientific) for approximately 1 second. Tissue sections were then dehydrated and cover-slipped as noted.

Apoptotic cell injury in the brain was visualized using rabbit anti-cleaved-caspase-3 (1:500, Cell Signaling Technology, Danvers, MA) as the primary antibody along with a Vectastain Elite Kit (Vector Laboratories, Burlingame, CA) and using the same protocol as with the staining of microglia. The presence of DNA fragmentation within cells was additionally determined using the TUNEL assay method as a second measure of apoptosis (Apoptag Red In Situ Apoptosis Detection Kit, Millipore, Billerica, MA). Tissue sections were deparaffinized as noted. After equilibrating in PBS for 5 minutes, tissues were incubated with proteinase K (20 μg/mL) for 15 minutes at room temperature, and then rinsed twice in PBS for 5 minutes each. Tissue sections were then covered with the provided equilibration buffer for ~2 minutes then incubated with terminal deoxynucleotidyl transferase enzyme (TdT enzyme) for 1 hour at 37° C. Enzyme activity was stopped by incubating the slides with the provided stop/wash buffer for 10 minutes at room temperature. Slides were rinsed 3 times in PBS, and then incubated with rhodamine-conjugated anti-digoxigenin antibody for 30 minutes at room temperature. Excess antibody was removed by 4 2-minute washes in PBS. Tissues were counterstained in 1 μL/mL HOECHST 33342 (Life Technologies, Carlsbad, CA) for 4 minutes, then rinsed twice in PBS for 5 minutes each. Tissues were cover-slipped using Pro-Long Gold Anti-fade (Life Technologies)) and stored at 4°C.

All analysis was done on coronal brain sections taken at the level of the mammillary bodies. Brain regions analyzed included the parasagittal grey matter, periventricular white matter, thalamus, and hippocampus regions CA1, CA3, and dentate gyrus. Grey matter regions were further divided into layers 1-3 and layers 4-6 for analysis, but results were combined for these sub-regions when no differences were found. Images were captured using a Leica DMRB transmitted light microscope (Leica-Microsystems, Wetzler, Germany) at 400x magnification. Identical illumination settings were used for all brain regions to allow for comparisons between regions as well as within regions. For all experiments, a minimum of 4 HPFs per brain region were captured. Microglia were identified by their characteristic morphology and dark, contiguous cytoplasmic staining and counted manually (Figure 3.1). Mast cells were identified by the metachromatic staining of secretory granules within their cytoplasm and again counted manually with the choroid plexus and leptomeninge regions additionally assessed (Figure 3.1). For caspase-3 analysis, image analysis software (Image Pro Plus 6.0, Media Cybernetics, Silver Spring, MD) was used to automatically count positively stained cells (Figure 3.2). Thresholds were set manually to include all cells with DAB-stained cytoplasm while at the same time ensuring no signal was scored as positive within negative control slides. For TUNEL analysis, a Zeiss fluorescence microscope was used to capture images (Figure 3.2). Image analysis software (Image Pro Plus 6.0, Media Cybernetics) was used to automatically count positively stained cells. Due to autofluorescence of red blood cells in nearly all wavelengths, cells were additionally filtered by size. Necrotic appearing cells were identified by their characteristic morphology with eosinophilic cytoplasm and pyknotic nuclei and again scored manually (Figure 3.2). Each HPF was scored on a 5-point scale based on the estimated percentage of necrotic appearing cells; with 0 = 0% necrotic cells, 5 = 1%-10% necrotic cells, 30 = 11%-50% necrotic cells, 70 = 51%-90% necrotic cells, 95 = 91%-100% necrotic cells as previously reported.[33] All tissue slides were coded with the experimenter blinded to animal treatment group.

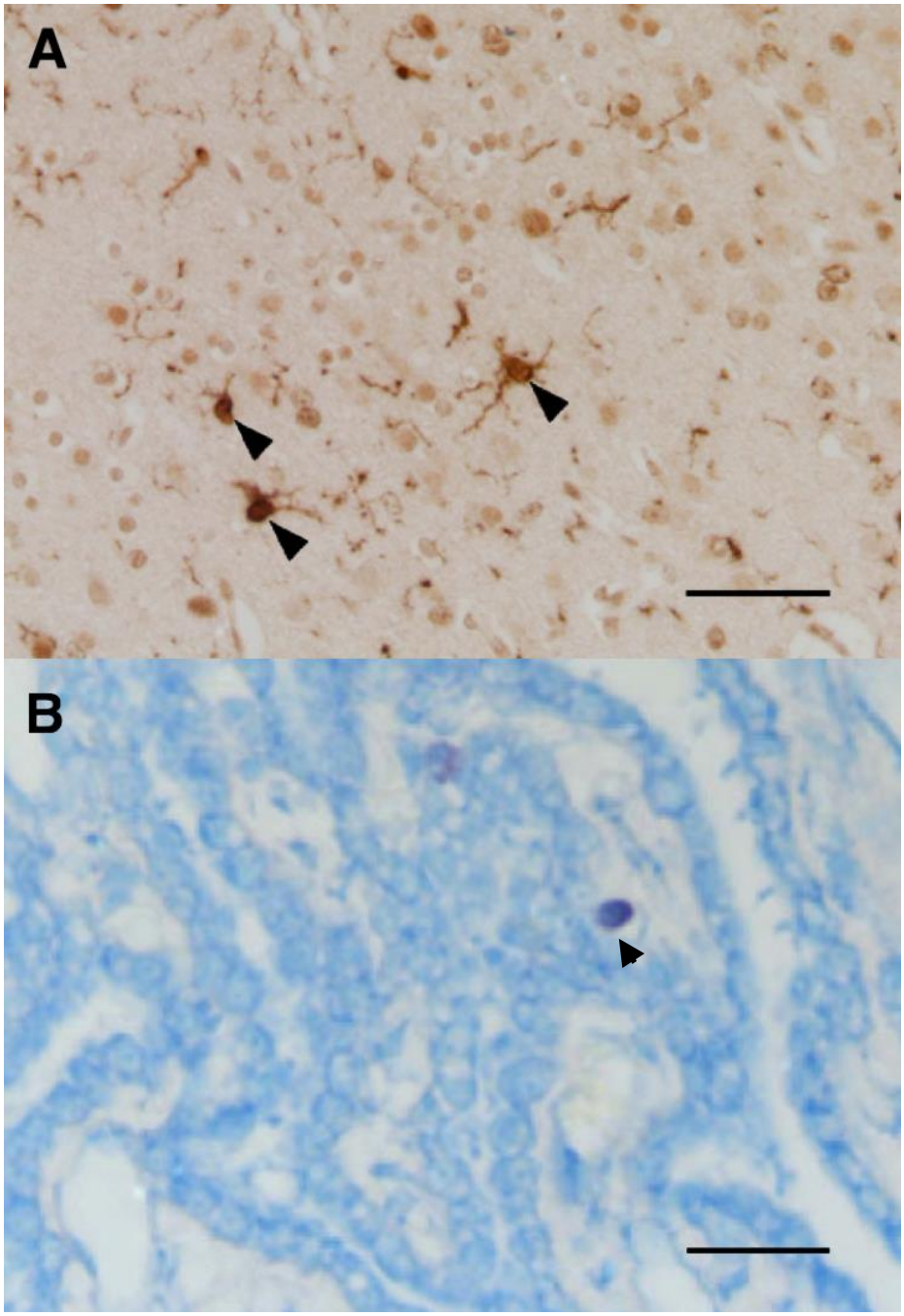

Figure 0.1. Representative photomicrographs (400X) of brain sections, A from the thalamus following anti-IBA1 immunohistochemistry showing microglia with dark contiguous staining (arrows); and B from the choroid plexus stained with toluidine blue showing mast cells characterized by their large metachromatic secretory granules within the cytoplasm. Scale bar = 20 μm.

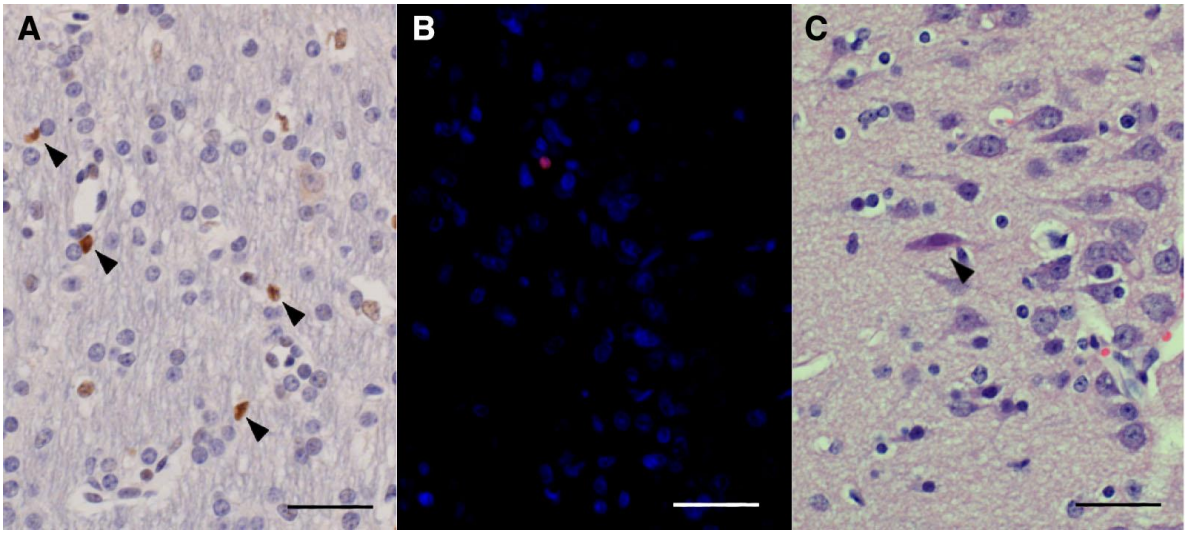

Figure 3.1. Representative photomicrographs (400X) of brain sections, A from the periventricular white matter following anti-cleaved-caspase3 immunohistochemistry and counterstained with hematoxylin showing contiguous dark brown cytoplasmic staining indicating the presence of cleaved-caspase3 (arrows); B from the dentate gyrus stained using the TUNEL assay method (red) and counterstained using Hoechst (blue) with the red fluorescence indicating DNA fragmentation and the blue fluorescence indicating normal cell nuclei; and C from the grey matter stained with H&E showing necrotic appearing neurons identified by their eosinophilic cytoplasm and loss of nuclear detail (arrow). Scale bar = 20μm.

3.2.3 Data Analysis

Differences in microglia, mast cell, cell necrosis, cleaved-caspase-3 positive, and TUNEL positive cell counts among the four experimental groups were analyzed using a one-way analysis of variance (ANOVA) test (Kruskal–Wallis) with Dunn's posttest for comparing each experimental group to the control group. Comparisons between brain regions were done using a one-way repeated measures ANOVA test (Friedman) with Dunn's posttest. All statistical analysis was done using Graphpad Prism (Graphpad Software, La Jolla, CA). All values shown are expressed as means ± SEM. Statistical significance was assumed for $p < 0.05$. The ECOG, ECG, FHR, blood metabolite and cytokine findings have been reported separately.[26,34,35]

3.3 Results

3.3.1 Fetal Arterial Oxygen Saturation, Blood Gases, pH and Lactate

Arterial oxygen saturation, blood gas, pH and lactate findings have been reported in Chapter 3, section 3.3.2. Please refer to Figure 2.4 (oxygen saturation, blood gas, pH) and Figure 2.5 (glucose and lactate) for these results.

3.3.2 Fetal Brain Inflammation

Microglia cell counts were analyzed at 48 hours of recovery after the repetitive UCOs as a measure of the brain inflammatory response. In the control group animals, regional differences were evident with the highest number of microglia in the white mater at 13.4 ± 1.4 cells/HPF, which was significantly higher than that of the grey matter, hippocampal CA1, and thalamus (all $p < 0.05$) (Figure 3.3). While this distribution of microglia cells across regions was also seen in the three UCO experimental groups, there were no significant differences for each brain region between the UCO group values compared to respective control values (Figure 3.3).

Mast cell distribution at 48 hours of recovery after the repetitive UCOs was additionally analyzed as a second marker of inflammation within the brain. Of note, mast cells were only found within the thalamus, the choroid plexus, and the leptomeninges. In the control group animals, regional differences were again evident with the highest number of mast cells in the choroid plexus at 0.9 ± 0.2 cells/HPF, which was significantly higher than in the leptomeninges at 0.1 ± 0.06 ($p < 0.05$) but not the thalamus at 0.3 ± 0.04 (Figure 3.4). While this hierarchal

distribution of mast cells across brain regions was generally seen in the three UCO groups, there again were no significant differences between any of the UCO and respective control group values (Figure 3.4).

3.3.3 Fetal Brain Injury

The presence of necrotic appearing cells at 48 hours of recovery after the repetitive UCOs was analyzed as a measure of overt cell injury within the brain. In the control group animals, low levels of necrotic-appearing cells were observed being somewhat higher and significantly different from zero in the parasagittal grey matter, dentate gyrus, and thalamus (Figure 3.5). In the three UCO groups, low levels of necrotic-appearing cells were again noted, but none of these experimental groups had values that were significantly changed compared to respective control values (Figure 3.5).

The presence of cleaved caspase-3 positive cells and TUNEL positive cells were analyzed as markers of apoptosis within the brain and thereby further measures of overt brain injury. In the control group animals, regional differences were evident with the number of cleaved caspase-3 positive cells in the dentate gyrus at 24.2 ± 5.0 cells/HPF, significantly higher than in the grey matter, CA1, CA3, and thalamus (all $p < 0.05$) (Figure 3.6). While this increase in dentate gyrus cell counts was also evident for the three UCO groups, there were no significant differences within each brain region between UCO group values, compared to

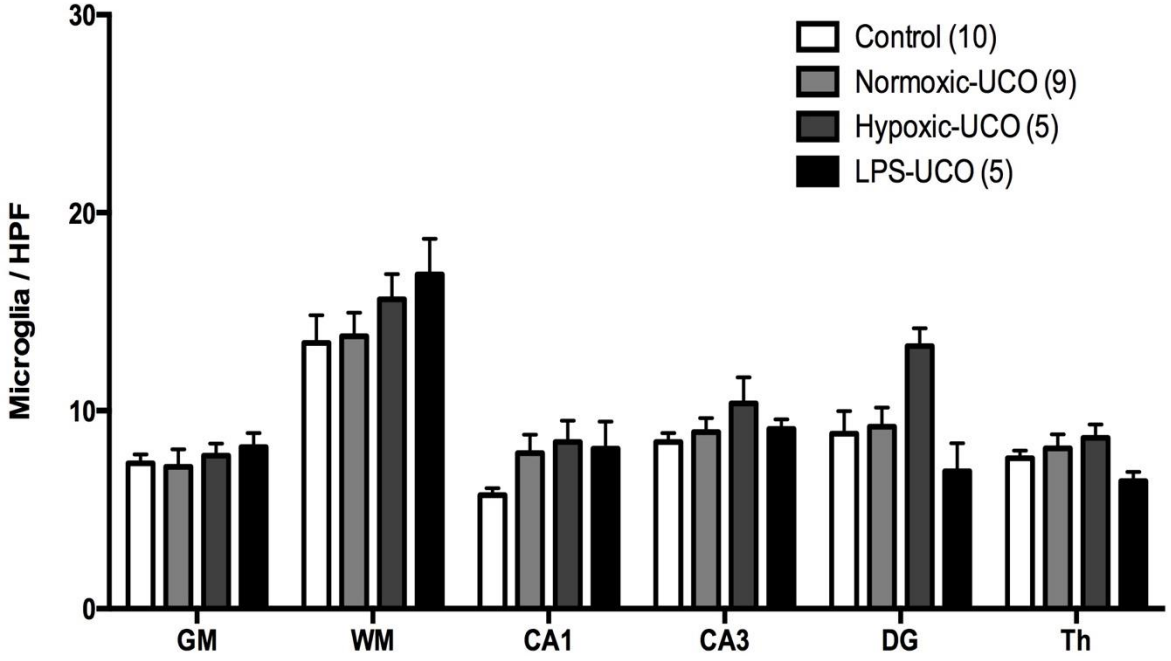

Figure 3.2. Bar graph of IBA1 positive microglia cells/high-power field (HPF) within the different brain regions of the control (open bars) and umbilical cord occlusion (UCO) (shaded bars) animal groups. GM = parasagittal grey matter, WM = periventricular white matter, DG = dentate gyrus, Th = thalamus. Values are mean ± SEM. Control, n = 10; Normoxic-UCO, n = 9; Hypoxic-UCO, n = 5; LPS-UCO, n = 5.

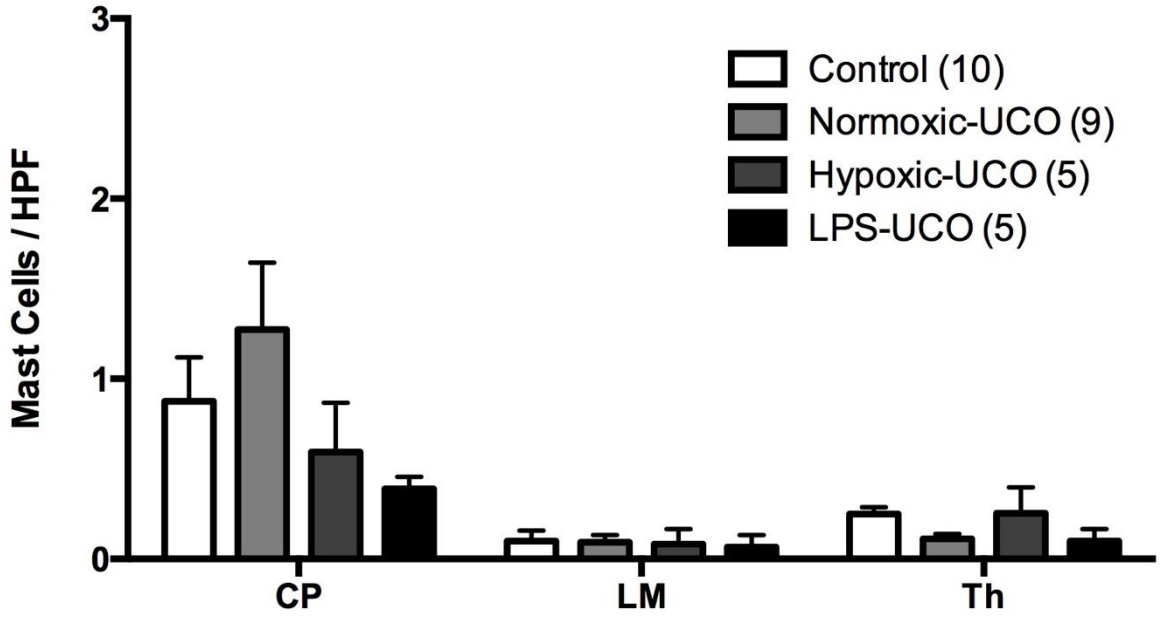

Figure 3.3. Bar graph of mast cells/high-power field (HPF) within the different brain regions of the control (open bars) and umbilical cord occlusion (UCO) (shaded bars) animal groups. Th = thalamus, CP = choroid plexus, LM = leptomeninges. Values are mean ± SEM. Control, n = 10; Normoxic-UCO, n = 9; Hypoxic-UCO, n = 5; LPS-UCO, n = 5.

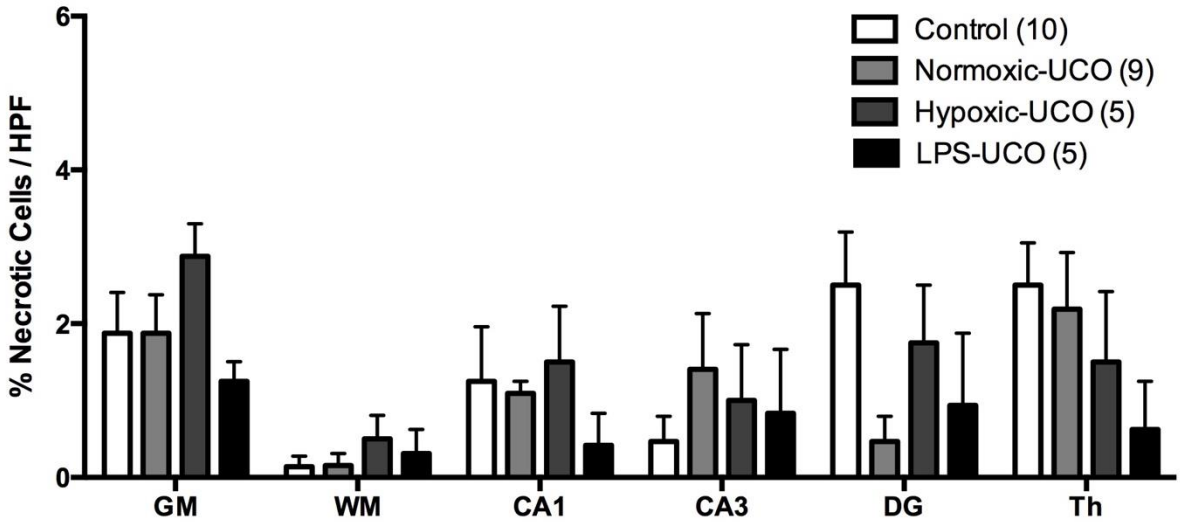

Figure 3.4. Bar graph of percent necrotic appearing cells/high-power field (HPF) within the different brain regions of the control (open bars) and umbilical cord occlusion (UCO) (shaded bars) animal groups. GM = parasagittal grey matter, WM = periventricular white matter, DG = dentate gyrus, Th = thalamus. Values are mean ± SEM. Control, n = 10; Normoxic-UCO, n = 9; Hypoxic-UCO, n = 5; LPS-UCO, n = 5.

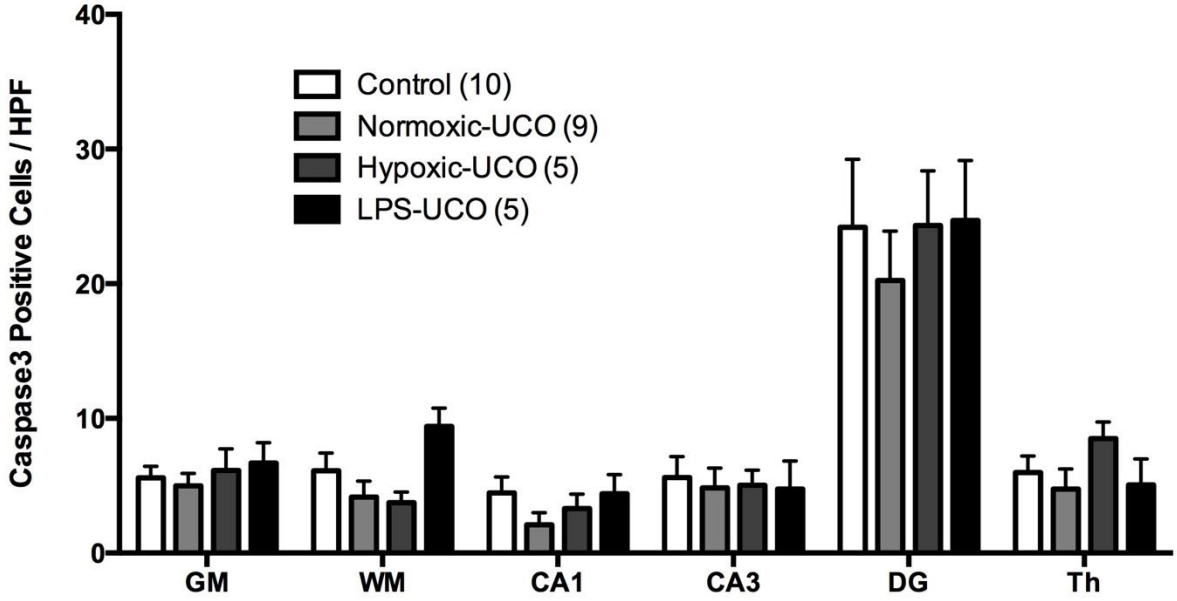

Figure 3.5. Bar graph of cleaved-caspase3 positive cells/high-power field (HPF) within the different brain regions of the control (open bars) and umbilical cord occlusion (UCO) (shaded bars) animal groups. GM = parasagittal grey matter, WM = periventricular white matter, DG = dentate gyrus, Th = thalamus. Values are mean ± SEM. Control, n = 10; Normoxic-UCO, n = 9; Hypoxic-UCO, n = 5; LPS-UCO, n = 5.

respective control values (Figure 3.6). The presence of TUNEL positive cells indicating DNA fragmentation as another measure of cell apoptosis was considerably less than that found for cleaved caspase-3. In the control group animals, very few cells were scored TUNEL positive with all brain regions having fewer than 0.10 cells/HPF and showing no regional differences (Figure 3.7). While the normoxic-UCO animals had TUNEL positive cell counts that were little changed from that of the control values, the LPS-UCO animals had TUNEL positive cell counts that were significantly increased in the hippocampal CA1 and the dentate gyrus at 0.12 ± 0.05 and 0.15 ± 0.05 cells/HPF, respectively (both $p < 0.05$) (Figure 3.7). Additionally, TUNEL positive cell counts in the CA1 region of the hypoxic-UCO group and the CA3 region of the LPS-UCO group were more than three-fold higher than respective control values, but these changes were not significant due to small animal numbers and population variance.

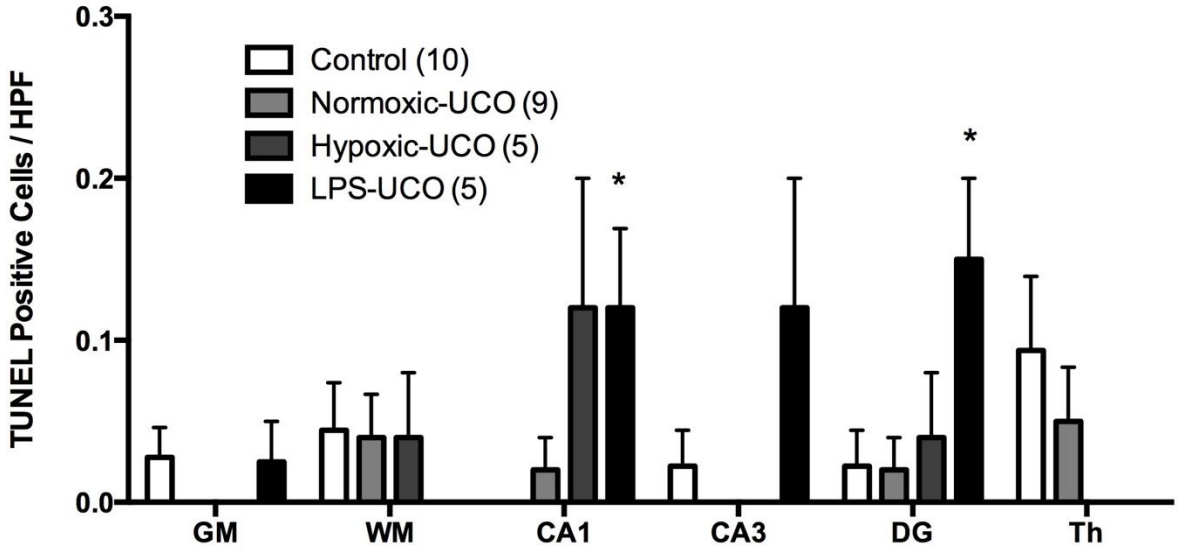

Figure 3.6. Bar graph of TUNEL positive cells/high-power field (HPF) within the different brain regions of the control (open bars) and umbilical cord occlusion (UCO) (shaded bars) animal groups. GM = parasagittal grey matter, WM = periventricular white matter, DG = dentate gyrus, Th = thalamus. * $p < 0.05$ compared to respective control value. Values are mean ± SEM. Control, n = 10; Normoxic-UCO, n = 9; Hypoxic-UCO, n = 5; LPS-UCO, n = 5.

3.4 Discussion

In the present study, near-term fetal sheep were subjected to repetitive UCOs of increasing severity leading to worsening acidemia as might be seen clinically during labour, with some of these animals also chronically hypoxic or subjected to low-grade inflammation as might be seen with fetal growth restriction and placental chorioamnionitis. We used a UCO paradigm consisting of 60-second UCOs occurring every 2.5 minutes, with 1 hour of mild-partial cord occlusions, followed by 1 hour of moderate-partial cord occlusions, then up to 2 hours of severe-complete cord occlusion. However, despite up to 4 hours of repetitive UCOs of worsening severity, 5 of the 20 UCO animals did not attain a severe degree of fetal acidemia with arterial pH < 7.00, although difficulty with complete occluder inflation was likely contributory in two of these animals. This differs from our previous study with 60-second UCOs of increasing frequency with 1 hour of complete UCOs every 5 minutes, followed by 1 hour of complete UCOs every 3 minutes, and then up to 2 hours of complete UCOs every 2 minutes with all 10 of the animals studied attaining an arterial pH < 7.00 within this time frame.[22] Moreover, the nadir arterial pH reached in the present study with up to 4 hours of repetitive UCOs of increasing severity at ~ 7.02 was somewhat higher than that previously seen with up to 4 hours of repetitive UCOs of increasing frequency at 6.90.[22] Accordingly, our 4 hour UCO paradigm with increasing severity resulted in less acidemia than that with increasing frequency. The major difference here is likely the time for recovery between complete occlusions at 90 and 60 seconds, respectively, during the severe UCOs when much of the pH change was noted, and indicating the importance of this variable in the progression of fetal acidemia.[36]

Microglia are the innate immune cells of the central nervous system and are involved in the phagocytosis of cellular debris, and induction of neuronal cell death during normal development.[37] However, when exposed to noxious stimuli such as inflammation and ischemia, microglia become activated which can lead to exacerbation of brain injury through the secretion of inflammatory mediators including reactive oxygen species, nitrous oxide, and pro-inflammatory cytokines.[38-41] In this regard, elevated microglia counts have been found within the dentate gyrus of human infants exposed to perinatal hypoxic-ischemia,[42] and in the white matter of the mid-term and near-term ovine fetus exposed to either hypoxic-ischemia or systemic endotoxemia,[43] and variably relating to the degree of brain injury. Additionally, we

have shown microglia counts to be increased in the white matter and hippocampus of the near-term ovine fetus at 24 hours recovery from repetitive UCOs with severe fetal acidemia and indicating a local inflammatory response within the brain to this insult.[22] In the present study, we found a regional hierarchy with microglia more numerous within the white matter for all four study groups, which is consistent with our previous report.[22] However, no change in microglia counts was seen for any of the brain regions at 48 hours recovery from the repetitive UCOs, either alone or in conjunction with pre-existing intrauterine hypoxia or low-grade inflammation, which differs from our previous findings.[22] Since the brain tissues were collected at 48 hours after the UCOs/LPS administration, any microglia inflammatory response from these stimuli might now be lessened or no longer evident compared to that we previously noted at 24 hours recovery from repetitive UCOs.[22] However, this seems unlikely since chronic endotoxin exposure in fetal sheep results in persistence of microgliosis within the subcortical white matter for several days after the LPS administration.[44] More likely then is that the degree of fetal acidemia resulting from the repetitive UCOs with nadir pH at ~ 7.00, 7.01, and 7.07 for the normoxic-UCO, hypoxic-UCO, and LPS-UCO groups, respectively, was not sufficient to evoke an inflammatory response with increased microglia activation as in our previous study where nadir pH reached 6.90.[22] This presumption is supported by the lack of a systemic inflammatory response in the normoxic- and hypoxic-UCO animals with arterial IL-6 levels also unchanged,[26] which can increase blood-brain barrier permeability to monocytes and other cellular and molecular inflammatory mediators.[9,11] While arterial IL-6 levels were increased at 1 hour recovery in the LPS-UCO animals and correlated with the degree of acidemia attained,[26] there was still no increase in brain microglia as measured 2 days later, although cell counts in the subcortical white matter were the highest amongst the 4 animal groups.

Mast cells are widely recognized as mediators of inflammatory processes through the secretion of a variety of biological molecules including histamine, prostaglandins, and cytokines, and have been implicated in the pathogenesis of inflammatory brain injury with increasing evidence for such with perinatal hypoxic-ischaemia.[20,22,45,46] In the immature rat brain mast cells are initially concentrated in the leptomeninges and choroid plexus, and following hypoxic-ischaemic insult show increased migration into the thalamus as well as degranulation supporting the contention that mast cells mediate inflammatory brain injury[20]. We also found mast cells to be increased in the choroid plexus and thalamus of the near-term ovine fetus at

24 hours recovery from repetitive UCOs/severe acidemia[22] further supporting a causal role for mast cells in newborn HIE. In the present study, mast cells were more numerous in the choroid plexus for all four study groups, which is consistent with our previous report.[22] However, similar to the microglia findings, no change in mast cell counts was seen for any of the brain regions studied in the normoxic-UCO, hypoxic-UCO or LPS-UCO groups when compared to controls. The reasons for this differential mast cell response from that of our previous UCO study[22] are likely to be the same as discussed for the differential microglia response, and implicate a coupling of both of these in the brain's local inflammatory response to perinatal hypoxic-acidemia along with some degree of systemic inflammation.

The current study showed low levels of necrotic-appearing cells in brain regions of the control group animals as identified using H&E morphology similar to that we[33] and others[47] have reported in studies of induced hypoxia in sheep. As such, it is possible that biologic low levels of necrosis exist in the developing brain, or alternatively, are the result of the inherent subjectivity of the scoring procedure. Additionally, the morphologic changes associated with standard H&E methods of identifying necrosis, including cell shrinkage and eosinophilia, can also occur as processing artifacts or nonlethal alterations in cellular morphology.[48] Despite up to 4 hours of repetitive UCOs in the normoxic-, hypoxic-, and LPS-UCO group animals, and resulting in severe acidemia in most of these animals with nadir arterial pH at or below 7.00, there was no significant effect on measured levels of necrotic-appearing cells within the brain when compared to respective control group values. In adult studies of cerebral ischemia, there is a reproducible sequence of changes, with an upper ischemic flow threshold of synaptic transmission failure but the maintenance of energy levels and a lower ischemic flow threshold of membrane failure that indicates energy depletion and is associated closely with the development of structural cell damage.[49] In studies of cerebral metabolism and ECOG activity in fetal sheep subjected to UCOs, we have shown the ability to rapidly enact mechanisms to protect energy levels for membrane integrity by increasing cerebral blood flow, substrate extraction, and anaerobic metabolism and decreasing non-essential energy usage when oxygen availability is acutely limited.[50,51] Of note, the animals herein studied also showed ECOG amplitude suppression during the UCOs with worsening acidosis indicating a decrease in synaptic energy use and with no difference in the timing for this between the UCO groups.[35] Accordingly, the near-term ovine fetal brain appears able to tolerate repetitive UCOs with

severe acidemia and nadir pH ~ 7.00 with no increase in overt necrotic cell injury, presumably through adaptive metabolic mechanisms such that the lower ischemic flow threshold of membrane failure with energy depletion was likely not reached.

Apoptosis, or programed cell death, is normally evident during the fetal/neonatal period of brain development in post-mitotic cells coincident with neuronal differentiation and synaptogenesis, which may relate to the establishment of axonal-target connectivity and competition for trophic factors produced by target cells.[52] Apoptosis in the fetal/neonatal brain can also be activated pathologically by hypoxic-acidemia resulting in selective neuronal loss, which can be exacerbated by chronic hypoxia.[53,54] The process of apoptosis involves the stimulation of intrinsic and/or extrinsic signaling pathways leading to the cleavage of caspase-3 into its active form, which together with other effector caspases targets multiple proteins for proteolysis resulting in the fragmentation of the cell's DNA and the dismantling of diverse cell structures.[55,56] Accordingly, immunoreactivity for cleaved caspase-3 as the active form of this effector caspase, and using the TUNEL assay method to detect fragmented DNA strands have become established markers of cellular apoptosis as used in the present study. In the control group animals, low levels of cleaved caspase-3 positive cells were seen in the brain regions studied but were 3-fold higher in the dentate gyrus, and with all of these levels considerably higher than the respective levels for apoptotic activity as measured using TUNEL. This higher level of apoptotic activity in the near-term ovine fetal brain as measured using cleaved caspase-3 has also been reported by others,[57] and likely relates in part to cleaved caspase-3 being evident earlier and longer during apoptotic cell death than DNA fragmentation which is a terminal event. However, recent study shows that caspase-3 activation does not always lead to DNA fragmentation, especially in the developing brain where it can play a nonapoptotic role in cellular differentiation and remodeling or neuroplasticity through selective removal of existing synaptic connections.[56] The present findings thereby suggest a substantial role for activated caspase-3 in regulating nonapoptotic processes in the developing brain and moreso in the dentate gyrus, consistent with the high level of neuroplasticity seen here into adulthood with a constant state of competing neurodegeneration and neurogenesis.[58]

Despite up to 4 hours of repetitive UCOs in the normoxic- and hypoxic-UCO groups and severe acidemia in most of these animals, there again was no significant effect on measured levels of either cleaved caspase-3 or TUNEL positive cells although the latter is likely to better reflect

apoptotic cell death within the developing brain as noted. While it has been held that brain injury in response to hypoxia is more likely to be apoptotic with milder insult and necrotic with severe insult, it is becoming evident that both occur as a continuum of cell death in proportion to the severity of hypoxic insult.[55] As such, the adaptive metabolic mechanisms enacted to protect against necrotic cell death are also likely to protect against apoptotic cell death in the normoxic- and hypoxic-UCO groups as studied. Moreover, the balance in the expression of anti- and pro-apoptotic genes determines apoptotic triggering in response to hypoxia, and it is possible that the mild- and moderate-partial UCOs have altered this in favour of anti-apoptotic gene expression by hypoxic conditioning, thereby increasing tolerance to the severe-complete UCOs.[55] However, the number of TUNEL positive cells was increased in the hippocampal CA1 and dentate gyrus regions for the LPS-UCO group animals compared to respective control values. While these animals did not show any evidence of a brain inflammatory response as studied, they did show an increase in plasma and amniotic fluid inflammatory cytokines that related to the degree of UCO-induced acidemia and indicating a fetal/placental inflammatory response.[26] Accordingly, it is likely that the LPS- and UCO-induced systemic inflammation together with the fetal hypoxic-acidemia contributed to the increased brain apoptosis with the relative expression of anti- and pro- apoptotic genes accounting for the regional differences in vulnerability.[55] This conjecture is also consistent with the synergistic interaction between inflammation and hypoxia in the pathogenesis of perinatal brain injury reported by others.[9,12,13] However, intra-amniotic LPS dosing alone similar to the present study has shown apoptotic cell counts in the ovine fetal brain to be selectively increased at 48 hours,[59] although a second study with lower LPS dosing but over 28 days showed no impact here.[60] Since the effects of intra-amniotic LPS alone were not studied which is a limitation, it is possible the apoptotic changes seen were primarily the result of the LPS, although it seems likely that the repetitive UCOs were also contributory given the relationship to the degree of resultant acidemia as noted.

In the present study, we have used an animal model relevant to human labour with repetitive UCOs leading to worsening fetal acidemia in the presence or absence of chronic hypoxia or simulated infection. Despite up to 4 hours of repetitive UCOs and severe acidemia in most of these animals with arterial pH to 7.00, there was no significant effect on measures of brain injury or inflammation, except in the LPS-UCO animals where TUNEL positive cells were

increased in the hippocampus. As such, the near-term ovine fetal brain shows remarkable tolerance for these cord occlusion insults and likely involving adaptive metabolic mechanisms, despite the severe acidemia noted.[50,51] This is consistent with the clinical literature whereby most infants with severe acidemia and arterial pH to 7.00 at birth will still be without neurologic complications.[1-3] However, in similar studies of repetitive UCOs but leading to more severe fetal acidemia with nadir pH to ~ 6.90, we found increased brain inflammation at 24 hours,[22] while de Haan *et al*[61] found increased infarction and selective neuronal loss at 72 hours. Accordingly, there is probably a narrow threshold between a degree of hypoxic-acidemia associated with no sequellae, and an insult causing cerebral impairment or even death, with the critical issue likely the maintenance of blood pressure and thereby tissue perfusion including that to the brain.[51,61] Moreover, this threshold in relation to worsening acidemia may be lowered in conjunction with fetal inflammation as previously proposed[9,12,13] and further supported by the present findings.

3.5 References

1. Goldaber KG, Gilstrap LC, Leveno KJ, Dax JS, McIntire DD. Pathologic fetal acidemia. *Obstet Gynecol.* 1991;78:1103–1107.

2. American College of Obstetricians. Neonatal encephalopathy and neurologic outcome, second edition. *Obstet Gynecol*. 2014;123(4):896-901.

3. Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. *Am J Obstet Gynecol*. 2008;199(6):587-95.

4. American College of Obstetricians and Gynecologists. Fetal heart rate patters: Monitoring, interpretation, and management; technical bulletin no. 207. Washington, DC: The College; 1995.

5. Ball RH, Parer JT. The physiologic mechanisms of variable decelerations. *Am J Obstet Gynecol.* 1992;166(6 Pt 1):1683-8; discussion 1688-9.

6. Anyaegbunam A, Brustman L, Divon M, Langer O. The significance of antepartum variable decelerations. *Am J Obstet Gynecol*. 1986;155(4):707-10.

7. Hoskins IA, Frieden FJ, Young BK. Variable decelerations in reactive nonstress tests with decreased amniotic fluid index predict fetal compromise. *Am J Obstet Gynecol*. 1991;165(4 Pt 1):1094-8.

8. Winkler CL, Hauth JC, Tucker JM, Owen J, Brumfield CG. Neonatal complications at term as related to the degree of umbilical artery acidemia. *Am J Obstet Gynecol*. 1991;164: 637–641.

9. Gotsch F, Romero R, Kusanovic JP, Mazaki-Tovi S, Pineles BL, Erez O, Espinoza J, and Hassan SS. The fetal inflammatory response syndrome. *Clin Obstet Gynecol*. 2007;50: 652–683.

10. Nitsos I, Rees SM, Duncan J, Kramer BW, Harding R, Newnham JP, and Moss TJ. Chronic exposure to intra-amniotic lipopolysaccharide affects the ovine fetal brain. *J Soc Gynecol Investig*. 2006;13:1–9.

11. Duncan J, Cock M, Suzuki K, Scheerlinck JP, Harding R, Rees S. Chronic Endotoxin Exposure Causes Brain Injury in the Ovine Fetus in the Absence of Hypoxemia. *J Soc Gynecol Investig*. 2006;13:87–96.

12. Eklind S, Mallard C, Leverin AL, Blomgren K, Mattsby-Baltzer I, Hagberg H. Bacterial endotoxin sensitizes the immature brain to hypoxic--ischaemic injury. *Eur J Neurosci*. 2001;13:1101–1106.

13. C Coumans ABC, Middelanis J, Garnier Y, Vaihinger H-M, Leib SL, Duering Von MU, Hasaart THM, Jensen A, Berger R. Intracisternal application of endotoxin

enhances the susceptibility to subsequent hypoxic-ischemic brain damage in neonatal rats. *Pediatr Res*. 2003;53(5):770-5.

14. Silveira RC, Procianoy RS. Interleukin-6 and tumor necrosis factor-α levels in plasma and cerebrospinal fluid of term newborn infants with hypoxic–ischemic encephalopathy. *J Pediatr*. 2003;143:625–629.

15. Chiesa C, Pellegrini G, Panero A, De Luca T, Assumma M, Signore F, Pacifico L. Umbilical cord interleukin-6 levels are elevated in term neonates with perinatal asphyxia. *Eur. J. Clin. Invest.* 2003;33(4):352–358.

16. Deng W. Neurobiology of injury to the developing brain. *Nat Rev Neurol.* 2010;6:328–336.

17. Hutton LC, Castillo-Meléndez M, Smythe GA, Walker DW. Microglial activation, macrophage infiltration, and evidence of cell death in the fetal brain after uteroplacental administration of lipopolysaccharide in sheep in late gestation. *Am J Obstet Gynecol*. 2008;198:117.e1–117.e11.

18. Kaur C, Rathnasamy G, Ling EA. Roles of activated microglia in hypoxia induced neuroinflammation in the developing brain and the retina. *J Neuroimmune Pharmacol.* 2013;8(1):66-78.

19. Jin Y, Silverman AJ, Vannucci SJ. Mast Cell Stabilization Limits Hypoxic-Ischemic Brain Damage in the Immature Rat. *Dev Neurosci*. 2007;29:373–384.

20. Silverman AJ, Sutherland AK, Wilhelm M, Silver R. Mast cells migrate from blood to brain. *J Neurosci*. 2000;20(1):401-8.

21. Prout AP, Frasch MG, Veldhuizen RAW, Hammond R, Ross MG, Richardson BS. Systemic and cerebral inflammatory response to umbilical cord occlusions with worsening acidosis in the ovine fetus. *Am J Obstet Gynecol*. 2010;202:82.e1–9.

22. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet.* 2008;371:75–84.

23. Becroft DMO, Thompson JMD, Mitchell EA. Placental Chorioamnionitis at Term: Epidemiology and Follow-Up in Childhood. *Pediatr Dev Pathol*. 2010;1:282–290.

24. Soothill PW, Nicolaides KH, Campbell S. Prenatal asphyxia, hyperlacticaemia, hypoglycaemia, and erythroblastosis in growth retarded fetuses. *Br Med J (Clin Res Ed).* 1987;294:1051–1053.

25. Xu A, Matushewski B, Cao M, Hammond R, Frasch MG, Richardson BS. The Ovine Fetal and Placental Inflammatory Response to Umbilical Cord Occlusions with Worsening Acidosis. *Reprod Sci*. 2015. [Epub ahead of print]

26. Green LR, Homan J, White SE, Richardson BS. Cardiovascular and metabolic responses to intermittent umbilical cord occlusion in the preterm ovine fetus. *J Soc Gynecol Investig*. 1999;6(2):56-63.

27. Richardson BS, Carmichael L, Homan J, Patrick JE. Electrocortical activity, electroocular activity, and breathing movements in fetal sheep with prolonged and graded hypoxemia. *Am J Obstet Gynecol*. 1992;167(2):553-8.

28. Gardner DS, Fletcher AJ, Bloomfield MR, Fowden AL, Giussani DA. Effects of prevailing hypoxaemia, acidaemia or hypoglycaemia upon the cardiovascular, endocrine and metabolic responses to acute hypoxaemia in the ovine fetus. *J Physiol.* 2002;540(Pt 1):351-66.

29. Kallapur SG, Willet KE, Jobe AH, Ikegami M, Bachurski CJ. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol.* 2001;280(3):L527–L536.

30. Kramer BW, Moss TJ, Willet KE, Newnham JP, Sly PD, Kallapur SG, Ikegami M, and Jobe AH. Dose and time response after intraamniotic endotoxin in preterm lambs. *Am J Respir Crit Care Med*. 2001;164(6):982-8.

31. Itskovitz J, LaGamma EF, Rudolph AM. Heart rate and blood pressure responses to umbilical cord compression in fetal lambs with special reference to the mechanism of variable deceleration. *Am J Obstet Gynecol.* 1983;147(4):451–457.

32. Rocha E, Hammond R, Richardson B. Necrotic cell injury in the preterm and near-term ovine fetal brain after intermittent umbilical cord occlusion. *Am J Obstet Gynecol*. 2004;191:488–496.

33. Durosier LD, Green G, Batkin I, Seely AJ, Ross MG, Richardson BS, and Frasch MG. Sampling rate of heart rate variability impacts the ability to detect acidemia in ovine fetuses near-term. *Front Pediatr.* 2014; 2: 38.

34. Frasch MG, Durosier LD, Xu A, Cao M, Matushewski B, Keenliside L, Louzoun Y, Ross MG, Richardson BS. Adaptive shut-down of EEG activity predicts critical acidemia in the near-term ovine fetus. *Physiological Reports*. 2015, In press.

35. Ross MG, Jessie M, Amaya K, Matushewski B, Durosier LD, Frasch MG, Richardson BS. Correlation of arterial fetal base deficit and lactate changes with severity of variable heart rate decelerations in the near-term ovine fetus. *Am J Obstet Gynecol*. 2013;208(4):285.e1-6.

36. Marín-Teva JL, Cuadros MA, Martín-Oliva D, Navascués J. Microglia and neuronal cell death. *Neuron Glia Biol*. 2012;7:25–40.

37. Yoshida T, Tanaka M, Sotomatsu A, Hirai S. Activated microglia cause superoxide-mediated release of iron from ferritin. *Neurosci Lett*. 1995;190(1):21-4.

38. Chao, C.C., Hu S, Molitor TW, Shaskan EG, Peterson PK. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol*. 1992;149(8):2736-41.

39. Giulian D, Baker TJ, Shih LC, Lachman LB. Interleukin 1 of the central nervous system is produced by ameboid microglia. *J Exp Med*. 1986;164(2):594-604.

40. Sawada M, Kondo N, Suzumura A, Marunouchi T. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. *Brain Res*. 1989;491(2):394-7.

41. Del Bigio MR, Becker LE. Microglial aggregation in the dentate gyrus: A marker of mild hypoxic-ischaemic brain insult in human infants. *Neuropathol Appl Neurobiol*. 1994;20(2):144-51.

42. Mallard, C., Welin, A.-K., Peebles, D., Hagberg, H. and Kjellmer, I. White matter injury following systemic endotoxemia or asphyxia in the fetal sheep. *Neurochem Res*. 2003;28(2):215-23.

43. Duncan JR, Cock ML, Suzuki K, Scheerlinck JP, Harding R, Rees SM. Chronic endotoxin exposure causes brain injury in the ovine fetus in the absence of hypoxemia. *J Soc Gynecol Investig.* 2006;13(2):87-96.

44. Lindsberg PJ, Strbian D, Karjalainen-Lindsberg ML. Mast cells as early responders in the regulation of acute blood–brain barrier changes after cerebral ischemia and hemorrhage. *J Cereb Blood Flow Metab*. 2010;30:689–702.

45. Skaper SD, Giusti P, Facci, L. Microglia and mast cells: two tracks on the road to neuroinflammation. *FASEB J*. 2012;26(8):3103-17

46. Keunen H, Blanco CE, van Reempts JL, Hasaart TH. Absence of neuronal damage after umbilical cord occlusion of 10, 15, and 20 minutes in midgestation fetal sheep. *Am J Obstet Gynecol*. 1997;176(3):515-20.

47. Schmued LC, Albertson C, Slikker W Jr. Fluoro-Jade: A novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res*. 1997;751(1):37-46.

48. Astrup J. Energy-requiring cell functions in the ischemic brain. Their critical supply and possible inhibition in protective therapy. *J Neurosurg*. 1982;56(4):482-97.

49. Richardson BS, Carmichael L, Homan J, Johnston L, Gagnon R. Fetal cerebral, circulatory, and metabolic responses during heart rate decelerations with umbilical cord compression. *Am J Obstet Gynecol*. 1996;175(4 Pt 1):929-36.

50. Kaneko M, White S, Homan J, Richardson B. Cerebral blood flow and metabolism in relation to electrocortical activity with severe umbilical cord occlusion in the near-term ovine fetus. *Am J Obstet Gynecol*. 2003;188(4):961-72.

51. Blaschke AJ, Staley K, Chun J. Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development*. 1996;122(4):1165-74.

52. Yue X, Mehmet H, Penrice J, Cooper C, Cady E, Wyatt JS, Reynolds EO, Edwards AD, Squier MV. Apoptosis and necrosis in the newborn piglet brain following transient cerebral hypoxia-ischaemia. *Neuropathol Appl Neurobiol*. 1997;23(1):16-25.

53. Burke C, Sinclair K, Cowin G, Rose S, Pat B, Gobe G, Colditz P. Intrauterine growth restriction due to uteroplacental vascular insufficiency leads to increased hypoxia-induced cerebral apoptosis in newborn piglets. *Brain Res.* 2006;1098(1):19-25.

54. Banasiak KJ, Xia Y, Haddad GG. Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog Neurobiol*. 2000;62(3):215-49.

55. D'Amelio M, Cavallucci V, Cecconi F. Neuronal caspase-3 signaling: not only cell death. *Cell Death Differ*. 2010;17(7):1104-14.

56. Yawno T, Hirst JJ, Castillo-Melendez M, Walker DW. Role of neurosteroids in regulating cell death and proliferation in the late gestation fetal brain. *Neuroscience*. 2009;163(3):838-47.

57. Perederiy JV, Westbrook GL. Structural plasticity in the dentate gyrus- revisiting a classic injury model. *Front Neural Circuits*. 2013;7:17.

58. Gavilanes AW, Strackx E, Kramer BW, Gantert M, Van den Hove D, Steinbusch H, Garnier Y, Cornips E, Steinbusch H. Chorioamnionitis induced by intraamniotic lipopolysaccharide resulted in an interval-dependent increase in central nervous system injury in the fetal sheep. *Am J Obstet Gynecol*. 2009;200(4):437.e1-8.

59. Nitsos I, Newnham JP, Rees SM, Harding R, Moss TJ. The impact of chronic intrauterine inflammation on the physiologic and neurodevelopmental consequences of intermittent umbilical cord occlusion in fetal sheep. *Reprod Sci*. 2014;21(5):658-70.

60. De Haan HH, Gunn AJ, Williams CE, Gluckman PD. Brief repeated umbilical cord occlusions cause sustained cytotoxic cerebral edema and focal infarcts in near-term fetal lambs. *Pediatr Res*. 1997;41(1):96-104.

Chapter 4

4.1 Summary

In this thesis we have used an animal model relevant to human labour with repetitive UCOs leading to worsening fetal acidemia in the presence or absence of chronic hypoxia or simulated infection. To our knowledge this is the first study to look at the inflammatory response in the placenta in response to UCOs in fetal sheep. Despite up to 4 hours of repetitive UCOs and severe acidemia in most of these animals with arterial pH to 7.00, there was no significant effect on measures of brain injury or inflammation. However, the hypoxic insult did result in infiltration of neutrophils into the maternal zone of the cotyledons. The link between UCO and neutrophil infiltration in the placenta was not elucidated, but may involve cytokines not studied in this thesis, or other biological molecules such as prostaglandins. The placenta is a possible source of the pro-inflammatory cytokines IL-6 and IL-1β seen in the fetal circulation and amniotic fluid respectively in our study. Further studies of the events occurring between the hypoxic insult and the infiltration of neutrophils in the placenta may identify new therapeutic targets or early markers of hypoxic brain injury.

4.2 Limitations and Future Directions

A limitation of this study is that it did not include an experimental group that was exposed to LPS alone, without UCOs and resulting acidemia. The effect of intraamniotic LPS on near-term ovine fetuses have been previously studied by Gavilanes *et al*.[1] Using a comparable dosage of 10 mg given at 123 days gestation, Gavilanes *et al*. found increased rates of apoptosis in the grey mater and hippocampus, as well as increased activated microglia in the hippocampus and caudate putamen after 48 hours[1], showing that that intraamniotic LPS alone can have significant and region dependent effects on brain injury and inflammation. Thus, the inclusion of this additional study group may have given us valuable insight on the LPS-UCO group. Additionally, Gavilanes *et al*. used flow cytometry for analysis of brain tissues, which is a far more sensitive method compared to those use in the present study. This may account for why they saw changes in apoptosis and activated microglia where we did not.

In the present study, repetitive UCOs with severe acidemia resulted in elevated neutrophil numbers in the zona intima of the placenta, but additional exposure to intraamniotic LPS did not have an additive effect. Since neutrophil infiltration into the fetal membranes is the

hallmark of chorioamnionitis, it is surprising that intraamniotic LPS exposure did not result in increased neutrophil numbers. This may be due to the fact that the extraplacental fetal membranes themselves were not analyzed but rather the fetal tissues adjacent to the cotyledons. Intraamniotic LPS exposure has been shown to cause increased cellular infiltration in the extraplacental fetal membranes[2,3], so analysis of those tissues may yield useful data on the placental inflammatory response in the context of repetitive UCOs.

An interesting finding in this study was the high levels of apoptosis in the near-term ovine fetal brain as measured using cleaved caspase-3, even in control animals. This finding was not corroborated by the TUNEL analysis of the same specimens, possibly due the fact that cleaved caspase-3 is evident earlier and longer throughout apoptosis compared to DNA fragmentation, which is a terminal event. However, it is more likely that not all these cells positive for cleaved caspase-3 will proceed to complete apoptosis. Studies have shown that in the developing brain, cleavage of caspase-3 can have nonapoptotic functions such as remodeling or pruning of existing neuronal connections with significance in early neuroplasticity, or may even have neuroprotective effects against ischemic insults .[4-6] Indeed, high levels of cleaved caspase-3 has been seen in the ovine fetus by other groups.[7] While the function of cleaved of caspase-3 in the near term ovine brain in unclear, the high levels present, especially in the dentate gyrus, suggests it has an important role in brain development, and may warrant further study.

4.3 Conclusions

In this thesis, an ovine model of repetitive UCOs was used to investigate the effects of severe fetal acidemia as might be seen clinically during labour on the placental inflammatory response and subsequent brain injury in the ovine fetus. Additionally, the effects of pre-existing chronic hypoxia or low-grade inflammation as might be seen with fetal growth restriction or histologic chorioamnionitis, were also examined.

Repetitive UCOs in the ovine fetus leading to severe fetal acidemia results in a placental inflammatory response as shown by increased neutrophils in the placental zona intima and elevated IL-1B in the amniotic fluid. When additionally exposed to intraamniotic LPS, a systemic fetal inflammatory response is seen as shown by elevated IL-6 in the fetal plasma.

Repetitive UCOs in the ovine fetus leading to severe fetal acidemia did not result in overt brain injury or inflammation, but when additionally exposed to intraamniotic LPS, an increased in apoptotic cell death was observed in the hippocampus.

This thesis provides an understanding of the fetal and placental inflammatory responses that occur in the near-term ovine fetus when exposed to repetitive UCOs with severe acidemia, and highlights the effect of underlying inflammation near the time of birth on the risk for subsequent brain injury.

4.4 References

1. Gavilanes AWD, Strackx E, Kramer BW, Gantert M, Van den Hove D, Steinbusch H, Garnier Y, Cornips E, Steinbusch H, Zimmermann L, Vles J. Chorioamnionitis induced by intraamniotic lipopolysaccharide resulted in an interval-dependent increase in central nervous system injury in the fetal sheep. *Am J Obstet Gynecol*. 2009;200(4):437.e1–8.

2. Kramer BW, Moss TJ, Willet KE, Newnham JP, Sly PD, Kallapur SG, Ikegami M, Jobe AH. Dose and time response after intraamniotic endotoxin in preterm lambs. *American Journal of Respiratory and Critical Care Medicine*. 2001;164(6):982–988.

3. Kallapur SG, Willet KE, Jobe AH, Ikegami M, Bachurski CJ. Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol*. 2001;280(3):L527–L536.

4. D'Amelio M, Cavallucci V, Cecconi F. Neuronal caspase-3 signaling: not only cell death. *Cell Death Differ.* 2010;17(7):1104–1114.

5. Gulyaeva NV. Non-apoptotic functions of caspase-3 in nervous tissue. *Biochemistry Mosc.* 2003;68(11):1171–1180.

6. McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E. Caspase 3 activation is essential for neuroprotection in preconditioning. *Proc. Natl. Acad. Sci. U.S.A.* 2003;100(2):715–720.

7. Yawno T, Hirst JJ, Castillo-Melendez M, Walker DW. Role of neurosteroids in regulating cell death and proliferation in the late gestation fetal brain. *Neuroscience*. 2009;163(3):838–847.

Curriculum Vitae

Name: Alex Xu

Post-secondary Education and Degrees:
Western University
London, Ontario, Canada
2007-2011 B. MSc.

Honours and Awards:
Western Scholarship of Excellence
2007-2008

Dept. of Obstetrics and Gynecology Graduate Scholarship
2011-2012

Western Graduate Research Scholarship
2011-2012, 2012-2013

Related Work Experience
Teaching Assistant
Western University
2011-2013

Publications:

Xu A, Durosier LD, Ross MG, Hammond R, Richardson BS, Frasch MG. Adaptive brain shut-down counteracts neuroinflammation in the near-term ovine fetus. *Front Neurol*. 2014;5:110.

Xu A, Matushewski B, Cao M, Hammond R, Frasch M, and Richardson BS. The Ovine Fetal and Placental Inflammatory Response to Umbilical Cord Occlusions with Worsening Acidosis. *Reprod Sci*. 2015.

Xu A, Matushewski B, Nygard K, Hammond R, and Richardson BS. Brain Injury and Inflammatory Response to Umbilical Cord Occlusions with Worsening Acidosis in the Near Term Ovine Fetus. *Reprod Sci*. 2015.