INVESTIGATION OF THE EFFECT OF INTRAUTERINE INFLAMMATION AND
INFECTION ON FETAL BRAIN INJURY USING HUMAN AND ANIMAL MODELS

By

Lindsay Alexandra Laurentia Patrick

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ABSTRACT

In recent years, increased focus has been placed on the role of intrauterine infection and inflammation in the pathogenesis of fetal brain injury leading to neurodevelopmental disorders such as cerebral palsy. At present, the mechanisms by which inflammatory processes during pregnancy cause this effect on the fetus are poorly understood. Our previous work has indicated an association between experimentally-induced intrauterine infection, increased proinflammatory cytokines, and increased white matter injury in the guinea pig fetus. In order to further elucidate the pathways by which inflammation in the maternal system or the fetal membranes leads to fetal impairment, a number of studies investigating aspects of the disease process have been performed. These studies represent a body of work encompassing novel research and results in a number of human and animal studies.

Using a guinea pig model of inflammation, increased amniotic fluid proinflammatory cytokines and fetal brain injury were found after a maternal inflammatory response was initiated using endotoxin. In order to more closely monitor the fetal response to chorioamnionitis, a model using the chronically catheterized fetal ovine was carried out. This study demonstrated the adverse effects on fetal white matter after intrauterine exposure to bacterial inoculation, though the physiological parameters of the fetus were relatively stable throughout the experimental protocol, even when challenged with intermittent hypoxic episodes.

The placenta is an important mediator between mother and fetus during gestation, though its role in the inflammatory process is largely undefined. Studies on the placental role in the inflammatory process were undertaken, and the limited ability of proinflammatory cytokines and endotoxin to cross the placenta are detailed herein.
Neurodevelopmental disorders can be monitored in animal models in order to determine effective disease models for characterization of injury and use in therapeutic strategies. Our characterizations of postnatal behaviour in the guinea pig model using motility monitoring and spatial memory testing have shown small but significant differences in pups exposed to inflammatory processes in utero.

The data presented herein contributes a breadth of knowledge to the ongoing elucidation of the pathways by which fetal brain injury occurs. Determining the pathway of damage will lead to discovery of diagnostic criteria, while determining the vulnerabilities of the developing fetus is essential in formulating therapeutic options.
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LIST OF ABBREVIATIONS

BBB: Blood-brain barrier
BDNF: Brain-derived neurotrophic factor
CI: Confidence interval
CNS: Central nervous system
CP: Cerebral palsy
CRP: C-reactive protein
DAPI: 4',6-Diamidino-2-Phenylindole (double stranded DNA staining)
ELISA: Enzyme-linked immunosorbent assay
FIRS: Fetal inflammatory response syndrome
GFAP: Glial fibrillary acidic protein
GFP: Green fluorescent protein
ICAM: Intracellular adhesion molecule
IL: Interleukin
IL-1ra: Interleukin-1 receptor antagonist
iNOS: Inducible nitric oxide synthase
IVH: Intraventricular hemorrhage
LAL: Limulus ameobocyte assay (for LPS detection)
LPS: Lipopolysaccharide
MBP: Myelin basic protein
MRI: Magnetic resonance imaging
NGF: Nerve growth factor

NK: Natural killer (cell)

NWMD: Neonatal white matter damage

OR: Odds ratio

PFA: Paraformaldehyde

PROM: Premature rupture of membranes

PVL: Periventricular leukomalacia

TNF: Tumor necrosis factor

TUNEL: Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling

UCO: Umbilical cord occlusion

UTI: Urinary tract infection

VLBW: Very low birthweight
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

I. Introduction to Cerebral Palsy

Improved techniques and equipment in neonatal care have led to an increase in the ability of preterm infants to survive and thrive, with the cut-off of viability now as early as 23 weeks gestation. This early survival, however, comes with highly elevated risks of developmental disorders such as cerebral palsy (CP).\textsuperscript{1} Trends in the prevalence of CP have varied with innovations in neonatal care, but the costs associated with life-long care continue to increase; direct and indirect health care costs of CP patients in Canada are estimated to be almost $400 million per year.\textsuperscript{2}

CP, with an overall incidence of 1.5-2.5 per thousand live births and a survival rate to adulthood of 90%, is classified by non-progressive lesions of the cerebral white matter leading to adverse motor development.\textsuperscript{1,3} Though the lesions in the brain leading to the diverse symptoms classified under the CP diagnosis do not themselves progress, the manifested symptoms show changes based on the affected child’s growth patterns and therapies.\textsuperscript{4} Diagnosis of CP is usually based on motor manifestations in early childhood due to abnormalities in the central nervous system (CNS).\textsuperscript{5} Thus, an inherent difficulty in the study of this disorder exists in the length of time required between recording of obstetric antecedents and diagnosis up to four years later. Because of the non-progressive nature of the CNS lesions causing CP, it has been found that some children “outgrow” the diagnosis, showing relatively little or no motor disability in the latter years of childhood, though they may suffer from other related developmental disabilities.\textsuperscript{3}

There are several subtypes of CP according to their characteristics and the number of
limbs involved in the pathology. For instance, the spastic form of CP is the most common manifestation of the disorder, and is characterized by hypertonicity, lack of protective responses, and delayed motor ability. Though not characterized by cognitive impairment, individuals with CP are more likely to develop intellectual deficits due to the ability of the causative cerebral lesion to affect non-motor areas. Approximately half of individuals with CP have an IQ less than 75, and one-third have a co-existing seizure disorder.

II. Causation of CP and white matter injury

While the causation of CP is often unknown, 70-80% of cases may be the result of intrauterine events. It has been estimated that, of cases where a causation for CP is known, 35% of cases are due to prenatal causes, 9% are due to intrapartum events (e.g. birth asphyxia), and 10% are due to postnatal causes (e.g. severe head trauma). This indicates that future treatment options to be evaluated should focus primarily on pre- and perinatal events. It is established that various cerebral pathologies correspond to the many categories and degrees of severity of CP, with white matter damage the underlying factor for all. Depending on the severity and region of the CNS damage, manifestations of CP can include aberrant movement, posture and balance, muscle tone alteration, and presence of involuntary movements.

Several known risk factors exist for CP, though the etiology of the brain injury is unknown in approximately 50% of cases. CP is considered to have a multifactorial origin, with several primarily prenatal factors working together to cause significant damage to the developing brain. The most prominent risk factor is preterm birth, which has a prevalence of 5-11% in
Preterm birth is associated with a myriad of subsequent challenges, including respiratory distress and abnormalities on neonatal ultrasonography. Approximately 25% of infants born at 23-25 weeks gestation are later diagnosed with CP, and those infants born with a birth weight <1500g are up to 100 times more likely to develop symptoms. This incidence drops to 3% for infants born at 27-28 weeks gestation, and drops still further to 0.1% at term (>37 weeks gestation or >2500g at delivery). Because of the larger proportion of term births, however, the term population comprises approximately half of all cases of CP.

Likewise, though infants born at less than 32 weeks gestation represent only 2% of live births, they represent a significant proportion of children with CP and 75% of all neonatal morbidities. In infants born at or before 30 weeks gestation, a major developmental disability will be present in 25% and 30-50% will have moderate developmental deficits.

“Preterm” is generally recognized to encompass those infants born before 37 weeks gestation, where term is 40 weeks. In recent years, improvements in preterm and neonatal care have led to an increase in survival of these infants. In addition, increased use of assisted reproductive techniques have led to an increased rate of multiple births, also associated with reduced gestation times. A recent cohort study found an almost doubled risk of childhood diagnosis of CP with threatened preterm labour or gestation of less than 27 weeks at delivery.

Thus, with the increase over past decades in the survival of very preterm infants, the number of cases of CP has increased dramatically, with neurologic impairments such as CP increasing by approximately 20%. This is most likely due to the increased ratio of preterm infants surviving to infancy. Preterm birth is known to cause the increased vulnerability of the
developing brain, and the pathways leading to brain injury can also cause initiation of preterm labour. It is therefore unclear at present whether preterm birth can be considered an independent risk factor, or more likely, whether it is intrinsically associated with the pathways of brain injury. Since treatments to prevent or halt preterm labour are not universally available at present, strategies to protect fetal development are the main focus of recent research strategies.

It is postulated that 70-80% of very preterm births are actually the result of intrauterine infections such as chorioamnionitis, which cause increased release of prostaglandins and other entities that initiate labour signals in the uterus (e.g. cervical ripening, rupture of membranes, uterine contraction). Thus, the risk of CP after preterm birth may be associated with infectious processes in the maternal, fetal or placental systems. Many bacteria have been implicated in the stimulation of preterm labour, and thus it is accepted that it is a generalized immune response to the presence of infection, rather than a response to a particular organism, that stimulates preterm labour. Bacteria may also act on the placenta to prematurely induce collagenase activity, which is usually only increased at term in order to cause rupture of the membranes via the breakdown of the extracellular matrix supporting the chorion and amnion. Data varies among retrospective and cohort studies, leading to differing conclusions on the relationship between preterm birth and chorioamnionitis. This infectious element contributes to the question of whether preterm birth is an associated but separate cause of fetal brain injury or whether it is a part of the pathway by which chorioamnionitis causes brain injury. It is postulated that the risk to infants due to prematurity is due to both the increased incidence of intrauterine infections and the inability of the preterm infant to mount an appropriate inflammatory response. Preterm infants generally develop a much stronger inflammatory
reaction when presented with an immune challenge such as chorioamnionitis, which may lead to self-injurious consequences.

Apgar scores, measures of neonatal well-being in the first minutes of life, appear to have little reliable predictive value for CP except at late testing points; a score of 0-3 (of a possible 10) at 1 minute was associated with CP in 1.5-20% of infants, while the same score at 20 minutes saw 57.1% of those infants later diagnosed with CP. Even higher Apgar scores at 10 minutes (<7 on 10-point scale) were associated with an increased risk of CP development (odds ratio, OR 5.98, 95% CI 2.72-13.2). Other measures of neonatal neurologic function include the Modified Dubowitz Score, a test of seven parameters related to normal neonatal posturing and reflexes. Poor performance on this test has shown an association with elevated proinflammatory cytokine IL-6 levels, but data on long-term implications is currently lacking.

A number of other gestational and neonatal complications are associated with adverse sequelae. Neonatal sepsis and seizures have been proven to be associated with increased risk of later CP diagnosis (OR 2.3, 95% confidence interval, CI 1.1-5.0 for sepsis and OR 14.0, 95% CI 1.7-61 for seizures). Placental abruption and subsequent preterm delivery has also been associated with an increase in CP diagnosis, mainly in the hemiplegic form of the disease (OR 7.20, 95% CI 1.63-31.9). In addition, it has been noted that males are more likely to have CP than females, though no apparent reasons exist for this difference in risk. It is also postulated that the medical facility in which the birth occurs may protect the infant from developing later neurodevelopmental problems, most likely due to the improved facilities for obstetric and neonatal care in tertiary health care centres. This may also be due in part to the mode of delivery, as Caesarian section is associated with a decreased incidence of PVL (OR
0.15, 95% CI 0.04-0.57) and is more likely to occur in tertiary health care centres. Interestingly, several studies have found that presence of preeclampsia decreases the risk of CP development, and it is currently unknown whether this occurs through a protective mechanism (e.g. concurrent magnesium sulphate treatments), or through protection via delivery without labour before extended exposure to microbiologic agents occurs.\textsuperscript{6,10,29} Case control studies indicate that several maternal and fetal factors do not necessarily have an association with increased CP risk; maternal age, race, prenatal care, alcohol or drug use, diabetes, herpes, or decreased fetal movements do not uniformly demonstrate increased independent risks with respect to CP.\textsuperscript{3,10}

III. Chorioamnionitis

In normal pregnancies, the amniotic fluid is a sterile environment. Chorioamnionitis occurs when the amniotic fluid and fetal membranes (the chorion and amnion) surrounding the fetus become infected. Clinical diagnosis rates vary greatly among diagnostic centres and gestational conditions, with a range in the literature of 1-56% depending on prematurity and premature rupture of the membranes (PROM).\textsuperscript{8,32-34} Commonly isolated microbial strains include \textit{Streptococcus}, \textit{Ureaplasma urealyticum}, \textit{Eschericia coli}, \textit{Candida}, \textit{Enterobacter} and \textit{Staphylococcus}.\textsuperscript{11,33-35} Microbial invasion of the amniotic cavity is more common in preterm presentation, and antibiotics or tocolytic interventions do not tend to halt the progression of labour or infection.\textsuperscript{33,36}

Although universal diagnostic criteria are lacking, the presence of maternal fever $>38^\circ$C, high maternal white blood cell count ($>15,000$ cells/mm$^3$), fetal tachycardia ($>160$bpm), uterine tenderness, purulent vaginal discharge, and high levels of maternal serum C-reactive protein
(CRP) (>20-50mg/mL) are all suggestive of clinical chorioamnionitis. While the presence of just one or two of these symptoms would alert the treating physician to possible infection, problems exist in that several of the diagnostic criteria are qualitative, variable in level required for diagnosis, or unavailable to some health centres. It is for these reasons that many cases of chorioamnionitis go undiagnosed. In addition, many cases of chorioamnionitis may be low-grade infections that exist in a subclinical presentation, giving little or no indication to the treating physician that anything is amiss. Studies have found the bacteria *U. urealticum* can persist in the amniotic fluid up to 2 months without the patient exhibiting clinical signs of chorioamnionitis. In cases where chorioamnionitis is symptomatic, however, case control studies have shown an identical odds ratio for the development of spastic CP with either fever >38 °C or diagnosis of clinical chorioamnionitis (OR 9.3, 95% CI 2.7-31). This result may be confounded by epidural or analgesic use, however, as these are known to increase maternal temperature. Diagnosis of clinical chorioamnionitis indicates need to proceed to delivery. In the cases of both diagnosed and undiagnosed (or subclinical) chorioamnionitis, further evidence of infection is usually found upon delivery and placental pathological examination.

Clinical diagnosis of chorioamnionitis increases the risk of CP in preterm and term infants, with an odds ratio in low birthweight infants of 6.8, and a 2 to 9-fold increased risk in all infants, including those born at term. It should be noted that these increased risks account for confounding variables, and exist independently of factors such as maternal age, race, socioeconomic status, smoking, oligo- or polyhydramnios, and previous pregnancy outcomes. Presence of clinical chorioamnionitis can also lead to a systemic inflammation of the umbilical cord (termed funisitis) or neonatal sepsis, both of which are associated with an increased
The isolation of bacteria in the amniotic fluid is associated with risks beyond CP development; maternal illness and sepsis, fetal loss, low birth weight and preterm labour are also associated with this complication. Almost 19% of infants exposed to intrauterine infection develop white matter injury in the form of periventricular leukomalacia; this number increases to 22% if the intrauterine infection is accompanied by PROM. A recent study found the majority of infants showing brain injury on neonatal ultrasound had been diagnosed with PROM. It is thought that some cases of severe chorioamnionitis may actually result from conservative treatment of PROM, since significant numbers of PROM cases are caused by presence of bacteria. However, a recent retrospective cohort found PROM to be equally prevalent in the obstetric records of both children later diagnosed with CP and those that were not.

Histological chorioamnionitis is diagnosed based on inflammatory lesions, vasculitis, and/or invasion of polymorphonuclear monocytes or neutrophils into the fetal membranes. This type of diagnosis, though retrospective with regards to the fetal environment, is a much more sensitive test of the conditions the fetus was exposed to during gestation. It is proposed that chorioamnionitis, and the histologic subtype in particular, may be caused either by bacterial ascension from the vagina or by transfer of inflammatory or bacterial agents (e.g. endotoxin) from the maternal bloodstream. The theory of bacterial ascension from the vagina is supported by evidence that, in cases where amniotic fluid showed microbial presence, these bacteria were also present on cervical swabs in every case. While the structure of the chorion is vascular and stratified, the amnion is a single layer of cells separated from the chorion, and therefore leaves the amniotic fluid vulnerable to infection once the chorion is
exposed to bacteria that have colonized cervical tissues.

A recent study found no evidence of histologic chorioamnionitis diagnosis in term births or preterm Caesarian sections, even when microbiological investigation found bacteria to be present on the membranes. This indicates that the presence of bacteria alone is not sufficient to cause the damage characteristic of a diagnosis of histologic chorioamnionitis, as length of exposure to the infectious agents may not have been sufficient to over-ride maternal and fetal defenses and cause lasting damage to the placenta. In addition to bacteria and bacterial by-products, a new class of genotoxins has been discovered to cause direct DNA damage and disruption of cell cycles in tissues within proximity of either pathogenic or commensal bacteria. This is an emerging field of research that may give additional insight into the mechanisms of injury involved in neurodevelopmental damage.

Recent research has focused on maternal blood markers of chorioamnionitis, since reliable and fast molecular markers in maternal serum may improve diagnostic capabilities. Maternal C-reactive protein tests are already widely available, but are not highly reliable based on specificity and sensitivity calculations, and testing for cytokines is only dependent on development of a faster clinical test. An additional cytokine, G-CSF, has been found to be 10 times higher in maternal blood in pregnancies with chorioamnionitis (with respect to control pregnant values). However, cytokines are rapidly degraded in maternal circulation, making measurements of significant values for diagnosis dependent on time of sample with respect to when the infection first established itself. It is known that several other cytokines are increased substantially in amniotic fluid samples in chorioamnionitis, but amniocentesis to collect fluid is not routinely performed, and may cause additional problems.
About half of all women who delivery prematurely show placental evidence of chorioamnionitis (range: 33-72%). As a “disposable organ,” the placenta is a phenomenal tool for assessing the fetal environment and for confirming diagnoses. A recent study found variable success rates in diagnosis of histologic and clinical chorioamnionitis; 68% of patients with histologic chorioamnionitis had not been diagnosed with clinical chorioamnionitis, but 62-76% of those that were diagnosed with clinical chorioamnionitis had histologic confirmation of the infection upon analysis of placental pathology. The presence of PPROM (preterm PROM) greatly increases the risk of chorioamnionitis; one recent study indicated a four-fold increase in the number of mothers with PPROM who later were found to have evidence of histologic chorioamnionitis. Risk of developing CP after histologic chorioamnionitis tends to carry a similar risk as the clinical diagnosis, with some studies indicating an approximate 9-fold increase in either diagnostic subset in term infants. However, no reliable correlation between severity of histologic chorioamnionitis and occurrence of periventricular leukomalacia (PVL) or fetal metabolic acidosis has been proven, and a recent study in premature infants who had histologic chorioamnionitis found no lasting motor or cognitive delays at the age of 2 years. A retrospective cohort study found similar levels of microbiologic infection in membranes and placentas of pregnancies that did or did not lead to children with CP, with only a significant increase of cases of E.coli isolation in the group where children were later diagnosed with CP. Further complicating matters, histologic lesions of the placenta caused by ischemic or other events during gestation or birth may be misclassified as histologic chorioamnionitis upon pathological examination.

It has been suggested that the correlation between clinical chorioamnionitis and adverse
neurodevelopmental sequelae reflects the fact that clinical manifestation of the infection represents a more severe or advanced infectious process, with demonstrable symptoms. Conversely, histologic chorioamnionitis, diagnosed only after delivery, may represent a weak or subclinical infection with a lessened maternal inflammatory response but may have had a less intense but longer-lasting adverse effect on the well-being of the fetus. Correlations between the diagnosis of histologic chorioamnionitis and the specific subtypes of CP diagnosis in childhood have been found. When term pregnancies were diagnosed with clinical chorioamnionitis and placental pathology confirmed the diagnosis, histologic chorioamnionitis was associated with a 12-fold increase of spastic CP and a 31-fold increase in spastic quadriplegic CP.

Various other methods of membrane or amniotic infection have been proposed, including introduction of bacteria during amniocentesis or the presence of bacterial vaginosis at the time of conception. In the latter case, failure to clear the already-colonized bacteria from the vagina before the fetus establishes itself in the uterus may lead to incorporation of the bacteria into the fetal membranes or amniotic fluid and subsequent chorioamnionitis and/or preterm birth. There is a 1.5 to 2.7-fold increased risk of clinical chorioamnionitis when bacterial vaginosis was present before conception. If bacterial vaginosis is present before conception, as it is in 10-20% of women of child-bearing age, treatment of the infection before conception may prevent future chorioamnionitis. Though not possible in unplanned pregnancies, regular screening for easily-detectable bacteria may lead to a decrease in the incidence of chorioamnionitis during pregnancy by eliminating the bacterial infection before conception. However, antibiotic treatment for bacterial vaginosis during pregnancy has not been shown to be associated with a reduction in preterm births.
There is also evidence that urinary tract infections (UTI) during pregnancy increase brain injury risk by 4-5 times, though some data indicate this damage tends to wane by one year of age unless the mother also had a fever during the UTI diagnosis. Ureaplasma urealyticum, a bacteria commonly isolated in chorioamnionitis, can persist up to 2 months in the amniotic fluid. Higher risk pregnancies subject to amniocentesis, such as those with maternal age >35 years, demonstrate an isolation rate of that particular bacteria of 2.8%. This further indicates stronger inflammatory responses in the mother may be detrimental to the fetal environment. Conditions such as bacterial vaginosis, UTI, and sexually transmitted infections during the first trimester have also been associated with a higher incidence of preterm delivery in the second trimester. Even infections remote to the reproductive tract, such as periodontal disease and appendicitis, have been associated with an increased risk of early labour. This is worrisome in that the inflammatory process in response to microbial invasion occurs quickly, and will persist and possibly elevate throughout the course of infection.

Lipopolysaccharide (LPS) is an endotoxin released from the cell wall of Gram-negative bacteria such as Streptococcus, U.urealyticum and E. coli, organisms commonly isolated in cases of chorioamnionitis. It has been proposed that it is this component of the bacterial chorioamnionitis infection that is most significant in initiating the inflammatory response to the invading organisms. In addition, studies have demonstrated that presence of LPS causes preterm labour or abortion in humans and animals, likely due to endotoxemia or stimulation of uterine contractility by phospholipase-mediated stimulation of prostaglandins. While LPS itself has been shown in rats to be too large an entity to cross the placenta, proximity of the infection to other portions of the fetal membranes may allow LPS entry into the fetal compartment or
stimulation of smaller inflammatory agents, most notably the proinflammatory cytokines. Studies in sheep, however, have shown that LPS can degrade the structure and size of placentomes, which may compromise the ability of the placenta to act as an effective barrier. Recent studies question the role of LPS as a direct effector of damage, after demonstrating a lack of consistent cytokine mRNA expression in the fetal brain.

It should be noted that infections of the amniotic fluid and fetal membranes can also spread around the maternal-fetal interface and within the fetus. The fetal component, termed the fetal inflammatory response syndrome (FIRS), is characterized by an inflammatory response by the fetus causing additive damage to additional developing organs. In essence, fetal swallowing or breathing movements can cause ingestion or inhalation of the infected amniotic fluid, leading to respiratory and gastrointestinal problems in addition to neurodevelopmental problems.

IV. Immunological aspects of the maternal-fetal systems

In the human, response to an invasive organism involves the innate and acquired immune systems. In the fetus, the innate system is of greater importance, due to the immaturity of the secondary defense systems until birth. Generally, the innate immune system provides a largely non-specific inflammatory response to pathogens, and acts as the first line of defense in the fetus and the adult. Because of this non-specific immune response in the fetus, a “bystander effect” can cause significant damage to tissues other than those that are initially subjected to an inflammatory insult. The initial maternal input to fetal nutrition and immune products begins at implantation through the degradation of decidual cells, and circulation between mother and fetus is established quickly such that circulation via the early placenta is usually initiated by two to
three weeks gestation. Once circulation is established, maternal blood is pumped into sinusoids at the placental interface, and diffusion across the villi allows nutrient and gas exchange between the conceptus and the mother, with venous entities on both sides bringing blood from this interface either toward the fetal heart or back into the maternal venous system. In addition to waste products to be disposed of by the maternal system, the fetus also sheds microvilli, macrophage-like (Hoffbauer) cells, placental “knots,” and aggregations of fetal capillaries.

It is known that hormones influence immunological capabilities in humans and animals. Previous studies in mice have found a higher bacterial colonization rate after inoculation when mice are pretreated and maintained with a progesterone supplement. The last trimester of pregnancy is associated with an increase in estrogen levels and a decrease in cell-mediated immunity. In addition, physiologic changes to bladder capacity and tonicity caused by uterine expansion and increased progesterone lead to increased risk of urinary tract infection, a causative factor for development of CP in the infant. Changes in glomerular filtrate may also influence this risk; entities found in the urine of pregnant women may be more conducive to bacterial growth in the urinary tract.

Although there is clearly more influencing immunity than hormone levels alone, it seems important to note that the increase in estrogens is accompanied by a decrease in natural killer cells late in gestation. This change in immune properties may be, in part, responsible for the apparent susceptibility to chorioamnionitis during the latter part of pregnancy. While the cervical mucus plug and fetal membranes help to protect the fetus and keep the surrounding amniotic fluid sterile, organisms are able to breach the barrier either via ascension from the
vagina or through maternal circulation into the placenta. Amniotic fluid itself contains a number of antimicrobial entities (e.g. the family of defensins, part of the innate immune system), in order to act as secondary defense against bacterial or viral invasion. Amniocentesis in patients with microbiologic chorioamnionitis, histologic chorioamnionitis and/or PROM showed elevated levels of these entities as compared to control (sterile amniotic fluid) cases, indicating an attempt to combat bacterial colonization of the amniotic fluid.

The blood-brain barrier (BBB) largely protects the brain from harmful infiltrates when established in the latter part of gestation. Animal studies, however, have indicated that presence of some leukocytes in the central nervous system may be present due to passage into the cerebral circulation before the tight ependymal epithelium of the BBB has been established. It is known, however, that the mature BBB also allows activated T cells and macrophages to cross. While microglia are essential to the brain in clearing away unnecessary debris and dead cellular material, an abundance of these cells or circulatory macrophages in the developing brain may have adverse consequences. Continued research into species-specific abilities of leukocytes to cross the BBB will clarify the interactions between peripheral and cerebral circulatory entities.

V. Proinflammatory Cytokines

Cytokines are naturally-present polypeptides released in increased amounts by many cell types in response to an inflammatory challenge such as endotoxins or bacteria itself. In regular development, cytokines are instrumental in cerebral processes such as neuronal development and proliferation, axonal growth, and synapse regulation. In fact, elevation of maternal serum cytokine levels is a predictor of both term and preterm labour.
of proinflammatory cytokines is also known to possess vasoactive effects, due to the cytokines themselves as well as the entities they stimulate (e.g. prostaglandins, leukotrienes, platelet activating factor). In response to the presence of infection, maternal and fetal tissues release excess amounts of these cytokines; these tissues include the placenta and membranes, uterus, umbilical endothelial cells, fetal brain microglia and astrocytes, and white blood cells (primarily macrophages and monocytes). It is interesting to note that cytokine expression has not been found in oligodendrocytes or neurons in cases of white matter lesions, though the effects of cytokines on these cells are pronounced.

Proinflammatory cytokines act on specific receptors of target cells, establishing a second messenger system within the cell. Target cells may further stimulate additional inflammatory responses in a positive feedback cycle or may stimulate prostaglandin and acute-phase protein release, leading to preterm labour and other complications. Cytokines are also known to cause neurologic disruption due to their stimulation of cell adhesion molecules, some of which promote attachment of infiltrating leukocytes. Cytokines can access the fetus or inflammatory conditions can cause the fetus to manufacture its own cytokines, creating a fetal inflammatory response. They can also influence other systems to malfunction, as is the case in cytokine stimulation of the hypothalamic-pituitary-adrenal axis to increase corticotrophin-releasing factor in the fetal brain after intrauterine exposure to inflammation.

A study of preterm infants found that 100% of children with CP had elevated amniotic fluid cytokines, while only 40% of controls did. Recent studies have indicated a correlation between adverse neurodevelopmental outcome and increased levels of cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 in umbilical cord blood, though consensus among these
findings is not complete. In addition, high maternal serum CRP (an acute-phase protein) concentration is correlated with increased amniotic fluid cytokine levels and PVL. Because of the relative ease of testing maternal serum CRP with respect to amniotic fluid cytokine levels in human studies, this test may be considered in future clinical testing to complement cytokine assay data. As previously noted, however, the sensitivity and specificity of the CRP test are poor, and thus CRP testing alone should not constitute grounds for diagnosis. The association between CRP and amniotic cytokines, however, is only valid in the vascular invasion phase of infection, and does not have any predictive value in terms of development of PVL. Studies have also demonstrated that amniotic fluid IL-1β predicts preterm labour, bacterial vascular invasion and chorioamnionitis, while TNF-α predicts neonatal sepsis. IL-6 has been found to increase at a later time point in the course of infection when compared to IL-1β and TNF-α, thus acting as a longer-term inflammatory stimulus. While these cytokines were associated with occurrence of inflammatory processes, they were not predictive of fetal brain injury. Other studies suggest that combining information on umbilical artery pH and umbilical artery IL-6 levels may be predictive of PVL.

In most cases of exposure to a bacterial stimulus or hypoxic episode, a response will be mounted by the individual that is proportionate to the challenge. When the body fails to respond appropriately to a challenge, the result is often progression to a severe disease state in which symptoms are present in the individual (e.g. blood clots, fever). Unfortunately, the fetus is unable to mount an appropriate response in most situations, and is further unable to show symptoms of inflammatory processes (other than tachycardia) without invasive testing. In some
cases, a genetic predisposition to increased inflammatory responses may accentuate the strength of the inflammatory cascade. In other cases, genetic variants of usually-commensal strains of *E. coli* have been indicated to secrete genotoxins that cause nuclear disruption and cell death.

As known pro-inflammatory mediators, TNF-α, IL-1β and IL-6 have been implicated in the pathogenesis of PVL and CP after inflammatory insult. When infection or an inflammatory event occurs, leukocytic cells that are capable of initiating or further stimulating the proinflammatory process are recruited to the site of the insult. While many cytokines may work together for one purpose, many cytokines are known to have redundant purposes. Evidence suggests that TNF-α is the initial cytokine to be stimulated, with a subsequent stimulation of IL-6 and IL-1β occurring due to the presence of both TNF-α itself and the continuing inflammatory stimulus. While varying levels of cytokines are found in fetal blood and other tissues, cytokine concentrations are significantly higher in amniotic fluid of cases with chorioamnionitis or neonatal sepsis. In addition, IL-6 has been found to be significantly higher in umbilical cord blood of neonates with PVL, with concentrations over 400pg/mL increasing risk of PVL 6-fold. These cytokines, all less than 50kDa, are able to move easily in the circulation and may be able to access membrane-protected environments such as the fetal compartment or, even further, the fetal brain. Even more deleterious is the fact that cytokines can weaken the BBB to the point that bacterial products themselves can pass through. However, one study found the increased amniotic and blood concentrations of IL-6 and IL-1β did not translate to an increased presence in neural cell cytoplasm of neonatal brains with PVL. The authors of that study put forth the hypothesis that the inflammation external to the fetus may
influence cell interactions and inflammatory processes even in the extracellular component of the brain. Another study found “normal” levels of cytokines in the amniotic fluid of most infants that later developed PVL. It should be noted, however, that the cut-off levels for cytokine categorization were quite high compared to other studies that have recorded cytokine values found in chorioamnionitis or subsequent brain injury.

Evidence of infection is not required for a continued proinflammatory response; studies have shown high cytokine presence in the absence of microbes in the amniotic fluid, accompanied by bacterial “footprints” indicating a previous or currently undetectable bacterial presence. Elevation of the proinflammatory cytokines leads to fetal “cytokinemia,” characterized by funisitis or chorionic vessel inflammation, and eventual diagnosis of histologic chorioamnionitis due to placental damage. These cytokines are also extremely potent; IL-1β requires only 5% receptor saturation for maximal activity. As with many proinflammatory mediators, the cytokines have proven difficult to monitor during pregnancy in amniotic fluid or maternal blood due to the fact that they are present in low levels and have a relatively short half-life before deteriorating or binding to soluble receptors. On postmortem analysis, TNF-α levels have been shown to be significantly increased in neonatal brains diagnosed with PVL, particularly early-stage PVL lesions. Amniotic fluid IL-1β, IL-8 and IL-6 were found to be significantly associated with neonatal PVL lesions in live infants. In the case of infants with FIRS or cytokinemia and later evidence of intraventricular hemorrhage, these proinflammatory cytokines have also been found in the cerebrospinal fluid. One paper suggests that the levels of proinflammatory cytokines present in the fetal system are actually a better indicator of subsequent neurodevelopmental problems than isolation of the bacteria that caused the
inflammatory response.

Cytokines, upon entry into the fetal system, cause leukocyte influx and may act to weaken the ependymal cell layer and allow blood leakage into the ventricles. If cytokines gain entry to the fetal brain itself, they are able to directly disrupt development of neurons and glia, in addition to stimulating further inflammatory responses in these cells. Proinflammatory cytokines may also cause cerebral vessel constriction and arterial blockage, causing further oxygen depletion to tissues. Inflammatory cytokines may also damage endothelial cells, causing disruption in blood flow throughout the brain as well as further recruitment of inflammatory agents. Cytokines are also involved in free radical toxicity and excitatory glutamate release, causing further tissue damage. The proinflammatory cascade is not accompanied by an increase in levels of anti-inflammatory cytokines or receptor antagonists, with clearly detrimental consequences.

It is thought that most cytokines present in cases of chorioamnionitis are derived from vaginal, placental and uterine tissues after exposure to ascending bacterial infections, though increasing focus has been placed on the role of the fetal inflammatory response in recent years. Damage to the fetal brain may occur due to the various toxic effects cytokines exert on CNS cells. For instance, cytokines are known to induce astrogliosis, damage oligodendrocyte development, and induce microglia to produce free radicals that can cause oxidative damage. IL-6 is also known to direct oligodendrocyte precursor development towards an astrogliosis pathway, thus depriving the developing brain of mature oligodendrocytes by this indirect pathway as well. In addition, cytokines may be stimulated by microglia and astrocytes in
the fetal brain itself once subject to inflammation or other trauma. Astrocytes, however, lack the LPS signal transducer Toll-like receptor-4, and therefore production and release of cytokines by astrocytes is due to inflammatory stimulation from sources other than the bacterial product LPS.

Recent research also indicates an interaction between cytokines and neurotrophic factors in development, and studies have found decreased levels of certain neurotrophins in the amniotic fluid in cases of chorioamnionitis and fetal CNS abnormalities. IL-6 is also an integral player in the ischemic response, inducing adhesion molecules (e.g. ICAM-1) and increasing oxidative stress. ICAM and VCAM, usually undetectable on the cerebral endothelium, may also increase the permeability of the BBB to monocytes and activated T cells, though it is known that these entities are able to cross the barrier in the absence of these adhesion molecules.

Recently, a number of genetically inherited cytokine polymorphisms have been identified. These polymorphisms include a single base pair polymorphism in the TNF gene that is associated with increased TNF-α presence, and polymorphisms in mannose-binding lectin associated with decreased ability to clear infections. This study found positive associations between cytokine polymorphisms and CP diagnosis. These alterations are potentially important in predicting cases in which the maternal system will be more susceptible to an ascending vaginal or cervical infection before or during pregnancy, and may partially account for the differences in inflammatory response mounted by the mother or fetus among pregnancies affected by chorioamnionitis.
VI. Perinatal white matter injury

Apoptosis is an important developmental regulator which can claim as many as 50% of developing embryonic cells over the full course of brain development. Indeed, apoptosis occurs regularly during axonal organization and glial proliferation, eventually leading to healthy and organized tissue in the normal fetal brain. When the fetus is subject to challenges in utero, however, the levels of apoptosis become unbalanced and harmful effects of inflammatory processes cause neural disorganization and necrosis in the susceptible fetal brain. Recent work has proposed that it is both the presence of inflammatory mediators and a lack of developmental protectors and autoregulation that contribute to the white matter damage characteristic of PVL and CP. PVL is most likely to occur between 23 and 32 weeks gestation, well before myelination is established (at 30-32 weeks), and is strongly associated with later adverse neurodevelopment outcome in the infant. The prevalence of PVL in infants born before 32 weeks gestation is between 5 and 15%. PVL, leading to CP in 38-93% of all cases and 60-100% of preterm cases, is a continuum of white matter damage usually involving focal white matter lesions at the outer edges of the lateral ventricles, but may also affect the centrum semiovale and the optic and acoustic radiations. While the lesions of PVL may begin within days of a fetal insult, tissue resorption and cystic lesions tend to take 10-20 days to develop. These small necrotic lesions in the telencephalic white matter damage are considered to be the pathologic marker of spastic CP. Because of the extensive nature of some necrotic lesions, it is likely that the fetus or neonate has been exposed to the insult causing the lesion for an extended period of time. It is proposed that focal damage may occur as a primary response to ischemic damage, caused in part due to the vulnerability of the “watershed” areas of white
matter, where blood perfusion pressure is the lowest. Approximately 40% of prenatally-caused CP cases may be due to vascular abnormalities in response to fetal challenges. Low perfusion situations lead to damage characteristic of PVL, while insults causing a high blood perfusion rate lead to another predictor of CP, intraventricular hemorrhage (IVH).

Susceptibility to PVL is due to a myriad of factors associated with the immaturity of the brain at 23 to 32 weeks (e.g. lack of white matter and endogenous protectors, immature circulatory and immune systems), and it is the nature and timing of the insult that leads to damage affecting various parts of the developing brain.

Different subcategories of CP have been linked to different types of white matter injury. For instance, the spastic diplegic form of CP has been linked to general PVL, while spastic quadriplegia appears to have a greater link to hypoxic events. A recent review indicated that a diffuse form of PVL may be caused by persistence of reactive oxygen or nitrogen species, leading to oligodendrocyte damage. Preterm birth and low birth weight also show selectivity in the type of CP diagnosed in childhood; in one study, very low birth weight (VLBW) infants (500-1500g) were all diagnosed with the spastic form of the disorder.

Genetic predispositions to circulatory difficulties are also associated with increased risk of brain injury, such as the Factor V Leiden mutation that predisposes to thrombosis and infarction. PVL lesions are not the only damage present in most cases of CP, but are often the best detected due to the focal regions of cell death present that can be detected by ultrasound. In addition, PVL and IVH lesions can be detected during the neonatal period, while CP cannot be diagnosed until at least one year of age.
The term neonatal white matter damage (NWMD) is often used to describe the spectrum of brain injury spanning from diffuse apoptosis to necrotic periventricular lesions. Diffuse white matter damage is also associated with neuromuscular disorders such as CP; approximately 2/3 of infants with focal PVL lesions also have evidence of diffuse brain injury. These infants will often demonstrate disorders of cognition and movement separate from a diagnosis of CP. Diffuse white matter injury is most often associated with “secondary energy failure,” a phenomenon caused by continued oxidative damage to the brain parenchyma in the hours or days after an acute injury occurs. In general, PVL lesions tend to be bilateral, leading to bilateral manifestation of CP symptoms, while hemorrhagic or secondary energy failure effects tend to remain unilateral in their focus and effects. Although lesions occur less frequently in later weeks of gestation, the basal ganglia and motor cortex are especially vulnerable in the third trimester.

The presence of chorioamnionitis in utero is associated with a doubling of the risk of development of PVL and associated neurologic abnormalities, such as intracranial hemorrhage and ventriculomegaly. Diagnosis of PROM has also been shown to increase lesions visible by magnetic resonance imaging (MRI) within 24 hours of birth, indicating a pre-existing insult at the time of delivery. In recent years, increased interest has been focused on the role of proinflammatory cytokines stimulated during chorioamnionitis in the pathogenesis of fetal white matter injury, though it is known that endotoxin itself can also permeate the BBB. Circulating proinflammatory cytokines, in response to an immune insult, are increased systemically and can cross the blood-brain barrier, thus directly disrupting the regular development of neuronal tissue.
or sensitizing the tissue to subsequent insults. Depending on the timing of the infection, cytokines may alter the composition of the BBB such that it is made more permeable or may cross earlier in gestation due to the immaturity of the barrier. Cytokines have been found in elevated levels in the white matter of infants who die at or shortly after birth with evidence of brain injury.

Interest in past years has focused on the integrity of the BBB both during development (as in the case of CP) and later in life (e.g. Alzheimer’s, Multiple Sclerosis). It is interesting to note that the BBB is not an immunologically privileged site, but rather is semi-permeable to leukocytes and inflammatory mediators even in the case of the healthy, fully developed endothelial BBB layer. Indeed, macrophages, microglia and T cells are found in almost every area of the brain, within the tissues as well as the cerebrospinal fluid. It is, as yet, unknown what mechanisms these immunologic cells use in order to gain passage into the tissues and ventricles. Microglia are functionally the equivalent of macrophages in the brain, performing protective duties in order to maintain a “clean” environment for optimal neural cell performance. Previous studies have shown that microglia are less abundant in brains of rat neonates exposed to LPS in utero, indicating the immune capabilities of the postnatal brain could be altered or compromised.

The PVL lesion is rarely detected in term infants, and is much more common in preterm infants born at less than 32 weeks gestation, with its highest incidence (15.7%) occurring at 28 weeks. Oligodendrocytes are especially vulnerable in the late second trimester, due to their transition from precursor cells to mature oligodendrocytes through several documented stages. Studies have demonstrated differential susceptibility to LPS and cytokine-induced insults among
these stages, with late-stage oligodendrocyte progenitors (the most prevalent at 28 weeks gestation) being the most vulnerable. Damage to oligodendrocytes is considered to be the main problem underlying motor disorders such as CP, since the absence of myelin can cause axonal signals to spread erroneously to neighboring regions, resulting in the aberrant limb and posture control indicative of CP. Depending on the extent of white matter damage, the disability associated with the lack of myelin can range from slight to severe, and can affect motor, cognitive or perceptual abilities. Recent studies have indicated that oligodendrocytes and their precursor cells are the main targets of inflammatory or ischemic damage, and that astrocytes are not similarly targeted. It has been proposed that the method by which the oligodendrocyte progenitor cells exhibit vulnerability is through the mitochondria, as studies have shown mitochondrial abnormalities and increased release of cytochrome c accompanying cell death after hypoxic challenge.

In severe PVL lesions, immunohistochemistry has revealed the strong presence of infiltrating macrophages, activated microglia, and reactive astrocytes (all capable of cytokine release). Hypertrophic astrocytes, stained with glial fibrillary acidic protein (GFAP) and associated with glial scar tissue, are abundant in brains with white matter damage. This observation reinforces the inflammation hypothesis of CP, as the hypertrophic form of this cell is produced only in response to insults such as inflammation or trauma. Microglia readily produce cytokines in response to inflammatory stimuli, and astrocytes produce TNF-α and IL-6 in the presence of IL-1. It is most likely the actions of this cytokine cascade, as well as associated oxidative and excitotoxic products (e.g. reactive oxygen species, glutamate) stimulated by the cytokines, that leads to the astrocyte proliferation and hypertrophy, thus
leading into another positive feedback cycle of inflammation.

In past decades, birth hypoxia was thought to be the major contributing factor to childhood CP. It is now recognized that fetal and neonatal hypoxic-ischemic episodes play only a contributing role in development of brain injury characteristic of CP. Fetal hypoxic-ischemic episodes occur more frequently than previously thought, with 16-29% of pregnancies demonstrating nuchal cord during late pregnancy, and 37% presenting nuchal cord at the time of labour. These events tend to be resolved without harm and even without any experience of fetal hypoxia, but in some cases can lead to fetal asphyxia, increasing the risk of subsequent damage. One study indicated a 71-fold increased risk of CP with intrauterine hypoxia or birth asphyxia diagnosis in term infants (OR 71.1, 95% CI 9.5-532). However, this study included a very wide range of asphyxia and hypoxia definitions, and this risk is most likely a sizeable overestimation. Cord blood gases showing acidemia in the fetus after suspected hypoxia also show a correlation with development of intracranial hemorrhage (pH<7.2, OR 3.3, 95% CI 1.1-10.3) but not CP. Antenatal hypoxic-ischemic events and hypotension can lead to increased inflammatory responses, oligohydramnios, growth restriction, and ultimately fetal brain injury. This decrease in oxygen available to the fetus causes acidosis, metabolic changes, and decreases in oxygen available to the tissues, specifically the CNS in this case. Hypoxic injury also leads to secondary energy failure and oxidative pathways that act in a positive feedback cycle similar to the proinflammatory process. Because hypoxic episodes stimulate an inflammatory response much the same as a bacterial infection, it is not surprising that high IL-6 levels in the cerebrospinal fluid of infants correlated with degree of brain injury. In addition, ischemia is associated with glutamate release, which is excitotoxic to the developing brain.
In support of this, hypoxic-ischemic models in the mouse have indicated that agents blocking the NMDA receptor are neuroprotective. In addition, hypoxic episodes decrease the availability of the antioxidant glutathione, leading to further production of reactive oxygen species. Research has indicated that it is the ischemia-reperfusion cycle that is most harmful to the developing brain, rather than the hypoxia alone. In both hypoxic ischemia and instances of inflammation during gestation, a common pathway involving cytokines and reactive oxygen or nitrogen species may lead to deleterious consequences (Figure 1-1). In the case of reactive species, it is the imbalance of metabolic detoxification and the reactive species, brought on by severe or repeated ischemic events, that causes oxidative or peroxynitritive damage.

Hypoxia and ischemia are also associated with the release of proinflammatory cytokines, and damage may occur by the release of the previously described cytokine cascades in addition to adhesion factors and chemokines. Just as proinflammatory cytokines are important in many normal physiological processes, reactive oxygen and nitrogen species are present in normally functioning cells and are important in cellular processes when present in moderate levels. The pathway by which hypoxic episodes or intrauterine infection act on the developing brain are similar in their final steps, though research suggests that the cytokines may be more harmful to the developing CNS tissues than the excitotoxic entities.
Figure 1-1: Representation of potential pathways by which intrauterine infection and hypoxic ischemia can influence fetal brain development through the actions of proinflammatory cytokines and oxidative damage.
VII. Previous studies of chorioamnionitis in laboratory animals

(i) Models of intrauterine infection

Our previous model of chorioamnionitis in guinea pigs involved the intracervical inoculation of guinea pigs with live bacterial broth at 70% gestation. While only 15% of fetal amniotic fluid sacs showed demonstrable microbiologic infection, there was a significant increase in white matter injury among all inoculated animals as compared to controls. The study further found increased levels of proinflammatory cytokines TNF-α, IL-1β and IL-6 in the fetuses whose mother received an intracervical bacterial inoculation, regardless of whether they showed microbiologic chorioamnionitis or their proximity to the cervix, where the bacterial broth was injected.

A study of intracervical inoculation of bacterial broth in 70% gestation rabbits had results similar to those found in our guinea pig study. In rabbits, only 9% of the fetuses showed microbiologic chorioamnionitis in the amniotic fluid, and 6% showed evidence of PVL upon histologic evaluation of the fetal brain. None of the control animals showed evidence of microbiologic chorioamnionitis or PVL.

A previous study in rats found that genital infection prior to breeding leads to adverse effects in pregnancy (e.g. fewer fetuses due to fetal resorption) and in neonates (e.g. decreased pup size). This was especially evident in dams that did not clear the bacteria from their vaginal tracts before or during gestation. This data is similar to human cases, where existing bacterial vaginosis before conception increased the incidence of chorioamnionitis during pregnancy. In rats, it was also found that naturally-occurring bacterial pathogens can traverse the placenta and establish an intra-amniotic infection. However, only fetuses of dams given vaginal bacterial
dosing before breeding demonstrated bacterial growth in the placenta, amniotic fluid, or fetal tissues. This study also found an inverse correlation between length of infection and isolation of bacteria from the vagina, indicating the maternal animal is eventually able to “clear” the infection.

(ii) Models of inflammation and cytokine mediators

Numerous models of infection or inflammation-induced cytokine induction have been performed in both pregnant and non-pregnant models, due to the association of a proinflammatory environment with diseases such as autism, rheumatoid arthritis, schizophrenia, pyelonephritis, and motor disorders such as CP. While the causation and timing of inflammation differs in each of these diseases, the common denominator is Th1-type cytokine upregulation.

When LPS is injected intraperitoneally to the pregnant rat, maternal serum cytokines have been found to increase at 1 and 6 hours post-injection; these increased cytokines include proinflammatory IL-1β, IL-6 and TNF-α as well as the anti-inflammatory IL-10. However, when amniotic fluid samples were analyzed, it was found that only the pro-inflammatory cytokines were increased in the fetal compartment. This study noted that not all pregnant rats injected with LPS reacted the same, but in the ones that did respond to the injection, all of them had significantly increased proinflammatory responses. This is one of the first studies to consider the individual animal’s ability to combat an inflammatory stimulus. While laboratory animals tend to be genetically homogeneous, different species and strains may have differential thresholds at which they are no longer able to effectively respond to an insult. In the human
case, this genetic variation is key to our survival, but may also affect the ability of the fetus to react appropriately to a gestational challenge. In contrast to other studies, this study also found that cytokine mRNA was only upregulated in the placenta and membranes, and not in the fetal brain. This result may be due to the relatively low dose of LPS given (100µg/kg) or to the strain of LPS used (O111:B4, rather than the more commonly used O55:B5).

The well-established ovine model has also been key in determining the effects of endotoxin on the ability of the fetus to maintain adequate cerebral oxygenation. Recently, it was found that pre-treatment with endotoxin directly into the fetal system caused severe oxygen deprivation upon subsequent hypoxic challenge. In vitro studies have complemented this research by investigating the cellular response of placental and cerebral tissue to hypoxic conditions or exposure to endotoxin. Ovine studies have also shown the extreme intolerance to LPS by this study animal; while rat studies commonly use 500µg-2mg/kg doses, the ovine model has been optimized for 67% survival to 3 days at 100ng/kg. These studies indicated a short-term rise in fetal heart rate and decrease in blood pressure which returned to baseline levels within 24 hours of endotoxin injection. Similar studies have shown varied results with respect to physiologic parameters such as oxygen content, blood pressure, cytokine levels and hematocrit, but all have demonstrated an increase in fetal brain injury after endotoxin exposure.

A study of viral injection modeling a possible causation of autism spectrum disorders found long-term upregulation of Th1-type cytokines (e.g. IL-1α and β, TNF-α, IL-6) but not Th2-type cytokines (e.g. IL-4, IL-5, IL-10) in the neonatal brain. The last trimester of human pregnancy is usually associated with a decrease in Th1 (cellular) immunity and an increase in
Th2 (humoral) immunity. The apparent emphasis on the Th1 system may indicate an aberrant response to inflammation even late in pregnancy, causing a greater cell-mediated immune response and subsequently causing damage or preterm labour due to the effects of the Th1 cytokines. This study also found that the upregulation in Th1 response occurred simultaneously with the increase in microglia and astrocytes in the brain, most likely indicating these cells as the source of the latent cytokine response. However, it must be noted that this study was performed on rats, and immune responses differ among species.

(iii) Models of fetal brain injury

In general, most studies using laboratory animals focus on postnatal brain development in rats and mice upon immune or hypoxic challenge. These animal models benefit from high sample sizes, short gestation, ease of breeding and manipulation, and resistance to stress responses characteristic of some other animal models. However, brain development in rats and mice is primarily postnatal, and the placenta differs greatly from that of the human, leading to experimental paradigms that may not translate to relevant data for research ultimately benefiting clinical treatment for humans (Figure 1-2). In the case of CP development, it is thought that damage is due not only to the response to injury but the failure to adequately control the response, and models should therefore also consider the differing fetal and neonatal immune capabilities.

The landmark study by Yoon et al. in maternal rabbits given an ascending intrauterine infection found that 6% of the fetuses of the inoculated mothers developed white matter injury. Since then, many studies have been performed using varying doses of bacteria or E.coli-derived LPS in rodents, and have presented data indicating the long and short-term consequences of
Figure 1-2: Brain growth spurts of seven mammalian species, expressed as first-order velocity curves of the increase in weight with age. The units of time for each species are as follows: guinea pig, days; rhesus monkey, 4 days; sheep, 5 days; pig, weeks; man, months; rabbit, 2 days; rat, days. Rates are expressed as weight gain as a percentage of adult weight for each unit of time. (Figure and caption reprinted with permission from Dobbing, J and Sands, J. 1979. Comparative aspects of the brain growth spurt. Early Human Development 3(1): 79-83.)
these varying doses. In rats, high doses (1-4 mg/kg) of intraperitoneal LPS in late gestation caused a rapid upregulation of cytokine mRNA in both the maternal and fetal brain, while a moderate dose (500µg/kg) demonstrated a 50% decrease in litter size but no obvious brain damage in survivors 10 days after injection. Despite the lack of obvious neonatal brain injury, glial fibrillary acidic protein (GFAP) staining was greater and myelin basic protein (MBP) staining was weaker in the LPS-treated group. This indicates that a change in neural composition was occurring, but had not significantly impacted the gross structure of the developing rat brain.

Intracerebral injection with LPS in neonatal rats demonstrated an increase in ventricle size, co-labelling of IL-1β with microglia, an increase in inducible nitric oxide synthase (iNOS) surrounding the ventricles, and an arrest of oligodendrocyte development accompanied by decreased MBP staining. This study injected LPS into the corpus callosum above the left ventricle, yet effects were noted similarly in both hemispheres. The authors suggest this is due to neuronal transit via the fibres of the corpus callosum, ventricular circulation, or some other secondary messenger system.

A study that combined experimental focal ischemic damage with injection of IL-1β found that the effect of added cytokine increased the damage produced by the ischemic insult. However, this result was only found when the IL-1β was injected into the ventricles, and not when it was injected into the cortex. A lack of protective effect of IL-1 receptor antagonist (IL-1ra) was also found when injected only into the cortex. This indicates the inability of these entities to freely move within the undamaged portions of the brain, while ventricular circulation allowed the cytokine greater access to damaged sites in order to contribute to the ischemic
damage. This conclusion is supported by the fact that receptors for IL-1 are present throughout the brain, including the cortex, and it is therefore not due to lack of receptivity to the injected cytokines.

In mice, initiation of an intrauterine infection with live bacteria was found to stimulate cytokine production in the uterus, placenta and fetal bodies at 3, 5, and 13 hours post-injection. It was found that uterine levels of the cytokines were up to 60 times higher than fetal levels, and the authors concluded that fetal and placental expression of cytokines were secondary in importance. However, the authors found that the fetus is the predominant source of IL-1ra, indicating a fetal response to maternal cytokine expression. However, levels of IL-1ra were insufficient to combat the IL-1 levels; levels of IL-1ra must be 10-1000 times higher than IL-1 levels in order to block its effects.

As previously discussed, brain injury may also occur due to ischemia in the fetal brain. This ischemia may be due to hypoxic episodes during gestation (e.g. nuchal cord) or due to stimulation by an inflammatory pathway causing oxygen deprivation by a number of intracellular mediators. When neonatal mice were given a glutamate agonist, it was found that an NMDA glycine-site inhibitor and a NOS inhibitor both independently protected against excitotoxic lesions. A free radical scavenger also showed a non-significant ability to abate damage caused by the glutamate agonist.

(iv) *In vitro* models of neural cell responses to inflammation and infection

Because inflammatory processes can influence proportions of various neural cell types, it has been suggested that animal studies should focus on staining of MBP and GFAP as early
markers of differential brain development. In studies of white matter damage, the impact on MBP staining is particularly emphasized, as it is the predominant protein (~30-50%) of the myelin sheath.

The time at which most periventricular brain injury occurs, 23 to 32 weeks gestation in humans, is the point at which late oligodendrocyte progenitor cells are the most abundant in the developing brain. These cells, developed from early oligodendrocyte progenitor cells, later progressively develop into immature and mature oligodendrocytes in the case of normal development. Recent studies, however, have shown that when subjected to a hypoxic challenge, late oligodendrocyte progenitors respond by either dying or accelerating along the developmental pathway prematurely. In vivo studies in rats have also shown late progenitor susceptibility to inflammatory damage. In the in vitro study, it was found that the early progenitor cells, though relatively resistant to cell death, lost the ability to label as oligodendrocyte lineage cells. These early progenitor cells, then, remained alive but questionable in their function, as they also failed to label for other neural cell lineages. Early progenitor cells subjected to an ischemic insult also demonstrated a loss of membrane integrity, indicating their susceptibility to other insults that may be associated with the ischemic insult. Immature oligodendrocytes, the stage following late progenitor cells, were found to be relatively resistant to the insult altogether. This is particularly relevant to our research, since the pathways of hypoxic and inflammatory damage both occur through proinflammatory mediators, and the presence of one of these insults may induce damage via the other insult.

Injection of interferon-δ and TNF-α into oligodendrocyte progenitor cultures has also
demonstrated a synergistic effect on cell death. In addition, cells tended to arrest their development upon exposure to these cytokines, showing dramatically decreased viable cell counts and continued markers of immaturity when compared to controls, even after accounting for the increase in cell death. While studies have shown that direct inoculation of oligodendrocyte cultures with TNF-α causes cell death, IL-1β does not have this effect. One study postulated that the actions of IL-1β are dependent on additional effectors due to their observation of the inability of this cytokine to work on neurons in a retrograde or polysynaptic fashion. It is therefore most likely that IL-1β exerts its damaging effects on the developing brain matter via its messengers, such as reactive oxygen species, nitric oxide (NO) release, and release of other proinflammatory cytokines. Because oligodendrocyte progenitors are migratory while later stages are more involved in stationery branching and myelin development, it is possible that migratory function of the late progenitor stage is what is most vulnerable to inflammatory conditions.

As previously discussed, cytokines may also have effects on neurotrophic factors responsible for the proper development of CNS tissues. When LPS was injected into rat brains during gestation, it was found that two neurotrophic factors, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), actually increased in the fetal brain, despite prior evidence that amniotic fluid levels decrease. Further studies must be done in order to determine whether the increased levels of these neurotrophic factors are helpful or detrimental to the developing brain. In a similar fashion to cytokines, moderate levels of these entities may be helpful in establishing neuronal population and synaptic junctions, but high levels may be disruptive to the immature circuitry.
VIII. Introduction to studies presented herein

As is evident from the review of the literature surrounding the causation of white matter injury in the fetus, there are many unknown factors involved in the pathogenesis of chorioamnionitis and the ensuing inflammatory response by mother and fetus. The previously-established guinea pig model of chorioamnionitis was helpful in proving the association between ascending bacterial inoculation, increased proinflammatory cytokines, and fetal white matter injury. The bacterial study, however, did not show a high rate of proven microbiologic infection in the fetal amniotic sacs, and research was therefore undertaken in order to determine the impact of a definitive inflammatory reaction. Chapter 2 details the study we undertook in order to control for the inflammatory process, in which we injected maternal animals with *E.coli*-derived endotoxin and utilized a time course in order to trace the patterns of amniotic fluid cytokines and fetal white matter injury.

While the LPS study involved a time course of fetal and maternal inflammatory response, the guinea pig as an experimental model has some drawbacks. Our primary reason for subsequently utilizing the ovine model was the ability to sample numerous physiologic parameters and collect a myriad of sera and tissues before the experimental endpoint (euthanization). Chapter 3 details a study performed involving chorioamnionitis in the 80% gestation ovine and the subsequent changes over time in physiology and fetal brain injury when further stressors (i.e. brief hypoxic episodes) are included as additional fetal challenges.

Research has not yet unequivocally proven the inability of LPS to cross the placenta in humans or laboratory animals, nor is the role of the placenta in mediating an inflammatory response clear. Chapter 4 presents a study undertaken in order to determine the effect of a bolus dose of LPS or cytokines IL-1β, IL-6 or TNF-α on fetal levels of cytokines. Using the *in vitro*
human placental perfusion set-up, we monitored and assayed fetal and maternal venous effluents in order to determine the placental role in cytokine production or passage, as well as the ability of LPS to cross the human placenta.

Our established model of chorioamnionitis in the guinea pig resulted in increased fetal white matter injury within an elapsed period of 2 days. In order to determine whether this model could be used for testing of possible treatment modalities, a post-natal behavioural study was undertaken in order to characterize the learning abilities and motor skills of these animals. Chapter 5 presents research undertaken in order to determine whether the behavior and learning abilities of guinea pigs exposed to infectious stimuli during gestation differed from control animals.

The primary goal of the research detailed herein was to further elucidate the associations and mechanisms by which infection and inflammation during gestation leads to a heightened proinflammatory response and eventual white matter injury in the fetal brain. The diagnosis of motor impairments such as CP in children is idiopathic in more than half the cases. It is our desire to determine the challenges that overwhelm the developing fetus’ defense system, such that treatment modalities and gestational markers of damage for early detection may aid in clinical management of motor disorders such as CP.
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CHAPTER 2: EFFECT OF MATERNAL INJECTION OF *E. coli*-DERIVED LIPOPOLYSACCHARIDE ON AMNIOTIC FLUID CYTOKINE LEVELS AND FETAL BRAIN INJURY IN THE GUINEA PIG

LA Patrick, EL Harnett, AE Farley, and GN Smith.

1 Department of Anatomy and Cell Biology, Queen’s University, and 2 Kingston General Hospital, Kingston Ontario.

INTRODUCTION

Cerebral palsy (CP), with a prevalence of 1.5-2.5 per thousand live births, is classified by a non-progressive lesion of the cerebral white matter leading to impaired motor development. Several known risk factors exist for CP, though the etiology is unknown in approximately 50% of cases. Chorioamnionitis increases the risk of CP approximately 3-fold in term infants, with an increase in the odds ratio in low birthweight infants to 6.8.

Endotoxins such as LPS are released from gram-negative bacteria during infection, and are known to have adverse effects in the absence of the bacteria itself. Endotoxins are known to increase levels of proinflammatory cytokines such as TNF-α, IL-1β and IL-6. They are also known to have detrimental effects on brain matter through neuroendocrine alteration in the hypothalamic-pituitary-adrenal axis. In addition, previous studies in rats have shown that prenatal exposure to LPS increases prematurity and decreases birth weight, thus increasing risk of fetal brain injury through these mechanisms.

A previous study where LPS was injected to maternal rats intraperitoneally found
increased fetal brain cytokine mRNA for TNF-α and IL-1β after only one hour. However, another study in rats found no increase in cytokine protein or mRNA in the fetal brain after maternal LPS administration, though this study used a much lower LPS injection concentration (50µg/kg vs. 500µg/kg in the previous study). Despite these potentially conflicting results in the measured mRNA levels in the fetal brain, it has been demonstrated that maternal systemic administration of LPS leads to increased levels of proinflammatory cytokines in the placenta, amniotic fluid, and fetal brain eight hours post-injection.

Previous studies have indicated that a threshold exists for the levels of LPS required before a substantial inflammatory response is initiated. This threshold may vary among animal models, and may also depend on the method and location of LPS infusion. When LPS is administered to the brain directly in rats, both LPS and cytokines (IL-6 and TNF-α) can be measured systemically in substantial amounts within 30-60 minutes. However, when LPS is administered systemically at the same rate of flow, higher levels of cytokines are stimulated. The authors of these results suggest that the degree of inflammatory response to LPS is, at least in part, regulated by the CNS. It has been shown that levels of cytokine receptors in the brain differ depending on age, species, and area of the brain investigated. In young mice exposed to LPS, the hypothalamus shows increased levels of IL-1 and IL-6 receptors and the hippocampus shows increased levels of TNF-α receptors. In the case of human brain development, it is known that the white matter is particularly vulnerable to insults in the late second trimester. It is as yet unknown what the pattern of expression of cytokine receptors in the fetal brain is at this time.
OBJECTIVE

The endotoxin LPS is released during infection with gram-negative bacteria, and is therefore an effective proxy treatment for simulating bacterial infection. In our previous model of bacterial inoculation in the guinea pig, it was determined that fetuses exposed to E.coli infection in utero had increased levels of selected proinflammatory cytokines in the amniotic fluid, and that the circulating levels of proinflammatory cytokines after intracervical inoculation were the same in the mother and fetus. In this study, endotoxin was administered intraperitoneally to the maternal guinea pig, and the effects on the fetus of this maternal inflammation were measured. This study was undertaken in order to determine whether the presence of maternal inflammation could induce similar levels of white matter injury in the fetus as our previous model of chorioamnionitis. This study was also undertaken in order to determine the profile of amniotic fluid cytokines and fetal white matter damage over time. We chose the guinea pig as a model for several reasons, including the close similarities in perinatal brain development and in the structure of the placenta as intermediary between mother and fetus. The hemochorial guinea pig placenta is the closest small laboratory animal model to the human in terms of the placenta; only thin layers of fetal cells lie between the maternal and fetal circulation in both the guinea pig and human.

We expect that maternal injection of LPS will elicit similar results to our previous guinea pig model of chorioamnionitis, with elevated levels of proinflammatory cytokines in the amniotic fluid and increased levels of fetal white matter damage.
METHODS

Animals (n=4-7 maternal animals, 13-24 fetuses at each time point) were bred in the Queen’s Animal Care facility after obtaining ethics approval from the University Animal Care Council. Lipopolysaccharide (O55:B5, Sigma L2880) was diluted with sterile saline and frozen in aliquots. Prior to use, the LPS aliquot was thawed and sonicated. LPS solution was injected intraperitoneally to maternal animals at approximately 70-75% gestation (days 45-50 of a 65 day gestation) at a concentration of 500µg/kg. This dose was chosen due to its effectiveness in inducing an fetal inflammatory response in a previous study performed on rats. The antibiotic Tribrissen 24% was administered each day at a dose of 0.05 mL/kg in order to prevent maternal sepsis. A small number of control animals (saline-injected, n=3-5) were sacrificed at selected time points in order to determine baseline values for the parameters studied.

At selected time points after LPS injection (t=1, 4, 8, 12, 16, 20, 24, 32, 40 and 48 hours), maternal animals were sacrificed using 2mL Euthanyl (1mL intraperitoneal followed by 1mL intracardiac upon sedation). A midline incision was performed and the uterine horns exposed. Placement of the fetuses was noted and fetuses were individually removed with membranes intact. Approximately 2mL of amniotic fluid was removed from each fetal sac by sterile 18guage needle and syringe, and was deposited in individually-labelled Eppendorf tubes. Maternal blood was obtained by cardiac puncture in the same manner, and all blood and amniotic fluid samples were centrifuged at 2000g for 3min. Maternal serum and fetal amniotic fluid samples were then pipetted from solid cell suspensions and blood products and placed in new, individually labelled tubes and were immediately frozen at –80 °C.

Upon removal of fetal membranes and placenta, fetal weight was recorded, and the
fetus was perfused through the aorta with 4% paraformaldehyde (PFA) in phosphate-buffered saline for approximately 4 minutes. The fixed fetal brain was then excised and placed in individual containers of 4% PFA for no less than 3 days. Fetal brains were then coronally sectioned, with the initial cut at the central sulcus. Serial sections from the midline were at intervals of 3-4mm, and the anterior and posterior sections of the brain were placed in separate tissue cassettes. These cassettes were then placed in 4% PFA and sent to the Department of Pathology for paraffin and microtome processing.

Enzyme-linked immunosorbent assay (ELISA) kits for the selected proinflammatory cytokines were obtained from R&D Systems, Minneapolis. Because a guinea pig serum kit does not exist, kits used were mouse-specific, having been assured of proven cross-reactivity by company technicians. ELISAs for IL-1β, IL-6 and TNF-α were performed on each amniotic fluid or maternal serum sample in duplicate, and some samples were repeated in multiple assays in order to confirm low inter-assay variability. Each sample was thawed in the 4°C fridge for 2-3 days before assay, and repeated freeze-thaw cycles were avoided. Standard curves and kit controls were performed for each assay set. The limulus amebocyte lysate (LAL) assay for LPS detection was performed on a number of amniotic fluid and maternal serum samples (QCL-1000, BioWhittaker, Walkersville MD).

Fetal brains were stained using both NeuroTacs (R&D Systems, Minneapolis) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, Roche, Laval QC) protocols, and were subsequently analyzed using either light microscopy (for NeuroTacs) or fluorescence microscopy with SlideBook v. 4.0 (for TUNEL). Slides were blinded to the researcher before images were taken.

Statistics were performed using GraphPad Prism v.5.00 for Windows.
RESULTS

The later time points of analysis (time intervals 32, 40 and 48 hours post-injection) were subject to maternal or fetal losses; half the maternal animals sacrificed at 48 hours aborted their fetuses within this time, and were thus excluded from the analysis. The final number of animals utilized for analysis was 55, having lost 15 animals from the study due to maternal or fetal deaths or abortions; none of the maternal losses were control animals. Fetal weights at 48 hours were found to be significantly lower than that of the controls and the animals at 8 hours post-LPS injection (Figure 2-1, ANOVA with Tukey post-hoc, \( p<0.05 \)). This is most likely due to small sample sizes at 48 hours, as we experienced the greatest number of maternal abortions or deaths at this time point. Preferred fetal weight range was 50-70g, based on fetal growth curve standards for 70% gestation, and our experimental fetal weights fell into this weight range.

Amniotic fluid ELISA analysis indicated some similarities in cytokine profiles between the two Interleukins; both IL-1\( \beta \) and IL-6 showed cyclic cytokine profiles over the time course of the experiment. As shown in Figure 2-2, IL-1\( \beta \) levels at 8 hours post-LPS were significantly higher than levels at most other time points and when compared to pooled control samples (ANOVA with Tukey post-hoc, \( P<0.05 \)). When comparing the time points at which matched control samples were also obtained, however, the levels of IL-1\( \beta \) at 16 hours post-LPS were significantly elevated (Figure 2-3, ANOVA with Tukey post-hoc, \( P<0.05 \)). The IL-6 cytokine profiles also showed cyclicity over the course of the experimental protocol,
Figure 2-1: Fetal weights (mean ± SEM) at time of maternal euthanization and removal of fetuses for perfusion. Fetal weights at 48 hours are significantly lower than that of the controls and the animals at 8 hours post-LPS injection (ANOVA with Tukey post-hoc, p<0.05). This is most likely due to small sample sizes at 48 hours, as we experienced the greatest number of maternal abortions or deaths at this time point. Sample size for each time point was 18-23 fetuses from 5-7 maternal animals.
Figure 2-2: Concentrations of proinflammatory cytokines IL-1β in amniotic fluid samples. Times indicate elapsed interval between injection of maternal animal with LPS bolus and sample collection. Asterisk indicates that the amniotic fluid levels of IL-1β at 8 hours after maternal LPS injection are significantly higher than pooled control values and all other time points except for the 20 hour group (ANOVA with Tukey post-hoc, p<0.05). Sample sizes for each time point were 10-23 fetuses from 5-7 maternal animals. Data is shown as mean ± SEM.
Figure 2-3: Comparison of control and LPS-exposed amniotic fluid IL-1β levels at selected time points. Asterisk indicates that amniotic fluid from sacs where maternal animal had received an intraperitoneal injection of LPS had significantly higher levels of IL-1β at 16 hours post-injection. Data is represented as mean with SEM.
but high variability in the values obtained prohibited any statistically significant elevations in comparisons with pooled controls or among time points (Figure 2-4, ANOVA with Tukey post-hoc, p<0.05). When matched controls were compared at selected time points, however, a significant elevation in IL-6 levels from control levels was found at 16 hours post-LPS (Figure 2-5, ANOVA with Tukey post-hoc, p<0.05). TNF-α levels remained low throughout the time course, with no significant increases over control values at any time point (Figure 2-6, ANOVA with Tukey post-hoc, p<0.05).

Collection of maternal serum was often problematic; in many cases we were unable to collect sufficient maternal blood to obtain adequate serum for assay. Therefore, while we obtained a sample size of 10-23 fetuses from 5-7 maternal animals for each time point, we were only able to measure maternal serum cytokines from 2-3 animals. Maternal serum ELISA analysis revealed that IL-1β and IL-6 levels were not significantly different when compared among time points or when compared to control values (Figure 2-7, P>0.05, ANOVA with Tukey post-hoc).

In our previous model of intracervical inoculation, a side study was completed in which we varied the interval between intracervical inoculation with *E.coli* and subsequent animal euthanization and sampling. Detailed methodology and results from that study were previously published. Because the current study’s rationale was based upon the results of that previous study, comparative analysis was performed. As shown in Figure 2-8, the current study using LPS mimicked the results of the *E.coli* inoculation study when comparing amniotic fluid IL-1β, the differences lying mainly in the time intervals used and the peak cytokine concentrations obtained.

Representative images for NeuroTacs and TUNEL models of cell death detection are
Figure 2-4: Amniotic fluid cytokine IL-6 levels at various intervals after maternal intraperitoneal LPS injection. There were no differences in cytokine levels at any of the time points, but a cyclicity in cytokine presence is evident. Sample sizes for each time point were 10-23 fetuses from 5-7 maternal animals. Data shown as mean ± SEM.
Figure 2-5: Comparison of control and LPS-exposed amniotic fluid IL-6 levels at selected time points.

Asterisk indicates that amniotic fluid from sacs where maternal animal had received an intraperitoneal injection of LPS had significantly higher levels of IL-6 at 16 hrs post-injection. Data shown as mean ± SEM.
Figure 2-6: Amniotic fluid cytokine TNF-α levels at varied intervals after maternal intraperitoneal LPS injection. There were no significant differences in amniotic fluid levels at any of the time points. Sample size at each time point was 11-23 fetuses from 5-7 maternal animals. Data shown as mean ± SEM.
Figure 2-7: Maternal serum cytokines (A) IL-1β and (B) IL-6 at various time points after intraperitoneal LPS injection. Note that sample sizes for these data points are very small (n=2-3 at each point). There were no significant differences in maternal serum cytokine IL-1β levels throughout the experiment, or when compared to control maternal serum cytokine levels (data not shown). Data shown as mean ± SEM.
Figure 2-8: Comparison of cytokine IL-1β LPS and intracervical *E.coli* inoculation time courses.

Although scales of time (y axis) and IL-1β concentration (x axis) are different, the same patterns of cyclicity over time were found in both studies. Asterisk indicates cytokine concentration is significantly greater than control values (not pictured). Data shown as mean ± SEM.
shown in Figure 2-9. Both staining protocols were used during the course of the experiment, though NeuroTacs was abandoned as a method due to high costs and inter-observer variability. Figure 2-10 shows the results of quantification of cell death using NeuroTacs in the subcortical and periventricular white matter at 20 and 48 hours after maternal LPS injection. While a modest increase in cell death was seen at 20 hours, the increase in cell death was significantly higher than control levels at 48 hours in the periventricular white matter (two-tailed t-test, p<0.05). TUNEL staining and immunofluorescence computer analysis confirmed increased levels of damage in the white matter with respect to control levels, but these increases were only significantly elevated at 20 and 24 hours post-injection (Figure 2-11, ANOVA with Tukey post-hoc, p<0.05). There was no difference between levels of white matter injury in the subcortical or periventricular white matter using the TUNEL method of cell death detection. (two-tailed t-test at each time point, p<0.05 for all).

Figure 2-12 shows comparative data from the current LPS study and the previous study of intracervical E.coli inoculation. When the brains of animals sacrificed at 48 hours post-LPS or post-bacterial inoculation are examined using the NeuroTacs system, the two models show a similar significant increase in periventricular white matter cell death compared to controls at 48 hours.

**DISCUSSION**

In this study, maternal injection with LPS resulted in increased proinflammatory cytokine concentrations and fetal brain injury within 48 hours of maternal injection. This inflammatory response and the subsequent white matter damage evident by both NeuroTacs and TUNEL analysis are indicative of a role for proinflammatory processes in instances of
Figure 2-9: Representative images of guinea pig periventricular white matter after staining with NeuroTacs (A,B) and TUNEL (C, D). With NeuroTacs, control staining (A) consists of plain blue counterstain included in the kit (40x). With TUNEL, control staining (C) involves blue DAPI marking of all double-stranded DNA (10x). Photo (B) shows a demonstrative NeuroTacs-positive cell (40x), with dark brown stain and aggregated nuclear material indicating that the cell is in the latter stages of dying. Photo (D) demonstrates TUNEL-positive cells expressing green fluorescent protein (GFP) as a marker of cell death (20x).
Figure 2-10: NeuroTacs staining of fetal brains after maternal LPS injection, as quantified by cells showing clear signs of cell death as a percentage of all cells in each field of view (FOV). Figure (A) represents counts in the subcortical white matter, and figure (B) represents counts in the periventricular white matter. Asterisk indicates significantly greater levels of cell death with respect to control. Control n=2 fetuses, 20 hour n=12 fetuses, 48 hour n=14 fetuses. Data shown as mean ± SEM.
Figure 2-11: TUNEL staining of fetal brains after maternal LPS injection, as quantified by cells signalling with GFP, the marker of cell death using the TUNEL protocol, as a percentage of all DAPI-signalling cells in each field of view (FOV). Figure (A) represents counts in the subcortical white matter, and figure (B) represents counts in the periventricular white matter. Asterisk indicates significantly elevated levels of cell death with respect to control levels (not pictured). Control levels were the same as levels of cell death in the 1 hour LPS groups for both areas investigated. Samples sizes were n=9-10 for each time point investigated. Data shown as mean ± SEM.
Figure 2-12: NeuroTacs staining of fetal brains after intracervical bacterial inoculation or maternal intraperitoneal LPS injection, as quantified by cells showing clear signs of cell death as a percentage of all cells in each field of view (FOV). Figure (A) represents counts in the subcortical white matter, and figure (B) represents counts in the periventricular white matter. Control animals received no bacterial inoculation or LPS injection, Exposed animals were fetuses from maternal animals that received an E.coli intracervical inoculation but did not show microbiologic chorioamnionitis, Infected animals were those fetuses whose mothers received an intracervical E.coli dose and did show microbiologic growth in their amniotic fluid, and LPS represents animals from the current study in the 48 hour interval group. Asterisk indicates significantly greater levels of cell death with respect to control. Data shown as mean ± SEM.
white matter disturbance during gestation. This study found that IL-1β in particular is a key inflammatory mediator in response to endotoxin, as amniotic fluid levels were increased at certain intervals after LPS injection. The failure of maternal serum cytokines to increase after endotoxin administration may have been caused by maternal Tribrissen antibiotic administration, though information on the effect of antibiotic on circulating levels of proinflammatory cytokines is currently unavailable. Though levels of IL-6 demonstrated similar increases and decreases as IL-1β during the study, variation in the raw values negated any statistically significant elevations from baseline. TNF-α raw values, on the other hand, showed very few deviations from baseline levels.

At 48 hours after LPS injection, white matter injury was similar to that found in our previous model of ascending bacterial injection. LPS use, while resulting in similar levels of white matter injury, initiated a proinflammatory cascade of a lower magnitude than in the case of live bacteria, and this occurred much more rapidly than with the use of intracervical E.coli inoculation. In the current LPS study, however, inflammatory mediators in the amniotic fluid were cleared by 48 hours, and maternal serum cytokines did not increase from baseline at any point. This contrasts with the results of our previous E.coli model, where cytokines were elevated at times beyond 48 hours post-inoculation, and maternal serum cytokines were elevated at certain time points as well. We suggest that the LPS injection causes a similar response as the E.coli model in certain aspects, such as cytokine profile cyclicity and periventricular white matter injury, while differing in the response of maternal cytokines and in the time taken for an inflammatory response to occur. This time lag in response with the E.coli model most likely reflects the fact that intracervical E.coli inoculation requires time to become established in the uterus and in the gestational sacs, whereas the response from LPS
injection is almost immediate. The similarities between the two models suggest that the common pathway by which fetal brain injury occurs likely involves elevated proinflammatory cytokine concentrations in the amniotic fluid, particularly IL-1β, rather than presence of bacteria. As noted, the first significant increase in levels of white matter injury in the periventricular white matter occurred at 20 hours post-injection of LPS, after the first spike in IL-1β (8 hours post-injection). It is suggested that IL-1β itself, or another entity stimulated by IL-1β, is a strong contributor to the white matter damage found in our studies.

Injection of LPS offers an added benefit over the use of live bacteria, in that concentrations delivered to the experimental animal are more precise and reliable, and the inflammatory reaction tends to begin more quickly than in cases where bacteria must breach the uterine cavity and colonize. Due to the increased severity of inflammation characteristic of direct LPS injection, as demonstrated by the high abortion and death rate in this study at 48 hours post-injection, it follows that severity of inflammatory response may influence the severity of subsequent brain injury. We did not find a difference in levels of white matter damage after 48 hours of LPS or *E.coli* exposure. Because we only investigated fetal brains at the common time point of 48 hours for these studies, it is impossible to tell whether the similarity in white matter damage found would persist at other time points.

A recent study did, in fact, demonstrate that there is an dose-dependent inflammatory response to LPS in guinea pigs. We based the LPS dose in our study on a similar study performed on rats, and have found in the literature that the dose used (500µg/kg) is a moderate concentration, depending on the animal species and protocol used. A previous study in rats where the maternal animals were given similar LPS doses (300 µg/kg) found that there were no fetal losses, significant changes in litter size or litter weight upon
Neonatal rats exposed to this dose of LPS in utero demonstrate an increase in caspase-3 and TUNEL staining in the white matter, accompanied by a decrease in myelin in these same areas. Further, a dose response study indicated that 300µg/kg LPS was the minimum dose required in order to stimulate a significant inflammatory response. Unfortunately, this information was unavailable at the time this study was undertaken, as this lower dose may have prevented some of the late-interval fetal and maternal deaths experienced in this study.

Previous studies in which the same dose of LPS (500µg/kg O55:B5) was administered to maternal rats and fetal tissues were subsequently collected and sampled found that at 2 and 8 hours post-injection, there were significantly elevated levels of all three cytokines (TNF-α, IL1β and IL-6) in the placenta and of IL-6 in the amniotic fluid. At a slightly lower dose in rats (300µg/kg), a continued increase in IL-1β mRNA expression in brains at postnatal day 1 in rats was found, though the expression of TNF-α at postnatal day 7 was decreased with respect to control animals. That particular study did not detail whether any signs of sepsis were present in the neonatal animals at the time of euthanization, and it therefore remains unclear whether the cytokine expression differences were due solely to the in utero inflammatory response to which the animals were subjected.

A study that gave low dose injections of LPS (100µg/kg) to rats found similar results to those found in our E.coli inoculation study, with maternal serum cytokines showing cytokine levels mimicking the fetal cyclical patterns but at much lower levels. It is possible that the low sample sizes of our maternal serum assay data resulted in a failure to demonstrate any significant elevations from baseline. Unfortunately, many of our maternal serum samples
were inadequate or contaminated with cellular debris (rendering it unusable), and our maternal serum data is thus lacking in sample size.

In this study and others, maternal injection with LPS causes increased proinflammatory cytokine presence and fetal brain injury within two days. Animal and human studies have demonstrated a correlation between chorioamnionitis and cytokines in the maternal serum, amniotic fluid, or fetal tissues. It has been reported in a number of studies that elevated levels of proinflammatory cytokines, stimulated by infection, are toxic on a cellular level and may lead to widespread damage in the developing fetus. In human studies, cord blood IL-6 and IL-8 but not IL-1β and TNF-α levels were raised in infants exposed to clinical chorioamnionitis. The authors suggest that the lack of the latter two cytokines indicates that they are more instrumental in systemic infections, such as neonatal sepsis. Our data supports this hypothesis, as the LPS injection used in this study mimics a systemic inflammation in the maternal animal, leading to a fetal or placental response in the form of elevated IL-1β levels in the amniotic fluid.

A previous study in rats where LPS was administered peripherally found that mRNA levels for the cytokines IL-1β and TNF-α were increased in certain brain areas, including the cerebral cortex, cerebellum and hippocampus. This same study found that, with few exceptions, anti-inflammatory cytokines and cytokine receptor antagonists were not increased in response to peripheral LPS dosage. Other studies have found maternal serum expression of both pro- and anti-inflammatory cytokines to be greatly increased after LPS administration intraperitoneally, but the amniotic anti-inflammatory agents do not similarly increase. Current knowledge on the ability of LPS, pro-inflammatory and anti-inflammatory cytokines
to cross the placenta is varied, with most studies suggesting a limited ability to do so. Presence of LPS in the maternal system may cause the placenta and fetal membranes to produce an inflammatory response, leading to cytokine presence in the fetus and amniotic fluid. The point at which the pro-inflammatory cytokine population overwhelms the anti-inflammatory mechanisms will differ depending on the nature of the insult, but it is most likely this point at which the developing fetal tissues may become compromised.

It was noted in many slides that, when a blood vessel was caught in cross-section, the epithelial cells lining the vessel were often TUNEL-positive. This observation, while not quantitative, supports the theory that inflammatory mediators are linked with intraventricular hemorrhage and cell death in the brain due to vessel compromise and subsequent leakage of blood into the ventricles. This is in contrast, however, to the hypothesis put forth by Kadhim et al., in which it is hypothesized that the lack of cell adhesion molecule and TNF staining on endothelial lining of blood vessels in neonatal brains with PVL may mean that the vascular circulation is less responsible for subsequent fetal brain injury than internal cerebral processes.

While comparison is impossible between *in vitro* and *in vivo* conditions and results, many histological studies support the hypothesis that inflammatory cytokines are the key mediators of inflammation in the fetal brain. A study in which levels of IL-6 and TNF-α were measured in rat microglial cell culture indicated that IL-6 levels were increased more than 10-fold at 6 hours post-LPS exposure and continued to increase up to 48 hours, while TNF-α was increased approximately 100-fold at 6 hours but decreased within 24 hours to levels still 50-fold higher than base levels. This data indicates that LPS and cytokine presence in the
fetal brain can cause a marked inflammatory response from microglia, leading to both oligodendrocyte damage and general inflammatory damage to both the neural tissue and the blood vessels within the brain. Further investigations of the effects of LPS on the fetal outcome of 70% gestation guinea pigs should involve unity of in vivo and in vitro techniques, as there is a paucity of research using the guinea pig in this area.

CONCLUSION

Intraperitoneal injection of 500µg/kg *E.coli*-derived LPS (O55:B5) results in an increase in the pro-inflammatory cytokine IL-1β and elevated levels of periventricular white matter damage. These results show some similarities to previous results obtained using an intracervical inoculation of live *E.coli* bacteria. This suggests that, in the absence of live bacterial infection, inflammatory cues in the fetal environment trigger pro-inflammatory pathways, eventually leading to fetal white matter injury through direct or indirect methods. Our results demonstrate a fluctuating inflammatory response profile in amniotic fluid cytokines, and it seems likely that it is the repeated or continuous inflammatory challenges to the developing fetus that result in eventual white matter damage.
Works Cited


CHAPTER 3: INTRA-AMNIOTIC INFECTION WITH E.COLI IN THE PRESENCE OR ABSENCE OF UMBILICAL CORD OCCLUSIONS IN THE PREGNANT SHEEP

Lindsay A. Patrick 1, Bryan Richardson 2, Shannon Hemstreet 2, Brad Matuszewski 2, Jac Homan 2, and Graeme N. Smith 1,3.

1 Dept. of Anatomy and Cell Biology, Queen’s University, 2 Dept. of Obstetrics and Gynecology, University of Western Ontario, and 3 Dept. of Obstetrics and Gynecology, Kingston General Hospital.

INTRODUCTION

Cerebral Palsy (CP) is the most common motor disorder affecting children, with a prevalence of 1.5-2.5 per thousand live births and a survival rate at adulthood of 90%. CP is classified by a non-progressive lesion of the cerebral white matter leading to adverse motor development. While multiple etiologies have been proposed, there is increasing focus on the interaction between chorioamnionitis and hypoxic-ischemic insults antenatally. Clinical chorioamnionitis, diagnosed by a combination of maternal fever, maternal and/or fetal tachycardia, purulent vaginal discharge and leukocytosis, is a risk factor for brain injury in both the preterm and term infant. Evidence of histologic chorioamnionitis upon pathological examination of the placenta and membranes after birth is found in up to 50% of premature births, though presence of the infection itself often goes undiagnosed clinically. We have previously demonstrated that intracervical inoculation of the pregnant guinea pig with E.coli was associated with fetal brain injury in the offspring. We also noted in that study that fetuses were adversely
affected after *E.coli* inoculation regardless of amniotic evidence of bacterial colonization, suggesting that subclinical infection and the inflammatory responses to remote infectious stimuli pose a risk to the developing fetus. Additional work in our lab indicates that a threshold exists for stressors such as LPS, beyond which defense systems are overwhelmed and the fetus sustains injury and possible death.

Evidence of perinatal hypoxia has been linked with 8-28% of cases of CP. Animal studies to date, however, have indicated that acute but brief periods of hypoxia in utero, while causing some neural damage, may not be the singular factor leading to the extent of damage noted during childhood examination. Prenatal nuchal cord, defined as the “entanglement of the umbilical cord around the fetal neck,” is a common cause of hypoxic episodes in human gestation (15-30% at delivery), and cases usually resolve themselves before the situation becomes problematic. A prospective study found that the presence of nuchal cord diagnosed prenatally did not lead to increases in fetal morbidity at or after delivery. It is proposed that compensatory mechanisms during gestational development of the fetal brain may overcome many instances of hypoxic damage due to cord compression or nuchal cord.

Due to the vulnerability of the developing fetal brain, either a heightened inflammatory response or severe hypoxic insults may cause serious neural damage. Epidemiologic studies have shown that chorioamnionitis and hypoxia together lead to a much greater incidence of CP (OR = 78, 95% CI 4.8-406) compared to either insult alone. In some cases, chorioamnionitis may be the causative factor in hypoxic ischemia in the fetus, as high levels of bacterial endotoxin may ultimately decrease placental vascular blood flow. A recent study demonstrated a
detrimental change in fetal ovine physiologic parameters with chronically administered LPS into the fetal venous system (e.g. decreased O₂ content, increased pCO₂), but these parameters returned to control levels within a day and remained as such throughout the experiment. This indicates that the fetal system may exhibit a hypoxic stress response after an initial insult, but may be able to adjust its physiological response to continued adverse conditions. In addition to vascular responses, overstimulation (or subsequent habituation) of neurons due to a build-up of the neurotransmitter glutamate in the synapses (from either over-production or failure to be cleared) may lead to sensitization of the fetal brain to subsequent insults, such as a proinflammatory cascade.

OBJECTIVE

In the current study, we utilized the well-established in utero fetal sheep model in order to determine the maternal and fetal response to intraamniotic bacterial infection in the presence or absence of acute hypoxic episodes. We hypothesized that the fetal brain injury following simultaneous exposure to chorioamnionitis and hypoxia would be greater than by either alone due to sensitization of the fetal brain. The objective of this study was to determine if chorioamnionitis, in the presence or absence of hypoxia, resulted in fetal metabolic, cardiovascular, or white matter abnormalities.

METHODS

I. Animal procedures

All work performed was done in accordance with University Animal Care and Veterinary Services standard operating procedures, and ethics approval was received from the University
Council on Animal Care at the University of Western Ontario. Maternal surgical and pre- and post-operative care have been previously described. Thirty preterm (118-120 days of 145 day gestation) fetal sheep were chronically instrumented according to the established protocol. Each fetus had a polyvinyl catheter placed in the right and left brachiocephalic arteries, cephalic vein, sagittal vein, and amniotic sac. A 20mHz piezoelectric crystal was secured above the sagittal sinus of the fetal brain to monitor Doppler flow. Each fetus was fitted with an inflatable silicone rubber cuff around the proximal umbilical cord. The antibiotic Crystapen (London Health Sciences Centre) was injected into the maternal and fetal veins at the time of surgery and on Day 1 of recovery. Experiments were initiated following a 3-day period of recovery.

II. Experimental groups

Animals were randomized into three treatment groups:

Group 1, umbilical cord occlusion (UCO) only, in which the umbilical cuff was inflated as described below,

Group 2, *E.coli* only group, in which *E.coli* broth was injected into the amniotic sac at the beginning of the experiment, and

Group 3, *E.coli* plus cord occlusion, in which both of the above treatments were administered.

III. Time course of experiment

The experimental protocol involved fetal environmental manipulations, monitoring and sample collection performed over a 96-hour period (Table 3-1). All experiments were started at the same time of day in order to eliminate confounders due to ovine sleep-wake schedule. Maternal arterial and fetal arterial blood were tested for glucose and lactate (YSI 2300, Yellow
Table 3-1: Schedule of sampling times throughout experiments. SSP=standard sampling procedures (fetal blood, maternal blood, amniotic fluid and fetal sagittal vein blood taken, Powerlab calibrated and measurements of fetal and maternal parameters taken for 10-20 minutes), AFC=amniotic fluid loop cultured on TSA plate, MRT=maternal rectal temperature taken.

<table>
<thead>
<tr>
<th>Date</th>
<th>Cord occlusions (Group 1)</th>
<th>Amniotic <em>E.coli</em> injection (Group 2)</th>
<th>Cord occlusions and amniotic <em>E.coli</em> injection (Group 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1S1, 10am Day 1</td>
<td>SSP, AFC, MRT</td>
<td>SSP, AFC, MRT, then injection of ~500CFU <em>E.coli</em> into amniotic catheter</td>
<td>SSP, AFC, MRT, then injection of ~500CFU <em>E.coli</em> into amniotic catheter</td>
</tr>
<tr>
<td>D1S2, 4pm Day 1</td>
<td>SSP</td>
<td>SSP, AFC</td>
<td>SSP, AFC</td>
</tr>
<tr>
<td>D1S3, 10pm Day 1</td>
<td>SSP</td>
<td>SSP, AFC</td>
<td>SSP, AFC</td>
</tr>
<tr>
<td>D2S1, 4am, Day 2</td>
<td>SSP</td>
<td>SSP, AFC</td>
<td>SSP, AFC</td>
</tr>
<tr>
<td>D2S2, 10am Day 2</td>
<td>SSP, AFC, MRT, then 2 minute cord occlusions each hour for 6 hours</td>
<td>SSP, AFC</td>
<td>SSP, AFC, MRT, then 2 minute cord occlusions each hour for 6 hours</td>
</tr>
<tr>
<td>D2S3, 4pm Day 2</td>
<td>SSP</td>
<td>SSP</td>
<td>SSP</td>
</tr>
<tr>
<td>D2S4, 10pm Day 2</td>
<td>SSP</td>
<td>SSP, AFC</td>
<td>SSP, AFC</td>
</tr>
<tr>
<td>D3S1, 4am Day 3</td>
<td>SSP</td>
<td>SSP</td>
<td>SSP</td>
</tr>
<tr>
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<td>SSP, AFC, MRT</td>
<td>SSP, AFC, MRT</td>
<td>SSP, AFC, MRT</td>
</tr>
<tr>
<td>D3S3, 6pm Day 3</td>
<td>SSP</td>
<td>SSP</td>
<td>SSP</td>
</tr>
<tr>
<td>D4S1, 2am Day 4</td>
<td>SSP</td>
<td>SSP</td>
<td>SSP</td>
</tr>
<tr>
<td>D4S2, 10am Day 4</td>
<td>SSP, AFC, MRT</td>
<td>SSP, AFC, MRT</td>
<td>SSP, AFC, MRT</td>
</tr>
<tr>
<td>D4S3, 6pm Day 4</td>
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<td>SSP</td>
<td>SSP</td>
</tr>
<tr>
<td>D5S1, 2am Day 5</td>
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<td>SSP</td>
<td>SSP</td>
</tr>
<tr>
<td>D5S2, 10am Day 5</td>
<td>SSP, AFC, MRT, euthanization</td>
<td>SSP, AFC, MRT, euthanization</td>
<td>SSP, AFC, MRT, euthanization</td>
</tr>
</tbody>
</table>

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Springs OH), pO₂, pCO₂ and pH (Radiometer Medical, Copenhagen) at each time point in the study. Samples of maternal arterial blood, fetal arterial blood, fetal sagittal vein blood and amniotic fluid were collected at each time point, and the volume (2-3mL) was replaced with sterile saline. Physiological parameters tested using Powerlab and Chart 5 instrumentation (AD Instruments, Colorado Springs CO) included true fetal blood pressure (fetal arterial pressure minus amniotic pressure), maternal blood pressure, fetal heart rate, and fetal sagittal vein Doppler velocity. Amniotic fluid was cultured on tryptic soy agar at selected time points in Groups 2 and 3 to monitor bacterial colonization, and in Group 1 to confirm sterility of amniotic fluid. Maternal rectal temperature was taken each day and food and water intake was monitored in order to determine whether the ewe was exhibiting signs of infection.

IV. *E.coli* culturing

Human-isolated *E.coli* bacteria was used, as this bacterium is often isolated in cases of chorioamnionitis. Two original *E.coli* samples were obtained from patients at Kingston General Hospital, and were transferred onto beads by Dr. Lewis Tomalty. These beads were frozen in vials at –80°C and shipped to the Valvano Microbiology lab at UWO. Growth curve experiments were performed in order to determine the viability of the strains after shipment (Figure 3-1). After determining the growth curves for each vial of beads was equal, one vial was selected and the beads from this vial were used for every experimental sheep in Groups 2 and 3.

Appendix I contains a detailed explanation of bacterial concentration calculations. Briefly, for each experiment requiring bacteria, an individual frozen bead was briefly immersed in approximately 20mL tryptic soy broth, and broth was incubated at 37°C overnight. The turbidity of the resultant bacterial suspension was then measured using spectrophotometry.
Figure 3-1: Growth curves obtained for each of the three frozen vials of *E.coli* culture beads after arrival at University of Western Ontario. Two cultures were from separately obtained patient samples at Kingston General Hospital, while the third vial (“Culture 1 transfer tube”) consisted of a separate batch of beads exposed to one of the initial cultures.
(Beckman Coulter, model DU530) at 600nm with a blank adjusted to and optical density (OD) of 0. Broth was diluted to an OD of 0.1 and in a side-arm flask, and was placed in the incubator at 37°C for one hour after recording an initial Klett value (Scienceware, Klett). Klett value after one hour was recorded and the resultant concentration in the side-arm flask was determined. The bacterial concentration was calculated using the standard approximation of 1 OD unit = $10^8$ bacteria = 147 Klett units. Serial dilutions of the bacterial broth were performed in sterile saline, and 4-5 tryptic soy agar plates were inoculated with 100µL of each of the lower dilutions ($10^3 - 10^0$). These plates were incubated overnight and were used to confirm bacterial concentration calculations. A volume of one of the diluted solutions was pipetted into a sterile glass tube such that the desired concentration (500 colony-forming units, CFU) was transported to the Animal Care Facility. Within 20 minutes of bacterial dilutions, experimental injection into the amniotic fluid catheter of the two groups that received *E.coli* doses (Groups 2 and 3) was performed immediately after Day 1, Sample 1 (D1S1).

V. Umbilical cord occlusions (UCO)

At the time the umbilical cuff was placed and secured to the fetus in surgery, it was inflated with sterile saline in order to determine the correct volume to achieve complete cord occlusion. In the two groups that received cord occlusions (Groups 1 and 3), occlusions were performed for two minutes each hour for six hours, beginning directly after Day 2 Sample 2. Fetal arterial blood was sampled and tested for glucose, lactate, pH, pO$_2$ and pCO$_2$ five minutes before each occlusion (baseline measurement) and 90 seconds into the occlusion. Powerlab measurements were recorded in Chart 5 (AD Instruments) throughout the period of occlusions in these groups.
VI. **Post-mortem procedures**

After the last measurement at Day 5, Sample 2 of the experiment, maternal animals were killed with 10mL of Euthanyl (Bimeda-MTC Animal Health, Cambridge, ON) intrajugular and fetuses were injected with an additional 2-3mL Euthanyl intracardiac upon removal from the uterus. Fetal brain and right lung were excised from both the instrumented fetuses as well as from any uninstrumented twin or triplet fetuses. Fetuses were weighed before dissection, and brain and lung were weighed after dissection. Sex of the fetus was recorded, and fetal brain was quickly dissected into brain regions. Serial slices of the right half of the brain were alternatively preserved by immersion fixation in 4% PFA in PBS or slow frozen in optimal cutting temperature (OCT) preservative resin. Left hemisphere was fast frozen in liquid nitrogen and immediately placed in a labelled bag and placed in a container of dry ice. Immersion fixed tissue remained in 4% PFA for 2-3 days, then secured in cassettes and sent for paraffin embedding.

VII. **Enzyme-linked Immunosorbent Assay (ELISA)**

Amniiotic fluid, fetal sagittal vein serum, fetal arterial serum and maternal serum samples were analyzed using an established protocol for ovine ELISA developed by Australia’s Commonwealth Scientific and Industrial Research Organization (CSIRO) and optimized by Dr. L.J. Yao of Dr. Ruud Veldhuizen’s lab, University of Western Ontario. Briefly, plates were blocked with assay diluent for 16-20 hours (BD Pharmingen, Mississauga, ON). The following day, plates were washed with 1x washing buffer (BD Pharmingen, Mississauga, ON) four times, and standards, controls, and samples were loaded. Standards were obtained from CSIRO and were serially diluted as recommended. Following another wash cycle, incubation periods in monoclonal (MAB 1001 for IL-1β, MAB 1004 for IL-6, Chemicon, Temecula, CA) and
polyclonal (AB 1838 for IL-1β, AB 1839 for IL-6, Chemicon, Temecula, CA) antibodies for 2 hours each. Substrate solution (BD Pharmingen, Mississauga ON) was followed by stop solution (BD Pharmingen, Mississauga ON), and plates were then read at a wavelength of 490 with a wavelength correction of 540.

VIII. Immunohistochemistry of fetal brains

Immunofluorescent staining was performed on fixed, paraffin-embedded sections of the fetal brain using TUNEL (Roche, product no. 11684795910) according to the established protocol accompanying the kit. Previous studies have indicated that the greatest levels of white matter injury occur at the external angle of the lateral ventricles and in the subcortical white matter, and therefore these areas were selected for immunohistological analysis. Slides were relabelled with new titles by another member of the research group such that the researcher was blinded to the animal number and treatment before microscopy. TUNEL-positive cells stained in the green emission spectrum (515-565nm), while DAPI-positive cell nuclei stained in the blue emission spectrum (~460nm). Four fields of view (FOVs) of the periventricular white matter at 20x and two FOVs of the subcortical white matter surrounding the central sulcus at 20x were taken for each slide using Slidebook (Olympus Imaging). All slides analyzed for the study were “Section 5” slides, a designation used during the post-mortem procedures indicating that the tissue was sagittally cut at the level of the central sulcus. All slide pictures were saved in colour as well as black and white files for the individual filters. The black and white GFP and DAPI files were then analyzed separately using Slidebook software (Olympus Imaging). For each picture, the program parameters were adjusted such that each white entity represented a cell that had been stained for either GFP or DAPI, depending on the file. In this manner, the computer
counted the number of cells stained per FOV at 20x from individual files, and values were assigned to the individual animal and brain area after unblinding the slides.

IX. **Western Blot**

In order to determine the viability of our antibodies and to determine the approximate protein content of the fast-frozen tissues collected at the time of autopsy, a series of Western Blot protein assays were conducted. Shavings of fast frozen lung, cortex, subcortex and cerebellum were collected on ice and were subsequently subject to standard Western Blot protocol. Anti-IL-1β and IL6 antibodies were used at concentrations of 1:50, 1:200, and 1:500 in order to determine threshold, and various exposure times were tested at each of these antibody concentrations. β-actin was added to each gel as an internal control.

X. **Statistics**

All statistics were performed using GraphPad Prism v.5.00. Comparisons between two variables used a two-tailed T-test. For multivariate analysis, parametric data groups were analyzed with one-way ANOVA tests with a Tukey post-hoc test, while nonparametric tests were performed with Kruskal-Wallis tests with a Dunn’s post-hoc test.

**RESULTS**

Of the 30 fetuses instrumented, 18 were included in the final analysis for the groups, including 7 in Group 1 (cord occlusions), 6 in Group 2 (*E.coli* administration), and 5 in Group 3 (both treatments). Two of the six fetuses in Group 2 and four of the five fetuses in Group 3 showed microbiologic evidence of *E.coli* growth; first evidence of bacterial growth in the
amniotic fluid usually occurred at 18-24 hours post-inoculation. Ten of the twelve animals not included in final analysis were excluded due to fetal death or maternal morbidity, while the final two were designated Controls due to failure of experimental protocol (non-functional umbilical cord occluder, inability to access microbiology facilities).

There was no difference in the fetal body weights between the groups or between male and female fetuses (Figure 3-2). In addition, there was no difference in the % weight of the brain or lung between groups after correcting for fetal body weight (Figure 3-2). For the purpose of statistical analysis, we normalized the data to the baseline values of each animal in order to decrease biological variation if there was a significant difference (p<0.05) between groups at the D1S1 measurement.

I. Physiological Parameters

As shown in Figure 3-3, physiologic parameters measured in fetal blood varied among the three groups. In data profiles for each measurement in the experiment (not shown), fetal blood profiles showed great variation over the course of the 4 days. At the end of the sampling protocol, Group 1 showed the largest changes from baseline levels, with increased pCO₂ and lactate, and decreased pO₂ and pH. Groups 2 and 3 showed little change from baseline conditions despite fluctuations throughout the course of the experimental protocol.

As demonstrated in Figures 3-4 and 3-5, fetal arterial blood levels of glucose and lactate showed variation throughout the course of the experiment, but groups did not significantly differ in any of the parameters studied, most likely due to the high intra-group variation. The elevated lactate levels demonstrated in Group 1 towards the end of the experimental protocol reflects the fact that a greater number of these animals had deteriorating health as the experiment went on.
Figure 3-2: Representation of fetal weights (in kg, mean ± SEM), and % weights of fetal brain and lung with respect to fetal weight. There were no differences in any of the three measurements between the groups (p<0.05 for all, ANOVA with Tukey post-hoc).
Figure 3-3: Selected physiologic parameters measured in fetal blood over the course of the experimental protocols. Asterisk denotes significant difference from normalized baseline, p<0.05, ANOVA with Tukey post-hoc, data shown as mean ± SEM.
Figure 3-4: Fetal arterial serum levels of glucose throughout the course of the experimental protocol. Baseline levels of glucose in Group 2 were significantly higher than baseline levels of Groups 1 and 3 at time D1S1 (p<0.05, ANOVA with Tukey post-hoc). Data shown as mean ± SEM.
Figure 3-5: Fetal arterial serum levels of lactate throughout the course of the experimental protocol. Levels of lactate in Group 3 were significantly higher than levels of Groups 1 and 3 at time D5S2 (p<0.05, ANOVA with Tukey post-hoc). Data shown as mean ± SEM.
This occurred in all three groups, but is most markedly expressed in Group 1, and a larger sample size would most likely eradicate this phenomenon. The poor health of the Group 1 fetuses is further demonstrated in the sagittal vein Doppler profiles (Figure 3-6). This data has been normalized in order to account for a significant difference between groups at D1S1. Thus, the numbers on the graph assume a start value of zero at D1S1, with all subsequent values reflecting a change from that baseline of zero. Figure 3-6 shows a significant increase in the Group 1 sagittal Doppler values near the conclusion of the experiment, further demonstrating the relatively poor health of the Group 1 fetuses at that time. Similarly to the case of the lactate values, we would expect that this increase would be eradicated by greater sample size.

Because of the expectation of increased physiologic stress in the group exposed to both environmental stressors, we compared the responses to the UCOs in Groups 1 and 3. With the exception of fetal heart rate, there were no significant differences in any of the physiologic parameters studied during the course of the UCOs (Figure 3-7). With respect to fetal heart rate, Group 3 appeared to have greater difficulty regulating heart rate during and between occlusions, though no difference between Groups 1 and 3 was ultimately found in the measurements taken after recovery.

II. ELISA

Unfortunately, the ELISA protocol currently available for ovine sera was not adequately sensitive for many of our samples. IL-6 detection was particularly difficult, with few readable values found in the amniotic fluid, and virtually no readable values in the fetal arterial serum or fetal sagittal vein serum.

As shown in Figure 3-8, the levels of amniotic fluid IL-1β increased modestly from
Figure 3-6: Fetal sagittal vein Doppler profiles over the course of the experiment (x denotes significant difference between CO and CE, * denotes significant difference between EO and CE). Levels have been normalized to baseline due to significant differences in initial measurements between groups. Data shown as mean ± SEM.
Figure 3-7: Fetal heart rate in response to umbilical cord occlusions throughout the course of the occlusions for Groups 1 and 3 (* denotes significant difference between groups). Data is shown as mean ± SEM.
Figure 3-8: Time course of amniotic fluid IL-1β levels. Group 2 tended to have the most readable values, but there were no significant differences in IL-1β levels within or among the groups. Data is shown as mean ± SEM.
baseline levels at D1S1, but did not differ significantly from baseline or from the other two study groups. IL-6 levels in the amniotic fluid did not change at any time point in the experiment, though this may be due in part to the paucity of readable values (Figure 3-9).

Due to the lack of readings available for IL-6 in the fetal sagittal vein or arterial sera, this data is not shown. IL-1β levels in the fetal artery were measured at selected time points during the experiment, and the experimental groups showed no intra- or inter-group variation (Figure 3-10). Sagittal vein IL-1β levels seemed to be present in lower levels in Group 3 at selected time points in the experiment, but again there was no statistically significant intra- or inter-group variation (Figure 3-11).

III. Periventricular Brain Injury

In the periventricular white matter and the subcortical white matter near the central sulcus, a baseline level of approximately 2.5% cell death per field of view was found in the 2 fetuses designated as controls (Figure 3-12). Though levels of cell death tended to be greater in the three experimental groups, a significant increase in cell death was only found Group 2, the group that received only *E. coli*. This difference was significant with respect to both the control group and the Group 1. Group 3 showed an intermediate level of cell death that was not significantly increased from baseline due to high variation. In Groups 2 and 3, there was no difference in levels of white matter injury with respect to the presence or absence of microbiologic chorioamnionitis (data not shown).

Figure 3-13 shows representative immunofluorescence images of the white matter of Groups 1, 2 and 3. Group 1 showed very little white matter damage overall, confined mostly to the cells closest to the ependymal layer. Group 3 showed diffuse white matter damage, while
Figure 3-9: Time course of amniotic fluid IL-6 levels. There were no significant differences in IL-6 levels within or among the groups. Data is shown as mean ± SEM.
Figure 3-10: IL-1β levels in fetal arterial samples at selected times during the experimental protocol. There were no significant differences within or among groups. Data is shown as mean ± SEM.
Figure 3-11: IL-1β levels in fetal sagittal vein samples at selected times during the experimental protocol. There were no significant differences within or among groups. Data is shown as mean ± SEM.
Figure 3-12: Fetal white matter injury in the periventricular and subcortical white matter as determined by TUNEL staining (* denotes significant difference between Group 1 and Group 2, as well as Control and Group 2). Data is shown as mean ± SEM.
Figure 3-13: Representative images of immunofluorescence staining using TUNEL in the periventricular white matter. (A) Co-localization of DAPI nuclear staining (blue) and GFP TUNEL staining (green, 40x). (B) Additional layering of TRITC caspase staining (red) in addition to DAPI (blue) and TUNEL stain (green, 20x). (C) Representative periventricular area from a Group 1 (cord occlusion only) animal (20x). (D) Representative periventricular area from a Group 2 (E.coli only) animal (20x). Bars indicate 50μm.
Group 2 showed diffuse and focal damage. In some sections, blood vessels were caught in cross-section, and it was noted that the epithelial cells of blood vessels also stained for TUNEL, indicating cell death.

IV. Western Blot

Our rudimentary foray into protein assays indicated that our antibodies did work at the concentrations we had been utilizing, and that the fast-frozen cerebral tissues contained abundant proteins (Figure 3-14). We found, however, that fetal lung did not show immunoreactivity to the IL-1β antibody.

DISCUSSION

Chronic catheterization of the fetal sheep is a well-established model used to investigate fetal responses to environmental manipulations. Through the use of this robust, long gestation animal model, we were able to take repeated blood samples as well as monitor the fetal environment. Compared to other ovine preparation models, fetal mortality was higher than expected in this study, most likely due to loss from *E. coli* overgrowth in the amniotic fluid. However, *E. coli* colonization in the amniotic fluid was not present in all Group 2 and 3 animals. Though the bacterial solution was injected directly into the amniotic cavity, it is possible that some animals failed to show colonization of the bacteria due to persistence of antibiotic treatment from the post-operative period, or were otherwise able to clear the inoculate.

One key advantage to the use of the ovine preparation is the unresponsiveness of the myometrium upon incision into the uterine walls, particularly with respect to primate uterine
Figure 3-14: Representative Western blots before β-actin addition after (A) 30 minutes and (B) 10 minutes of exposure. Lanes 1, 2, 5 and 6 contain lung tissue, while lanes 3, 4, 7 and 8 contain cerebral cortex. The band at 24kDa is the IL-6 marker, while IL-1β is detected at 29kDa in sheep. Note that IL-1β does not appear to be present in lung tissue, while IL-6 appears to be found in both tissue types.
In comparison to the human or guinea pig, the ovine placenta has a much thicker barrier separating maternal and fetal circulations. We avoided the problem of placental diffusion of bacteria or inflammatory agents in this case by injecting the bacterial broth directly into the amniotic fluid sac, thereby bypassing any impediment the placenta may have caused. The use of bacteria rather than endotoxin injection was favoured in this case due to the extreme sensitivity of the ovine fetus to \textit{E.coli} endotoxin, in addition to more closely mimicking the clinical case of chorioamnionitis.

The objective of our study was to determine if chorioamnionitis in the presence or absence of hypoxia resulted in metabolic and cardiovascular changes in the fetal sheep. We did not find that fetuses exposed to both intra-amniotic infection and umbilical cord occlusions performed poorly as measured by physiological parameters such as arterial blood pH and sagittal vein Doppler velocity, though we did find increased levels of white matter injury in Group 2, the fetuses exposed to \textit{E.coli} alone.

After hypoxic-ischemic insult to the fetus, the brain may be protected from damage by activation of compensatory mechanisms, during which neuronal mitochondria buffer intracellular calcium uptake and receptor antagonists block NMDA and AMPA receptors from allowing further influx of calcium. It has been proposed that hypoxic damage disrupts both neuronal membrane integrity and “secondary energy failure,” a process in which the failure of the fetal brain to overcome initial insult leads to academia, prolonged decreases in energy availability, and eventual cell death. In addition, the presence of proinflammatory cytokines may weaken the blood brain barrier in the fetus (or themselves may cross it), allowing greater infiltration by macrophages, which may then activate microglia and in turn produce even more cytokines.
The threshold at which brain injury occurs after hypoxic challenges may be quite high, however, as instances of nuchal cord occur frequently in pregnancy with very few instances of long-lasting ischemic damage.\textsuperscript{11, 22, 23}

We noted that our results tended to show a cyclicity in the parameters studied over the course of the experiment, which supports the suggestion that persistent or recurrent episodes of stress may be the primary cause of fetal compromise.\textsuperscript{21} Previous studies in the ovine fetus found increased pCO\textsubscript{2}, mean arterial pressure, and fetal heart rate, and decreased O\textsubscript{2} content/saturation and pH on the first day of exposure to endotoxin, with recovery to baseline values thereafter.\textsuperscript{14, 18} Likewise, we found an altered ability of our Group 3 animals in heart rate regulation during the course of the UCO protocol with respect to the animals that had not received \textit{E.coli}, though levels returned to baseline after recovery. This is in agreement with data obtained by Peebles \textit{et al.}, who did not see any lasting differences in fetal heart rate after endotoxin administration.\textsuperscript{5}

The time at which the experiments were performed, at approximately 85\% gestation, is a time when human pregnancies often experience variable-type fetal heart rate decelerations.\textsuperscript{9} Also in agreement with studies by Peebles \textit{et al.}, the sagittal blood flow was maintained in our experiments, despite speculation that inflammatory processes and hypoxic episodes would lead to vasodilation and hypoperfusion of the brain.\textsuperscript{5}

We noted fetal brain damage in the subcortical and periventricular white matter, indicating widespread neural effects after fetal challenges such as chorioamnionitis. It has previously been shown that even neurons distant from the site of greatest white matter injury can show signs of cellular stress.\textsuperscript{14} Another study has shown marked changes in cellular structure and function upon electron microscopy of the fetal brain after LPS administration, including
evidence of apoptosis, phagocytosis, and chromatin condensation. In our study, we noted in several slides that when a blood vessel was caught in cross-section, the epithelial cells lining the vessel were often TUNEL-positive. This observation, while not quantitative, supports the theory that inflammatory mediators are linked with intraventricular hemorrhage and cell death in the brain due to vessel compromise and subsequent leakage of blood into the ventricles.

In cases of cross-tolerance, an individual is exposed to different insults with effects on similar organ systems or tissues and experiences diminished damage due to pre-stimulation of protective mechanisms. Initial analysis in our study indicates that exposure to *E. coli* 24 hours before UCOs does not appear to be detrimental to the fetus, in that the Doppler velocity readings and blood gas profiles remain stable. This contrasts with the heightened Doppler velocity values found in Group 1 and the slowing of Doppler velocity found at the end of the experiment for Group 2. Previous studies concur that a time lag is necessary in cases of preconditioning, suggesting that this indicates protein synthesis is necessary for the ability of the organism to strive after subsequent insult. Though the pre-treatment with *E. coli* before UCOs may not be detrimental in this case, it has been noted that the compensatory mechanisms responsible for this preconditioning may be overwhelmed with repeated exposure to similar insults.

It was expected that the CO group would function as a sort of control in terms of brain injury, as previous studies have found that acute but limited episodes of fetal hypoxia in the sheep model are generally well tolerated and do not result in marked levels of brain injury, though they may result in some protein alterations and tissue remodelling. In addition, proinflammatory cytokines in cases of hypoxic ischemia are somewhat balanced by the release of anti-inflammatory cytokines after this insult, while these same anti-inflammatory cytokines fail
to increase after a bacterial insult. Although previous studies have indicated that a single dose of bacterial endotoxin does not cause significant fetal brain injury, we expected moderate levels of fetal brain injury to be present in this study after *E. coli* injection as the bacteria remained free to colonize throughout the course of the experiment, thus continuing to cause immune stimulation and possible brain injury in this model. In addition, constant LPS infusion over an extended period has been shown to cause histologic chorioamnionitis and increased infiltration of activated microglia and macrophages into the fetal brain, primarily the subcortical white matter.

A link between prenatal insults such as histologic chorioamnionitis and bronchopulmonary dysplasia in infants has been established. It is therefore interesting to note the lack of cytokine immunoresponsiveness in the fast-frozen fetal lungs using the Western blot procedure. While the inflammatory response after chorioamnionitis tends to cause increased proinflammatory cytokines in many fetal organs, we speculate that IL-1β may not be the key mediator of subsequent lung damage leading to respiratory problems in the neonate.

**CONCLUSION**

In this study, we were able to chronically instrument the ovine fetus such that we were subsequently able to manipulate the fetal environment with either bacteria, hypoxic episodes, or both insults. By using the ovine model, we were able to take serial samples throughout the course of the experiment in addition to fetal tissues at the conclusion of the experiment. The data collected in this experiment to date indicate high variability and/or cyclicity in the levels of physiologic parameters during the course of fetal challenges, with brain injury following
bacterial exposure. It is interesting to note that the fetus exposed to bacteria and UCOs was less adept at heart rate regulation, but showed non-significant levels of white matter injury on post-mortem analysis. This leads us to question the influence of fetal circulatory regulation in the case of gestational insults, though variation in this study may also be due to relatively small sample sizes. Our data indicate a neutral or slightly protective effect of previous bacterial inoculation in the face of subsequent UCOs, adding credence to the theory that the fetus exposed to one challenge may actually be better able to respond to subsequent challenges.


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CHAPTER 4: EFFECT OF MATERNALLY-INFUSED LPS OR PROINFLAMMATORY CYTOKINES ON CIRCULATING CYTOKINE LEVELS IN THE FETUS USING THE IN VITRO HUMAN PLACENTAL PERFUSION SET-UP

Lindsay A Patrick 1, Laura Moore 1, Anne Farley 1 and Graeme N Smith 1,2.

1 Department of Anatomy and Cell Biology, Queen’s University, and 2 Department of Obstetrics and Gynecology, Kingston General Hospital.

INTRODUCTION

It is accepted that there is a link between intrauterine infection and white matter injury in the fetus. In the human case, it is known that the most frequent method by which infectious agents gain access to the fetus is through ascension from the vagina. 2 Placental and umbilical inflammation have been linked to an increased risk of PVL in the infant. 1,3 In preterm infants, intraventricular hemorrhage and PVL, both of which are linked to CP development, are present much more frequently in infants whose placentas and membranes showed evidence of inflammation. 4 In addition, increased levels of LPS and/or proinflammatory cytokines are associated with an increased risk of preterm labour/delivery and hypoxic ischemia systemically and more importantly, cerebrally. 5 The addition of the risks of preterm delivery and cerebral hypoxic ischemia further increases the challenges to fetal brain development.

A previous guinea pig model of chorioamnionitis in our laboratory demonstrated that exposure to an induced intrauterine infection using E.coli inoculum induces increased levels of proinflammatory cytokines in both the amniotic fluid and maternal blood, regardless of
subsequent growth of the bacteria in the amniotic fluid. This increase in cytokines was accompanied by an increased incidence of subcortical and periventricular white matter damage, which in humans most often is associated with cerebral palsy. A further study in our lab was performed, and demonstrated that the presence of the \textit{E. coli} inoculum itself was unnecessary in order to produce increased cytokines and brain injury, as a maternal injection of lipopolysaccharide (LPS) also induced increased levels of cytokines and fetal brain injury (see Chapter 2).

Despite this link between exposure to infectious agents, increased cytokines and fetal brain injury, it is as yet unknown what role the placenta has in the inflammatory process. Particularly, it is unknown if endotoxin and maternal cytokines cross the placenta and/or the placenta is a source of cytokine production that can lead to elevated levels in the fetus and subsequent brain injury. Trophoblast cells can produce proinflammatory cytokines in response to an inflammatory process, meaning that either the bacterial presence or the presence of the cytokines can initiate further cytokine production by the placenta. In a recent \textit{in vitro} study by Ma \textit{et al.}, they found that both trophoblasts and subsequently differentiated syncytiotrophoblast cultures showed a dose-dependent proinflammatory response to stimulation with LPS. In addition, this same group noted that placental tissues differentially regulated particular cytokines after LPS exposure, with the greatest levels of production occurring for the cytokines TNF-\(\alpha\), IL-6 and IL-10.

Studies investigating the passage of proinflammatory cytokines or endotoxin from maternal to fetal circulation have had mixed results, with most indicating an inability of LPS or cytokines to cross the placenta, or the ability to cross only when present on the maternal side in large quantities. While one study using the \textit{in vitro} human placental set-up indicated that
cytokines cannot transfer across the placenta, another found bidirectional transfer of IL-6. The latter of the two studies, however, used a closed system of perfusion, meaning solutions were continuously recirculated through the system, which may affect the results.

The human placenta, termed villous haemo-monochorial, is composed of two layers of fetal cells between the fetal and maternal circulations in late gestation: a trophoblast cell layer and one syncytiotrophoblast layer. This barrier acts as a sieve in order to allow gases and nutrients through from maternal to fetal circulations, but blocks large molecules and plasma proteins, except those for which specific transport proteins exist (e.g. glucose, immunoglobulins, amino acids).

In addition to the maternal-fetal transport, the fetus transfers used materials and other products back to the maternal system. Because the fetally-derived syncytiotrophoblast layer is in direct contact with the maternal system, fetal trophoblast cells are often found circulating in the maternal bloodstream. For this reason, there is ongoing research into fetal markers of inflammation that may be easily detectable in maternal blood.

**OBJECTIVE**

It is known that the placenta can produce proinflammatory cytokines in response to inflammation or bacterial challenge. However, it is unknown to what extent maternal circulating cytokines and bacterial products (e.g. endotoxin) can cross the human placenta and/or stimulate placental production of cytokines. Most studies to date have utilized animal models of LPS without determining the ability of LPS to cross the placenta. This study was undertaken in order to determine what contribution the maternal system makes to cytokines and endotoxin in fetal
circulation.

We hypothesize that the lipopolysaccharide (LPS) will stimulate production of proinflammatory cytokines by the placenta such that circulating fetal levels will increase from their baseline levels. We further expect that proinflammatory cytokines TNF-\(\alpha\), IL-1\(\beta\) and IL-6, injected on the maternal side of the placenta, will subsequently be increased on the fetal side.

**MATERIALS AND METHODS**

All experiments were performed on term human placentas obtained from elective Caesarean sections at Kingston General Hospital, which were transported on ice and perfused within 30 minutes of delivery. Preparation of fetal and maternal perfusion media and the precise methodology of the *in vitro* human placental perfusion setup has been previously described. Briefly, a matched peripheral chorionic artery and vein pair for a single cotyledon were canulated; the fetal side was then placed facing downwards suspended in saline in the perfusion apparatus. Two catheters were pierced into the intervillous space of the maternal side in order to deliver maternal perfusate solution, while a third catheter collected the venous effluent on the maternal side.

The fetal arterial line was connected to a Powerlab transducer that continually recorded pressure in the line, thus allowing us to monitor the flow of the perfusate and any variation in vessel dilation. The fetal perfusate was bubbled with 95% nitrogen to simulate the relatively hypoxic conditions of the fetal circulation, while the maternal perfusate was bubbled with 21% oxygen. Maternal arterial flow was maintained at 1.7mL/minute, while fetal flow was maintained at 2.9mL/min. The perfusion system was an open system, in that perfusate solutions were not recycled. Before the experiment was begun, it was necessary to ensure the cotyledon was
properly canulated and was being perfused by the maternal arterial lines. This was achieved by measuring oxygen content in the fetal artery and vein. Once the fetal vein oxygen concentration had an increase of approximately 20-40mmHg over fetal arterial levels, it was established that the oxygenation in the fetal vein was being delivered from the maternal arterial line, and our set-up was perfusing correctly. Once perfusion matching had occurred and pressure in the fetal system was stable, samples of each of the four lines (maternal and fetal arterial and venous) were taken every 5 minutes until 15 minutes in order to establish baseline conditions.

Each placenta received a bolus dose of either *E.coli*-derived LPS, TNF-α, IL-1β or IL-6 in the maternal arterial line. *E.coli*-derived LPS (O55:B5, Sigma) was diluted into aliquots such that each cotyledon would receive an approximate dose of 500µg/kg. This was the dose used in our previous guinea pig study and was modeled after a study of LPS injection in rats to cause neonatal brain injury. Estimating the average weight of a cotyledon at 40g, we therefore injected bolus doses of 20µg LPS during the experimental protocol for that set of experiments. Cytokine doses to be used were determined by analyzing information from previous human studies on amniotic fluid, maternal and fetal serum cytokine levels in control cases as compared to cases in which the infant had chorioamnionitis or periventricular leukomalacia. In the case of TNF-α, the literature studied found an average 11pg/mL concentration in control cases as compared to an average 72pg/mL concentration in non-control cases. In the case of IL1β, previous studies had found an average 10pg/mL concentration in control cases as compared with 360pg/mL in non-controls. The literature found an average 1pg/mL concentration in controls, compared with 66pg/mL in non-controls for IL-6. We thus injected a bolus dose of 100pg TNF-α, 400pg IL-1β, or 100pg IL-6.

The bolus dose of either LPS or cytokine was added after 15 minutes of baseline
measurements, followed by an immediate sampling of the 4 lines annotated as time zero. Samples were taken every 5 minutes up to the 15 minute point, then every 15 minutes up to the two hour point, at which point the experiment was stopped. The perfused cotyledon was weighed in order to determine the exact concentration per unit weight of LPS or cytokine the placenta had received.

Samples of maternal and fetal arterial and venous effluents were assayed using specific human ELISA kits from R&D Systems for TNF-α, IL-1β and IL-6. Levels of LPS in the effluents were quantified using the limulus amoebocyte lysate (LAL) kit (QCL-1000, Cedarlane Laboratories).

Data were analyzed using two-tailed t-tests and one-way ANOVA with Tukey post-hoc. Statistics were performed using GraphPad Prism 5.00 software.

RESULTS

The average cotyledon weight for all experiments (n=40) was 25.32±2.115g. When analyzed in separate treatment groups, there were no differences in the average cotyledon weights (ANOVA with Tukey post-hoc, p<0.05). The pressures measured in the fetal line of placentas did not change over the course of the experiment for any treatment, or within treatment protocols (Figure 4-1).

I. **20µg LPS bolus dose:**

Assays for IL-1β and IL-6 indicated fetal venous levels to be significantly lower than maternal venous levels at almost all time points measured; fetal levels were 10-50% of the
Figure 4-1: Perfusion pressures measured during the course of the placental perfusion experiments. N=10 placental perfusions for each bolus dose of LPS or cytokine. Pressures remained relatively stable within the 2 hour experimental window, and there were no significant differences in pressures among experimental protocols. Data is shown as mean ± SEM.
concentration of maternal levels. As shown in Figure 4-2, however, IL-1β levels in the fetal venous effluent tended to mimic the cyclic patterns in the maternal venous effluent, though on a much smaller concentration scale. In contrast, IL-6 in the maternal and fetal venous lines remained relatively steady throughout the experiment, though fetal concentrations of IL-6 showed some variation (Figure 4-3). TNF-α levels in maternal and fetal venous effluents did not demonstrate the same characteristics; maternal venous levels only increased from baseline at t=120 min, and maternal and fetal venous concentrations of TNF-α were similar in magnitude (Figure 4-4).

Several arterial samples were also assayed in order to confirm low levels of cytokine in perfusate lines. As shown in Figure 4-5, maternal and fetal venous levels of IL-1β were elevated from their respective arterial levels. Only maternal venous levels were significantly increased from arterial levels in the case of IL-6.

LPS injected into the maternal line was found in the maternal and fetal venous effluent samples taken immediately after LPS had been injected into the placenta, and in the maternal venous effluent ten minutes after the injection (Figure 4-6). Fetal venous samples at subsequent times were not statistically different from the baseline fetal arterial levels (two-tailed t-test, p<0.05).

The maternal venous effluent samples measured at time zero (MV4) and ten minutes into the experiment (MV6) showed significantly higher levels of LPS than the arterial maternal levels, (ANOVA with Tukey post-hoc, p<0.05). Fetal venous effluents measured at t=15 and 30 minutes (FV7 and FV8) were not significantly elevated from arterial levels. MA and FA n=2, MV n=5, FV n=6.
Figure 4-2: Cytokine IL-1β levels in maternal and fetal venous effluents after a bolus dose of LPS at time zero. Logarithmic scale was used in order to demonstrate the similarity in cytokine trends. Asterisks indicate times at which maternal or fetal venous IL-1β levels were significantly different than baseline levels (two-tailed t-test, p<0.05).
Figure 4-3: Cytokine IL-6 levels in maternal and fetal venous effluents after a bolus dose of LPS at time zero. Logarithmic scale was used in order to demonstrate cytokine trends. There were no significant changes from baseline levels in either maternal or venous lines.
Figure 4-4: Cytokine TNF-α levels in maternal and fetal venous effluents after a bolus dose of LPS at time zero. Logarithmic scale was used in order to demonstrate cytokine trends. Levels of TNF-α were increased from baseline in the maternal venous effluent (two-tailed t-test, p<0.05).
Figure 4-5: Comparative assay levels of IL-1β and IL-6 at t=5 minutes after LPS bolus injection into the maternal arterial line. There were significant increases in the levels of both cytokines in the maternal venous line when compared to arterial levels, but a significant increase in IL-6 was observed in the maternal venous line only.
Figure 4-6: Limulus amoebocyte assay results for detection of LPS in selected maternal and fetal arterial and venous effluents after a bolus dose of 20µg LPS was injected into the maternal arterial line. The fetal venous effluent measured immediately after LPS addition was significantly elevated from fetal arterial levels at the same time point (two-tailed t-test, p<0.05). The maternal venous effluent samples measured at time zero (MV4) and ten minutes into the experiment (MV6) showed significantly higher levels of LPS than the arterial maternal levels, (ANOVA with Tukey post-hoc, p<0.05). Fetal venous effluents measured at t=15 and 30 minutes (FV7 and FV8) were not significantly elevated from arterial levels. MA and FA n=2, MV n=5, FV n=6. Data is shown as mean ± SEM.
II. 400pg IL-1β bolus dose:

As shown in Figure 4-7(A), we found almost universally elevated levels of IL-1β in the maternal arterial and venous lines 15 minutes after IL-1β was injected into the maternal arterial line. These levels were significantly higher than the fetal arterial and venous concentrations of IL-1β (ANOVA with Tukey post-hoc, p<0.05). Fetal venous levels of IL-1β were not significantly elevated from baseline (fetal arterial) levels (ANOVA with Tukey post-hoc, p<0.05).

In the IL-1β-dosed placentas, levels of fetal venous cytokine were approximately half of the maternal venous levels throughout the experiment. Figure 4-7(B) shows a similar pattern as was found in the IL-1β profiles after LPS bolus dosing, in that the fetal venous IL-1β levels tend to mimic the maternal venous levels, though on a smaller scale. Fetal venous levels of TNF-α and IL-6 remained relatively stable throughout the experiment after a bolus dose of IL-1β, though small increases in their concentrations occurred at 5 and 15 minutes (respectively) after the bolus dose was injected into the maternal line (Figure 4-8).

III. 100pg TNF-α bolus dose:

The maternal venous effluent showed a 1200% increase in TNF-α levels (from baseline) 5 minutes after the TNF-α bolus dose, most likely indicating pooling of the added cytokine on the maternal side. There was otherwise little variation in the maternal venous TNF-α levels, which remained 20 to 30-fold higher than fetal venous concentrations (data not shown). As seen in Figure 4-9(A), levels of all three cytokines remained relatively stable throughout the course of the experiment, with no significant variation due to TNF-α bolus (ANOVA with Tukey post-hoc, p<0.05).
Figure 4-7: Cytokine assays for IL-1β detection after bolus dosing with IL-1β. (A) Representative values of arterial and venous line IL-1β at t=15 minutes after cytokine bolus was injected into maternal arterial line. Maternal arterial samples demonstrated almost universally high levels of cytokine, and maternal artery and vein samples were significantly higher than levels of cytokine in the fetal artery and vein samples (ANOVA with Tukey post-hoc, p<0.05). A modest but non-significant increase was observed in the fetal venous samples compared to fetal arterial samples. (B) Time course of IL-1β levels after a maternal arterial bolus dose of IL-1β. Note the similarity in venous effluent cytokine levels. Asterisks indicate venous lines significantly increased over baseline levels (two-tailed t-tests, p<0.05). Data shown as mean ± SEM.
Figure 4-8: Concentrations of all cytokines after a bolus dose of IL-1β into the maternal arterial line. Note that transient non-significant increases in IL-6 and TNF-α occur in the fetal venous effluent at 5 and 15 minutes after the bolus dose of IL-1β. Data is shown as mean ± SEM.
Figure 4-9: Fetal venous cytokine profiles after (A) maternal TNF-α bolus dose, and (B) maternal IL-6 bolus dose. There was high variation in IL-1β values in both experiments, but levels of this cytokine remained lower than the stimulated levels after IL-1β bolus dose.
IV. 100pg IL-6 bolus dose:

IL-6 levels in the maternal and fetal venous lines remained relatively stable throughout the course of the experiment in which a bolus dose of IL-6 was administered, though statistically insignificant peaks of IL-6 occurred in the fetal venous line at 30 and 90 minutes after IL-6 injection (ANOVA with Tukey post-hoc, p<0.05). Maternal levels of IL-6 were approximately 20-fold greater than fetal levels, and venous levels did not show significant variation at any of the time points assayed (data not shown, ANOVA with Tukey post-hoc, p<0.05). As shown in Figure 4-9(B), none of the three cytokines measured in the fetal venous circulation after an IL-6 bolus dose showed substantial variation over the course of the experiment. TNF-α levels in the fetal venous effluent were almost universally too low to be detected by the ELISA, and the lowest threshold value for the assay kit was therefore ascribed to these samples (0.12pg/mL).

Overall cytokine trends:

IL-1β levels in LPS-dosed placentas appear to be cyclic, increasing in the maternal venous effluent and the fetal venous effluent in turn, but TNF-α showed little variation in response to this treatment. IL-6 profiles indicated insignificant variability in the fetal venous line. Increases in the levels of fetal venous IL-1β from baseline were apparent 60 and 120 minutes after LPS infusion, while maternal venous levels indicated a more rapid IL-1β response.

IL-1β levels in the fetal venous effluent appear to mimic maternal venous levels after a bolus dose of IL-1β as well. IL-1β in the maternal and fetal venous lines increased from their respective baseline levels at time points within 30 minutes of cytokine infusion.

Placentas dosed with TNF-α and IL-6 showed little discernable response in any of their cytokine concentration profiles. Maternal venous effluent TNF-α showed a large peak in TNF-α
levels 5 minutes after the bolus doses were administered, likely indicating nearly immediate pooling of the cytokine in the maternal venous system.

**DISCUSSION**

Where other studies found the ability of IL-6 to cross the placenta bidirectionally or the inability for any cytokines to cross, we saw an increase in IL-1β in the fetal venous line after maternal arterial injection of the cytokine. Our studies also showed an increase in IL-1β in fetal venous effluents after a bolus dose of LPS to the maternal artery. This contrasts with previous reports of IL-6 as the most actively-secreted cytokine after endotoxin exposure. It is our conclusion that stimulation by IL-1β or endotoxin causes proportional presence of IL-1β on the fetal side. We cannot definitively say that IL-1β crosses the placenta; radiolabelling studies must be performed to determine the origin of the cytokine in the fetal venous effluent.

The potential for IL-1β to cause significant damage upon arrival in the fetal compartment is great. IL-1β is known to be a potent early-stage stimulator of proinflammatory responses. TNF, other interleukins, adhesion molecules and iNOS are all known to be upregulated by IL-1. IL-1β is also known to have a wide variety of direct detrimental effects on the fetus, including increased permeability of the BBB.

Our LAL assay data indicates the ability of LPS to cross the placenta upon injection into the maternal arterial line in a bolus dose. This data, however, is based on very small sample sizes, and further research into the transplacental passage of LPS should be considered. In a study of radiolabelled LPS into the pregnant rat, autoradiography demonstrated strong presence of the LPS in the maternal organs, the placenta, and tissue surrounding the amniotic sacs within 2
hours of LPS injection. This same study, however, was unable to find detectable levels of LPS using autoradiography in the fetus, and Gamma counting indicated negligible levels of radiation in some fetal tissues. Similar radiolabelling studies using the in vitro human placental perfusion set-up would complement data from the LAL assay.

In the case of both interleukins, fetal venous levels were significantly lower than maternal venous levels. This is most likely because maternal blood, upon coming in contact with the placenta, gives nutrients and gases to the placental tissue itself before these materials are passed on to the developing fetus. In this study, we are unable to conclude whether it is placental blockage or break-down of the materials that is occurring; evaluation of placental tissue for cytokine presence or mRNA upregulation for proinflammatory mediators would supplement the data obtained here.

Trophoblastic over-production of cytokines and compounds with effects on vasculature is known to occur in chorioamnionitis as well as other diseases of pregnancy, such as pre-eclampsia. A marked increase in proinflammatory cytokines also occurs at the onset of labour in normal pregnancies, the bulk of which are likely produced by the placenta. Numerous clinical and laboratory studies have characterized the association between increased proinflammatory cytokines and adverse outcomes (e.g. preterm labour, chorioamnionitis), though research has not shown a strong predictive value of maternal serum cytokines for diagnostic purposes. Knowledge of the ability of cytokines to cross the placenta or stimulate production of cytokines and other molecular markers will allow further elucidation of the mechanisms by which a maternal inflammatory response leads to fetal injury.

Significant attenuation of placental production of the proinflammatory cytokine IL-6 has
been found in cases where the antioxidant N-acetyl-cysteine is injected before and/or after LPS exposure, suggesting a possible role of antioxidants in stemming the proinflammatory cascade at the placental level. In vitro studies indicate that both control and LPS-treated placental tissues respond to anti-inflammatory steroids such as dexamethasone by reducing their proinflammatory cytokine output, but there are many other effects of steroid treatment that must be weighed against this possible treatment avenue.

CONCLUSION

This study indicated an inability of some cytokines to cross the placenta or induce a notable response in the fetal compartment. This study also found, however, that IL-1β presence in the maternal vasculature induces a proportional presence of IL-1β on the fetal side. Our data also found IL-1β profiles to be the most varied following a maternal dose of LPS. The highly potent nature of IL-1β as a proinflammatory mediator, coupled with the data obtained in this experiment, necessitate further study into the wide-ranging detrimental effects to the fetus after IL-1β presence is increased. Further research should also investigate the ability of LPS to cross the placenta, as well as the long-term (non-bolus) responses to inflammatory triggers.


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CHAPTER 5: POSTNATAL EFFECTS OF INTRAUTERINE EXPOSURE TO EXPERIMENTAL CHORIOAMNIONITIS IN THE GUINEA PIG

Lindsay A Patrick, Erica Harnett, Anne Berndl, Anne Farley and Graeme N Smith.

1 Department of Anatomy and Cell Biology, Queen’s University, and
2 Department of Obstetrics and Gynecology, Kingston General Hospital.

INTRODUCTION

It is widely accepted that infection during gestation can induce a proinflammatory cytokine release, inducing an inflammatory cascade that can lead to preterm birth and white matter injury characteristic of neurodevelopmental disorders such as cerebral palsy (CP). There are many manifestations of CP, depending on the limb(s) affected and the level of movement ability in the limbs and in the body as a whole. These manifestations depend on the location and severity of the causative white matter lesion. For instance, diplegic CP refers to two affected limbs, and the spastic form of CP generally involves an “excess of muscular action.” Within the subcategories of CP, some generalizations in movement characteristics can be made. For instance, it has been noted that most movements in spastic CP patients are slower, involve more muscles and positions than in a normal movement, and involve less planning before the movement is undertaken. The slower nature of the movements is thought to be an adaptive response to the disability, allowing similar end results (e.g. catching a ball, avoiding an obstacle) by beginning the pattern of movement sooner than those without a disability.

While the brain injury causing CP may be extensive enough that it also affects other areas
of function, a recent study found a relatively low incidence of behaviour problems in young adults with the disorder. In contrast, a large proportion of children and adolescents with CP have been reported to have intellectual or cognitive difficulties, though these deficits range widely in nature and scope. When a diagnosis of clinical chorioamnionitis has been made, 35% of infants <2000g and 22% of infants >2000g are later diagnosed with CP or exhibit symptoms of delayed development. In addition, a recent study found that the Revised Pre-screening Developmental Questionnaire scores for infants born <32 weeks gestation without evidence of chorioamnionitis were significantly lower at the age of one year than those born at term (P<0.001).

The Gross Motor Function Measure (GMFM) uses assessments of various motor activities and postures in order to determine motor function and its changes over time. This test can be used to assess high and low functioning subjects alike in order to give an objective score or rank. For instance, a recent study found children with CP who were high functioning (primarily hemi- and diplegic CP) had an average GMFM of 86.5±23.3, while children with more severe CP manifestations (primarily diplegic and quadriplegic CP) had an average GMFM of 39.77±34.4.

While many studies have revealed increased activation of proinflammatory cytokines and apoptosis in the brain of subjects exposed to infection in utero, few prospective studies have been performed in which the neonatal outcome of prenatally-induced infection is quantified. Previously, we established a guinea pig model whereby chorioamnionitis, accompanied by an increase in amniotic fluid proinflammatory cytokines, leads to increased levels of fetal white matter damage. The current study was undertaken as an extension of the established fetal model in order to determine the extent to which the brain injury accompanying prenatal exposure
to infection leads to demonstrable behavioural characteristics. Establishment of a reliable behavioural model can thus be utilized in the development of therapeutics for the prevention of fetal brain injury through the proposed mechanism. It is expected that treatments for the disruption of the pathway leading from chorioamnionitis to fetal brain injury will lead to decreased levels of white matter damage accompanied by improved behavioural characteristics.

METHODS

Animals:

Animal use was approved by the Queen’s University Animal Care Committee. Dunkin-Hartley guinea pigs were obtained from Charles River Canada. For breeding purposes, three females and one male were placed in a cage together for approximately 2 weeks, the length of the guinea pig estrous cycle. Females were subsequently housed in groups of 3 without a male present. Animals were provided with food and water ad libitum.

Bacterial Inoculation:

Human E.coli isolate was incubated in tryptic soy broth, and then diluted to $10^8$ colony-forming units (CFU) using sterile saline. Serial dilutions were performed to $10^1$ CFU, and 10µL of each dilution was plated on blood agar to determine accuracy of dilutions and integrity of bacterial strain. Because growth on plates could not be determined for 24 hours, calculations of dose for each intracervical inoculation were based on the most recent plating results. It is for this reason that inoculations ranged from 1000-2500 CFU, as dilution precision or bacterial growth varied.
Maternal guinea pigs were intracervically inoculated with 1000-2500 CFU *E. coli* at approximately 70-75% gestation (day 45-50 of 65 day gestation). Guinea pigs were anaesthetized during the inoculation procedure using the 5% Isoflurane induction box and maintenance at 4% by mask. A nasal speculum and hysteroscope were used in order to visualize the cervix, thus ensuring that the bacterial solution was inoculated past the cervix for spread into the uterus. Control guinea pigs were anaesthetized but not inoculated, since inoculation with even sterile saline could have introduced regular vaginal flora past the cervix. Maternal guinea pigs were monitored until anaesthetic wore off, and returned to individual cages. Maternal guinea pigs were then allowed to carry fetuses to term, deliver, and wean pups for approximately 20 days postnatal.

**Behavioural Monitor Testing:**

For behavioural monitor testing, pups were kept at the Botterell Hall facility but were housed as litters without the maternal animal after 20 days of age. The ActiMot Motility Monitor (TSE Systems, Germany) apparatus is described fully in Appendix II, but is described briefly here. The behavioural testing apparatus consisted of a 92x92cm enclosed box with 32x32 infrared sensors, connected to ActiMot software on a nearby computer. An additional strip of sensors was affixed to a slightly higher portion of the monitor box in order to record movement in the z axis in addition to the x and y axes. This upper strip was adjusted to account for animal height in order to ensure normal movement did not interrupt the upper beam. Any movement by the animal disrupted beams in the x, y and/or z axes, and the ActiMot software interpreted this beam interruption as animal presence and movement. The software was checked each day to set parameters for time of trial and demarcation of the “center area” of the monitoring box.
Trials were performed by guinea pigs at 50 and 65 days of age, individually, and in 60-minute segments. Trials took place in a darkened room, in order to eliminate extraneous distractions. Computer analysis captured all movements and performed spatial analysis so no operator bias existed and no human distraction was present during trials. Behavioural parameters analyzed included time moving, distance traveled, rearing, rotations, time spent in center, and hyperactivity.

Morris Water Maze Testing:

At 50 and 65 days after birth, control and infection-exposed pups were subjected to spatial and memory behaviour testing using a Morris Water Maze protocol coupled with the ActiMot spatial analysis system (TSE Systems, Germany). This equipment is fully described in Appendix II, but is described in brief here.

A specialized video camera was mounted on the ceiling above a 6-foot diameter, 4-foot deep basin, which was subsequently filled almost entirely with water. The camera was connected to a computer that had VideoMot system software installed, such that the area recorded by the camera was defined and split into segments for analysis based on user-defined parameters. Software recording of each animal trial was recorded using an attached VCR, and tracks recorded by the software were saved for further analysis. A hidden Plexiglass platform was sunk into the pool, with 1.5-2 inches water covering the platform surface, a 30x30cm square.

Several static objects in the room (cages, cords on wall, net on wall) acted as spatial orientation cues for the animals, such that points of reference were available. At the beginning of each trial day, the pool area as a whole and the individual quadrants and platform we used for analysis were redefined in order to account for slight movements, and lighting was readjusted in
order to eliminate glare while maintaining video and monitoring quality. Two research team members were present for most testing, such that an animal handler and computer operator were able to perform separately. On days when only one researcher was present, the VideoMot’s remote start/stop button was utilized in order for the animal handler to control the timing of the software recording.

Each day, four 60-second (maximum) tests were performed in which the animal was placed into the water pool with each of the four quadrants as a starting point. Animals were placed onto the hidden platform (always located in Quadrant 1) at the beginning of the day, and again at the end of each trial where the animal did not find the platform. Each trial was stopped immediately once the animal mounted or tried repeatedly to mount the hidden platform. Trials were a maximum of 60 seconds as guinea pigs tended to become too tired to properly swim after 90-120 seconds. A minimum of 5 minutes rest was given to each animal between their trials each day. Seven days of these tests were performed on each guinea pig pup at 30-36 days of age and/or 50-56 days of age, and results were analyzed using measures of learning and retention abilities.

Analysis:

Pups were euthanized with 2mL Euthanyl (1mL intraperitoneal, followed by 1mL intracardiac) within 24 hours of the behavioural analysis on postnatal day 65. Pups were then perfused through the aorta with 4% paraformaldehyde, and brains were removed and preserved in 4% paraformaldehyde at 4°C until sectioning. After sectioning perfused brains into 4mm segments and embedding in paraffin, blocks were cut for slides. Slides were immunostained using NeuroTacs stain for detection of cell death (R&D Systems, Minneapolis). Computer
imaging with MCID software (GE Healthcare Niagara Inc.) was used in order to quantify levels of staining in the periventricular white matter and the white matter surrounding the central sulcus at 25X magnification.

All statistics were performed using GraphPad Prism 4.0 or 5.0 in order to calculate parametric statistical values. A Student’s t-test was used in comparing two variables, and a one-way ANOVA with Tukey post-hoc was performed for comparison of larger numbers of samples.

RESULTS

Vaginal membranes of the guinea pig are only physiologically open during the period of estrous, every 16 days on average. Gestation was therefore estimated within a 2-7 day period based on the time at which vaginal membranes were partially or fully open. Gestational age estimations for the bacterial inoculation were confirmed after birth, and all neonatal weights fell in the normal term range.

Behavioural Monitor:

We used nine Control maternal animals and five E.coli-Exposed maternal animals to obtain 20 control male, 8 control female, 8 E.coli-Exposed male, and 7 E.coli-Exposed female pups. Upon comparison of the data obtained for the Control and E.coli-Exposed animals, it was found that there were no differences between the Control and Exposed animals for the variables measured. Table 5-1 shows the average values obtained for the control and exposed groups, unstratified by age or gender.

Upon stratification of the Control and Exposed groups with respect to age, it was found that the Control and Exposed groups converged with age in the amount of time spent in the
Table 5-1: Average Control and Infection-exposed values for each variable quantified using the ActiMot tri-axis motility monitor. Control n=28 pups, exposed to infection n=15 pups; unpaired t-test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Average ± Standard Deviation</th>
<th>Exposed Average ± Standard Deviation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in center (%)</td>
<td>2.59±4.20</td>
<td>7.73±12.50</td>
<td>0.142</td>
</tr>
<tr>
<td>Rotations</td>
<td>49.11±17.22</td>
<td>40.18±23.36</td>
<td>0.206</td>
</tr>
<tr>
<td>Distance travelled (m)</td>
<td>76.24±31.08</td>
<td>61.54±34.34</td>
<td>0.178</td>
</tr>
<tr>
<td>Time moving (sec)</td>
<td>49.55±18.61</td>
<td>41.37±24.16</td>
<td>0.265</td>
</tr>
<tr>
<td>Rearing</td>
<td>109.98±45.57</td>
<td>89.23±68.58</td>
<td>0.304</td>
</tr>
<tr>
<td>Hyperactivity (sec)</td>
<td>14.05±6.96</td>
<td>11.40±7.33</td>
<td>0.259</td>
</tr>
</tbody>
</table>
center of the apparatus, while the behaviours diverged with respect to rearing frequency with increased age (Table 5-2). In addition, it was found that within the group exposed to infection, time moving, distance traveled, and rearing behaviours were all age-dependent. As shown in Table 5-3, the results obtained upon separating groups by gender indicate no sex differences in behaviours among the control or exposed groups.

**VideoMot and Morris Water Maze:**

The sample sizes used for Water Maze testing are displayed in Table 5-4. One male animal in the *E.coli*-Exposed group showed a deficit in swimming ability, and was excluded from the study. An additional male pup in the *E.coli*-Exposed group suffered from suspected seizures after three days of testing, and was also excluded from the study. The effect of excluding these animals would be a bias towards insignificance of results if the seizures were due to extreme neurologic disturbance. One Control female became unwell with possible sepsis and was also excluded from the study.

Due to the complicated nature of behavioural testing, and the Morris Water Maze in particular, data was stratified for analysis in a number of ways in order to account for age, gender, gestational age at inoculation, and improvement with respect to starting position in the pool. For the purpose of the data presented, “Early Testing” refers to water maze testing beginning at 30 days of age and proceeding for the subsequent 6 days, while “Late Testing” refers to the same protocol but initiated at post-natal day 50. “Retention Testing” refers to offspring that were tested at the Early Testing stage but were subsequently tested at the Late Testing stage as well.

Figure 5-1 demonstrates the differences between both the control and *E.coli*-Exposed
Table 5-2: Age analysis of Control and Infection-exposed animals for selected variables in the ActiMot tri-axis motility monitor. Control day 50 n=23, control day 65 n=23, exposed day 50 n=15, exposed day 65 n=8; paired t-test. Asterisk indicates P≤0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>50 days</th>
<th>65 days</th>
<th>P value, Control vs Exposed</th>
<th>P value, Control, 50 vs 65 days</th>
<th>P value, Exposed, 50 vs 65 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in center (%)</td>
<td>1.13±1.41</td>
<td>5.79±10.20</td>
<td>0.050*</td>
<td>2.48±4.54</td>
<td>4.41±7.37</td>
</tr>
<tr>
<td>Rotations</td>
<td>46.48±22.64</td>
<td>38.04±26.18</td>
<td>0.158</td>
<td>50.98±28.63</td>
<td>54.96±28.15</td>
</tr>
<tr>
<td>Distance travelled (m)</td>
<td>70.21±31.13</td>
<td>57.50±36.15</td>
<td>0.137</td>
<td>81.12±47.50</td>
<td>87.44±39.74</td>
</tr>
<tr>
<td>Time moving (sec)</td>
<td>7.72±3.32</td>
<td>6.48±4.10</td>
<td>0.165</td>
<td>6.68±4.48</td>
<td>9.90±4.41</td>
</tr>
<tr>
<td>Rearing</td>
<td>101.3±67.49</td>
<td>79.50±64.87</td>
<td>0.163</td>
<td>110.4±58.20</td>
<td>157.1±52.58</td>
</tr>
<tr>
<td>Hyperactivity (sec)</td>
<td>12.74±6.83</td>
<td>10.73±8.04</td>
<td>0.217</td>
<td>15.32±10.84</td>
<td>16.5±8.38</td>
</tr>
</tbody>
</table>
Table 5-3: Gender analysis of Control and Infection-exposed animals for the selected parameters. Control males n=22, control females n=6, exposed males n=8, exposed females n=7; unpaired t-test. Hyperactivity is defined by the ActiMot program as periods of time where the guinea pig moved at velocity greater than 20m/s.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Males</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control, Average± Std.Dev.</td>
<td>Exposed, Average± Std.Dev.</td>
<td>$P$ value</td>
<td>Control, Average± Std.Dev.</td>
<td>Exposed, Average± Std.Dev.</td>
<td>$P$ value</td>
<td>$P$ value</td>
<td>$P$ value</td>
<td>$P$ value</td>
<td></td>
</tr>
<tr>
<td>Time in center (%)</td>
<td>2.63±4.44</td>
<td>9.05±12.66</td>
<td>0.200</td>
<td>2.48±3.53</td>
<td>6.22±13.13</td>
<td>0.492</td>
<td>0.929</td>
<td>0.679</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotations</td>
<td>49.58±19.12</td>
<td>46.68±27.32</td>
<td>0.788</td>
<td>47.21±16.35</td>
<td>32.75±16.79</td>
<td>0.145</td>
<td>0.769</td>
<td>0.252</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distance travelled (m)</td>
<td>78.18±33.47</td>
<td>69.03±40.68</td>
<td>0.581</td>
<td>69.15±40.68</td>
<td>52.97±25.70</td>
<td>0.256</td>
<td>0.459</td>
<td>0.373</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time moving (sec)</td>
<td>51.34±20.03</td>
<td>45.63±28.63</td>
<td>0.614</td>
<td>46.58±16.44</td>
<td>36.50±18.82</td>
<td>0.325</td>
<td>0.564</td>
<td>0.475</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rearing (# per hour)</td>
<td>113.26±46.58</td>
<td>63.75±88.83</td>
<td>0.476</td>
<td>103.0±45.56</td>
<td>91.25±41.90</td>
<td>0.646</td>
<td>0.684</td>
<td>0.909</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperactivity (sec)</td>
<td>14.43±7.56</td>
<td>13.13±8.88</td>
<td>0.718</td>
<td>12.67±4.60</td>
<td>9.43±4.97</td>
<td>0.249</td>
<td>0.488</td>
<td>0.334</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5-4: Sample sizes used in Morris Water Maze testing stratified by age at time of testing and gender. All animals subjected to Early Testing were later tested for Retention Testing, therefore sample size statistics are identical. Animals tested at 50 days postnatal (Late Testing) were not previously tested at an earlier age. Information on pup gender for several litters in the Early/Retention Testing group was unfortunately lost during testing.

<table>
<thead>
<tr>
<th></th>
<th>Water Maze Early Testing (30 days postnatal)</th>
<th>Water Maze Late Testing (50 days postnatal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>E.coli-exposed</td>
</tr>
<tr>
<td>Males</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Females</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Total pups</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>Total maternal animals</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 5-1: Results of Early and Late stage testing using the hidden platform water maze protocol, all starting quadrants combined for each day of testing. There were no discernable changes in the abilities of the control or *E.coli*-exposed animals to learn the task at Early stage testing (30-36 days postnatal), while some deficit was apparent in *E.coli*-exposed animals at Late stage testing (50-56 days postnatal). Asterisks indicate a significant difference between both Early Testing groups and the Late testing *E.coli*-exposed group on days 5 and 7 of testing protocol. Data is shown as mean ± SEM.
groups and between these treatment groups at both Early and Late Testing stages. No significant differences were found between the control and *E.coli*-Exposed groups at either time point, but a significant difference was found in the ability of the Late Testing *E.coli*-Exposed animals to attain the same improvements over the course of experimental testing. These Late Testing *E.coli*-Exposed animals showed a significant delay in ability to find the platform on days 5 and 7 of the experimental protocol, which their younger counterparts and the Late Testing control peers did not demonstrate.

Figure 5-2 demonstrates data comparing the two classes of animals at postnatal ages 50-56, those who had previously had water maze training at postnatal days 30-36 (Retention Testing groups) and those who were being evaluated for the first time (Late Testing groups). Animals who had had previous water maze training performed significantly better than those who had never been exposed to the water maze environment. One exception to this finding was that the control animals of the Late Testing group improved by the end of the testing protocol (Days 6 and 7), such that there was no statistical difference in the time they spent in the water as compared to the Retention groups. In contrast, the *E.coli*-Exposed Late Testing group continued to spend more time in the water than the other groups, as demonstrated by their paucity in learning ability already outlined in Figure 5-1.

As shown in Figure 5-3, the animals that took part in Early Testing showed an ability to retain the ability to find the platform upon their second round of testing (Retention Testing) two weeks later. A small statistically insignificant learning curve exists during Retention Training that is abolished after only one day of Retention testing. There was no difference between the treatment groups in the abilities of the animals to perform well on the task.

Figure 5-4 shows Late Testing data stratified by animal gender, and demonstrates that
Figure 5-2: There were no differences between the Late Testing groups or the Retention Testing groups. The control Late Testing group took significantly longer to find the platform than both Retention groups until Days 6 and 7, at which point the Control group showed the same water maze abilities as the Retention groups. The *E.coli*-exposed Late Testing group spent a significantly greater time in the water than the Retention Test groups at all time points. Pound symbol indicates only *E.coli*-exposed Late Testing group is significantly different than Retention groups, while asterisk indicates significantly elevated swimming times in both Late Testing groups as compared to the Retention groups. Data is shown as mean ± SEM.
Early Testing groups and their later Retention tests

Figure 5-3: The Early Testing groups were later subject to Retention Testing two weeks after their initial trials ended. No difference was seen between treatment groups at either time testing point, and the Retention Testing times were the same as those attained by the animals in their initial testing sessions by Day 4. No difference was found in the learning retention abilities of the *E.coli*-exposed and control animals in their retention trials. Data is shown as mean ± SEM.
Figure 5-4: Control and *E.coli*-exposed Late Testing groups separated into gender categories. There were no significant differences in water maze performance between genders in any of the testing protocols. Data is shown as mean ± SEM.
there were no significant differences between male and female pups. This result was also found upon analysis of Early Testing and Retention Testing; no differences existed between males and females in any of the testing protocols.

It was suggested that performance on the Morris Water Maze may be dependent on the starting quadrant, and that the most notable changes may take place between the first and second day of testing. We therefore analyzed a subset of data from Early Testing of the pups where a comparison was made between Days 1 and 2 of the testing protocol when the pup was started in the 2\textsuperscript{nd} quadrant of the pool. Figure 5-5 shows demonstrative data of the results we achieved upon data stratification in this manner; no changes were found between groups based on starting quadrant when consecutive early days of testing were analyzed. Further analysis was performed in order to investigate the overall improvement on the task, comparing the first and last day of training when pups were started in particular quadrants. Again, no difference was found in performance (Figure 5-6).

Because variations in the exact gestational age at bacterial inoculation were present, data were analyzed in order to account for the differences among litters in their bacterial exposure time. Actual inoculation date varied from days 39 to 55 of the 65-day gestation (60-85\% gestation), with a mean inoculation date of 72\% gestation. Very few of our animals were inoculated at the very early or very late ends of the inoculation spectrum, making statistical analysis of differences difficult. Figure 5-7, however, shows that it is unlikely that there are any differences in timing of inoculation with respect to overall task improvement in the median ranges of inoculation, those corresponding with our desired target of 70-75\% gestation when the brain is becoming most vulnerable. It should be noted that the control animal values were all combined for the purpose of this graph simply because the control animals were not inoculated at
Figure 5-5: Improvement in time to find the platform from day 1 to day 2 of early (30-day) trials with a second-quadrant start. No significant differences were found in this analysis or other similar analyses stratifying starting quadrant in consecutive early days of testing protocols.
Figure 5-6: Improvement in time to find the hidden platform in the water maze in the Early Testing protocol. There were no significant differences between groups with respect to starting quadrant. Data is shown as mean ± SEM.
Figure 5-7: Average time to find hidden platform in the Morris Water Maze in early (30-day) trials in *E. coli*-exposed guinea pig offspring. Inoculation was performed at estimated gestational age of 45-50 days based on estrous timing, and actual inoculation times represented here are based on actual birth day of litter. Data is shown as mean ± SEM.
all, and the duration of anaesthetic these animals underwent would be insufficient to cause any alterations in fetal well-being.

**Fetal white matter analysis:**

A total of 10 guinea pig pup brains were analyzed in both the Control and *E. coli*-Exposed groups after behavioural testing was completed at 56 days. Two Control and two *E. coli*-Exposed pups included in these numbers were from the Tri-axis motility monitoring group, with the remainder comprised of the Water Maze testing group. The groups were combined due the identical pup age and prenatal treatments. The quantification of white matter cell death in the periventricular and subcortical white matter in this study was consistent with our previous studies; pups exposed to *E. coli* inoculation during gestation had significantly more cell death evident upon analysis than did controls (Figure 5-8, one-tailed T-test, *p*=0.03).

Behavioural testing results were combined because animals were subject to the same prenatal environment and were euthanized at the same postnatal age. There was no significant difference in cell death levels between the two areas of the brain examined (subcortical vs. periventricular white matter), nor were there gender differences, and therefore data was combined. The difference in white matter cell death was significant upon combined comparison of control animals (*n*=29 FOVs) and *E. coli*-exposed animals (*n*=30 FOVs, Student’s one-tailed T-test, *p*=0.03).

**DISCUSSION**

While our results with the ActiMot tri-axis motility monitor indicate a tendency for the *E. coli*-treated pups to display different stress responses, it is difficult at this time to determine
Figure 5-8: Quantification of white matter cell death in the subcortical and periventricular white matter in animals tested in the Morris Water Maze and the Tri-axis Motility Monitor. Behavioural testing results were combined because animals were subject to the same prenatal environment and were euthanized at the same postnatal age. There was no significant difference in cell death levels between the two areas of the brain examined (subcortical vs. periventricular white matter), nor were there gender differences, and therefore data was combined. The difference in white matter cell death was significant upon combined comparison of control animals (n=29 FOVs) and E.coli-exposed animals (n=30 FOVs, Student’s one-tailed T-test, p=0.03). Data is shown as mean ± SEM.
whether these observations would become statistically significant with greater numbers of animals analyzed. It is possible that the post-natal age at which we performed the motility monitor tests was not a suitable time for determining substantial behavioural or motor differences in guinea pigs. In human studies, it has been found that most mild cases of CP diagnosed at early ages (1-2 years) have substantially or completely resolved by the age of seven. The point at which our guinea pig pups would show the greatest developmental changes was estimated and utilized in these studies, but it is possible that earlier testing (e.g. 10-15 days postnatal) may have altered our results.

In our Morris Water Maze testing, we did not see any differences in the abilities of the control animal to perform the task with respect to the animals that were exposed to intracervical E.coli inoculation during gestation. While learning disabilities are not a characteristic of cerebral palsy diagnosis, children with the disorder are far more likely to have intellectual deficits. A recent study reported that a wide range of intellectual and cognitive disabilities are experienced by 66% of young adults with cerebral palsy, though these range from mild to severe deficits in memory, attention, and concentration. Our study had two male animals in the E.coli-Exposed groups who had difficulties; one animal suffered seizures and one did not demonstrate ability to swim. While one female Control animal was excluded due to ill health, it is interesting to note that the more severe issues were found only in the E.coli-Exposed male group. In humans, infants of male gender are more likely to have cerebral palsy. In our studies, we did not find a correlation between gender and performance on behavioural tasks.

Because very few studies utilize the guinea pig model for behavioural testing paradigms, it is difficult to compare our findings with those of other researchers. One research area where guinea pigs are used for behavioural monitoring and Morris Water Maze testing is that of fetal
alcohol syndrome. Results from those studies tend to demonstrate fetal hyperactivity in the monitoring apparatus and variable deficits in abilities using the Morris Water Maze. Our findings in this study are consistent with data found in studies of schizophrenia after infection in pregnancy. While there are many other factors involved in the etiology of schizophrenia, it has recently been suggested that the pathways by which brain damage occurs in the prenatal environment is very similar, whether resulting in motor or psychological deficiencies after birth. When LPS is injected to late-gestation maternal rats, pups show increased activity in behavioural monitoring tests upon amphetamine injection when compared to controls. While we did not use the same methodology, we did see a tendency towards greater movement in our behavioural monitor testing after gestational bacterial exposure. In addition, neonatal rats given a viral injection in order to model causation of autism spectrum disorders showed abnormalities in locomotion in open-field tests after short and long intervals after injection. These animals also showed cytokine upregulation and increased cell death in the neonatal brains. In contrast to our results, a study in adult mouse offspring who were exposed to inflammation in pregnancy found decreased activity and greater stress responses in the elevated plus maze, and decreased levels of anxiety when confronted with an unfamiliar cagemate.

One study in hemiparetic spastic CP cases found that affected children had difficulty on particular tasks when increased association between perception and movement were required. Abilities in children with motor disorders have varied among studies depending on the nature of the task, and it is interesting to note that the guinea pigs in our study were able to fare as well as the non-affected animals in the Morris Water Maze. This finding is in agreement with human behavioural studies that have indicated the ability of an affected individual to accommodate for
their atypical movement in order to complete a task successfully. It is thought that the difficulty in movement characteristic of spastic CP, the most common subtype of the disorder, is due in part to the white matter damage of the brain proper and in part to altered synaptic transmission to muscles along the spinal tracts.

Prevention of gross motor deficits may increase the child’s overall well-being, as overall self-confidence improves with increased perception of physical ability. This may carry further benefits, in the form of behavioural improvement, for the child’s family and educational providers. Parents of children with CP have reported higher stress levels with respect to their child than parents of children with Down’s Syndrome, though mothers of Autistic children reported the highest levels of stress.

CONCLUSIONS

Tri-axis motility monitoring of guinea pigs exposed to E.coli inoculation during gestation shows the potential for elucidating specific aberrant behavioural characteristics as compared to control animals. Control and E.coli-Exposed animals performed similarly on the Morris Water Maze, though two male Exposed animals and one female Control animal had adverse outcomes external to behavioural testing. Animals exposed to chorioamnionitis in utero demonstrated significantly greater levels of white matter injury in the subcortical and periventricular white matter upon histological analysis. Further testing of postnatal behavioural characteristics should focus more intently on motor responses and movement analysis.
Works Cited


6-1: MECHANISMS OF FETAL BRAIN INJURY

I. Role of Infection and Inflammation

As demonstrated in our studies and others, there is a link between evidence of chorioamnionitis and subsequent fetal white matter injury. It is essential that uniform diagnostic criteria be established, as the infection is likely under-diagnosed in the clinical setting. Clinical chorioamnionitis is independently associated with a doubling of the risk of abnormal neonatal ultrasound results after adjusting for other conditions (e.g. PROM, gestational age). Histologic chorioamnionitis is also associated with an increased diagnosis of white matter lesions on neonatal ultrasound. Links between infection and adverse sequelae are not limited to cerebral palsy; schizophrenia and bronchopulmonary dysplasia have both been associated with prenatal indications of infection. In addition, viral infections such as toxoplasmosis and herpes have been linked to fetal brain injury via other injury pathways.

In response to infectious stimuli, a cascade of inflammation ensues, utilizing cytokines, chemokines, coagulation factors, and other entities to mediate the resultant damage. In some cases, genetic polymorphisms may cause a predisposition to increased inflammatory responses or an alteration in binding proteins, accentuating the strength of the inflammatory cascade.

As demonstrated in Figure 6-1, the presence of intrauterine infection can influence the development of fetal stress and eventual white matter injury by a number of methods. Primary among those methods are the endotoxin-mediated initiation of the proinflammatory response, the changes in vascular performance. In vitro studies have been particularly useful in elucidating the
**Figure 6-1**: Representation of potential pathways by which intrauterine infection and hypoxic ischemia can influence fetal brain development through the actions of proinflammatory cytokines and oxidative damage.
mechanisms by which neural matter, especially oligodendrocytes and their precursors, are
affected by these prenatal disturbances. In vitro and in vivo studies have indicated that bacterial
infections in the uterine environment contribute to localized hypoxic environments in the
immature fetus. The immature fetus does have mechanisms to protect against these insults by
preserving blood flow to the vital organs, but continued inflammatory signals eventually
overcome these protective measures.

Several studies have shown that a single insult to the fetus (hypoxic or inflammatory)
may not cause lasting fetal injury, but it has been suggested that subsequent insults may
overwhelm the defensive resources of the developing fetus. Likewise, it is possible that
stimulation of the inflammatory process causes sensitization to insults in utero, such that any
subsequent insult (e.g. subsequent cytokine peaks and/or hypoxia) can induce damage such as
the white matter injury seen in our studies. As shown in our ovine and guinea pig models of
chorioamnionitis and inflammation, amniotic fluid cytokines appear to fluctuate over the course
of an inflammatory response. Fetal white matter injury was found to follow these inflammatory
processes, indicating a role for these cytokines in the causative pathway. Further work in our
research group indicated that a threshold level of endotoxin is required in order to induce
significantly elevated levels of fetal brain injury while maintaining health of the animal.

In addition to the detrimental effects on neurodevelopment, inflammatory cascades can
cause further damage to the fetus due to initiation of preterm labour signaling. It is known that
certain pro-inflammatory cytokines (the interleukin family, for example) can stimulate
prostaglandin production, leading to initiation of other labour signals and uterine contraction. As
previously stated, the resultant preterm labour is also associated with an increase in fetal
vulnerability and is itself a risk factor for fetal and neonatal problems such as PVL and the
resultant cerebral palsy.

II. Role of Hypoxia-Ischemia

Over the past decade it has become clear that clinical signs of hypoxia at the time of birth are not uniformly associated with adverse neurodevelopmental outcomes. Indeed, hypoxia in the perinatal period may have its place in the causal pathway to brain injury, but associations with subsequent brain injury are weak. Given the lack of uniformity in diagnostic criteria for infection or asphyxia, it is difficult to make clear conclusions regarding the causal pathways and associations with adverse neurodevelopmental sequelae.

In the past, events and signs that were later proven to have little predictive outcome or association with adverse neurodevelopment indicated a diagnosis of intrapartum hypoxia or asphyxia. These indications included early Apgar scores, abnormal fetal heartbeat during labour, and nuchal cord. While these events should be tracked for persistence or co-existing factors (such as acidosis), our research and the research of others reveals there may be a stronger association with intrauterine infection than with transient hypoxic effects during gestation or labour. Indeed, even a diagnosis of neonatal encephalopathy in the neonatal period does not indicate definite long-term morbidity, though risks are certainly increased. While this diagnosis is associated with elevated levels of childhood death, a large proportion of survivors have no identifiable neurologic disorder.

In cases of hypoxia and resultant ischemia, there are a number of recognized responses by the human fetus. These include circulatory redistribution to preserve blood flow to the critical cardio-respiratory organs and cranium, depletion of cerebral vascular resistance, and localized
hypotension. These steps act in concert with other mechanisms, such as the use of alternative internal energy sources, to preserve cerebral integrity. It is only when these initial resources are overcome that the fetal defence system is overwhelmed and the brain becomes vulnerable to ischemic damage. Research indicates that the length of the ischemic insult must be prolonged and severe to cause lasting damage.

As shown in Figure 6-1, the adverse consequences of hypoxia and ischemia can affect neuronal and glial survival by alterations of membrane and synapse integrity after continued challenge. Research has shown, however, that it is unlikely that hypoxic episodes alone are the primary cause of most cases of white matter damage. While survival mechanisms exist to help the developing brain counteract these insults and promote neuronal and glial survival, continued hypoxic-ischemia eventually leads to calcium disturbances and glutamate build-up, at which point damage to the neuronal environment is inevitable to some degree. Hypoxia in the cerebral tissues can be caused, to some extent, by inflammatory processes brought on by infections such as chorioamnionitis. Endotoxemia is known to cause cerebral hypotension due to proinflammatory cytokines disrupting endothelial function. Conversely, ischemic processes can initiate signalling that contributes to the inflammatory response in the tissues, leading to both positive and negative feedback cycles. Retrospective human studies have shown, however, that there is no association between ischemic indicators such as acidosis with evidence of funisitis or histologic chorioamnionitis.

Our study of hypoxic episodes in the ovine fetus indicated that, despite poor physiologic responses to cord occlusions, ovine fetuses did not demonstrate increased levels of white matter injury. However, fetuses subjected to chorioamnionitis and hypoxic episodes tended to show
increased levels of white matter injury than those subjected to cord occlusions alone, but did not show significant impairments in physiologic responses to hypoxic stress. This indicates that a pre-existing stressor (in this case, chorioamnionitis) may aid the fetus in adjusting to subsequent stressors, a process known as preconditioning.  

III. Role of Developmental Vulnerability

As shown in Table 6-1, species vary widely in the degree of brain development achieved by the time of birth. The time of the brain growth spurt generally coincides with rapid neuronal reorganization, axonal growth and proliferation, and myelination. In the human, this period occurs roughly between the gestational ages of 28 and 34 weeks. It is partially for this reason that premature infants make up a much larger proportion of cases of neurodevelopmental damage. Indeed, infants born before 32 weeks show a much higher incidence of adverse sequelae; 75% of infants born before 32 weeks show neonatal morbidities. It should be noted, however, that all periods of fetal nervous system development are vulnerable to insults, and the period of the brain growth spurt is therefore only one period during which the developing fetal brain can be substantially harmed. Early embryological development is a period of intense vulnerability of all fetal systems, and effects of early gestational problems cannot be discounted in the etiology of later brain injury. It may be the case that teratogenic or envirotoxin exposure early in gestation functions as a preconditioner and causes developmental changes in the ability of the fetus to deal with environmental stressors later in pregnancy.

It is thought that the relative permeability of the BBB during early and mid-gestation may contribute to the vulnerability of the developing brain. Though it is known from animal studies that many cellular mediators (e.g. monocytes, activated T cells) can cross the intact BBB, the
Table 6-1: Percentage of adult brain weight achieved by various species at birth. Adapted from

<table>
<thead>
<tr>
<th>Species</th>
<th>% Adult weight at birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey</td>
<td>76</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>61</td>
</tr>
<tr>
<td>Sheep</td>
<td>53</td>
</tr>
<tr>
<td>Man</td>
<td>27</td>
</tr>
<tr>
<td>Pig</td>
<td>25</td>
</tr>
<tr>
<td>Rabbit</td>
<td>15</td>
</tr>
<tr>
<td>Rat</td>
<td>12</td>
</tr>
</tbody>
</table>
extent to which this occurs in humans is largely undefined. The ability of an inflammatory response to weaken the ependymal cell interactions may lead to greater levels of damage to the developing brain due to infiltration of inflammatory mediators into the cerebrospinal fluid.

It is thought that inflammatory tissue injuries in general are similar in the broad aspects of etiology; that is, cellular damage due to chronic inflammation or degeneration is always due to inappropriate initiation or cessation of molecular signalling. While degenerative neurologic diseases can occur at any stage of life, it is possible that the fetus is especially vulnerable to these molecular signalling errors due to the fact that its systems are largely incomplete in development.

IV. Role of Pro-inflammatory Cytokines

A recent study indicated that TNF-α alone did not affect the ability of oligodendrocyte precursor cells to differentiate into mature oligodendrocytes, nor did it increase the levels of cell death in the \textit{in vitro} cell population. This study found that only in the presence of IFN-δ did the TNF-α cause sustained damage to the developmental well-being of the cells. Studies such as this are integral to answering the question of possible mechanistic pathways, as the \textit{in vivo} system cannot be similarly controlled in order to account for synergistic interactions such as these. This ability to control cell environment has allowed cell culture studies to provide proof of the direct effect that cytokine interactions can have on the vulnerable fetal system.

Levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α have been shown to be present in elevated levels in the amniotic fluid of fetuses showing neural abnormalities on neonatal ultrasound. IL-1 receptor antagonist did not similarly increase in these cases, though IL-1 is known to be an inducer of this entity as well. Our studies in the guinea pig indicated
that IL-1β and IL-6 in particular are elevated in the amniotic fluid after a maternal inflammatory response is initiated. Our studies in the placenta indicated an inability of these cytokines to cross the similarly-structured human placenta. The ability of varied cell types, including trophoblasts and leukocytes, may therefore be responsible for the cytokines present in the fetal system and amniotic fluid via positive feedback cycles and initiation of downstream inflammatory processes.

V. Characteristics of Fetal Brain Injury

While pathways causing brain damage are unquestionably intertwined, there are characteristics of brain injury that are more common depending on the primary prenatal insult. For instance, focal lesions surrounding distal blood vessels are more often associated with asphyxict damage from persistant hypoxia, while focal lesions surrounding the ventricles are more likely to be associated with inflammatory damage. Likewise, focal periventricular hemorrhage is most often associated with damage in only one cerebral hemisphere, while periventricular leukomalacia is more likely to affect both hemispheres.

6-2: FUTURE DIRECTIONS: PREVENTION OF FETAL BRAIN INJURY

I. Treatment of preterm labour

Due to the medical and social implications of CP as a childhood disorder, and also due to its approximate $5 billion per year cost in the US alone, it is important to study the pathways by which the disorder develops in order to find complementary treatments to halt development of the disease. Any treatment option used during pregnancy must be carefully assessed, as fetal development, particularly development of the CNS, is so sensitive to environmental factors. In addition, endogenous factors that cause damage at high levels may actually be needed for
particular developmental steps in lesser doses (e.g. proinflammatory cytokines, apoptosis promoters), and thus the blockage of any endogenous compound or the addition of maternally-administered drugs must have carefully weighed risks and benefits. Consideration must also be given to the effectiveness of the route of administration (e.g. maternal vs. fetal) and the timing of the administration (e.g. before vs. during insult).

As preterm birth is the leading risk factor for the development of motor disorders such as CP, it is intuitive that tocolytic agents be assessed for their efficacy in preventing the preterm labour. Only recently has a proven treatment for prolonging labour been established, and use of this tocolytic is only available in selected centres at present. However, it should be noted that studies indicate threatened preterm labour is associated with an increased incidence of childhood CP diagnosis. This association is most likely due to the extended fetal exposure to bacteria or inflammatory products that triggered the preterm labour signals when physicians are able to prolong delivery. Thus, prolonging pregnancy may actually cause more damage than delivering early in some cases, depending on the length and severity of inflammatory exposure and the gestational age of the fetus. In addition, antibiotic administration in cases of PROM may lead to fewer diagnoses of clinical chorioamnionitis, but the inflammatory process may already be established in the fetal compartment. Because antibiotics cannot access the amniotic fluid in meaningful amounts, the fetus may still be subject to inflammatory mediators from aspiration of the amniotic fluid even when bacteria have been eliminated.

II. Attenuation of the inflammatory response

The phosphodiesterase (PDE) family of cyclic nucleotides is key in uterine contractility,
inflammation and neuronal development, and the PDE4 sub-group are known to be integral in inflammation control. In mice, it has been found that LPS-induced preterm labour is attenuated by co-injection with a PDE4 inhibitor, most likely through both direct actions and indirect blocking of downstream mediators of PDE4 such as TNF-α. In fact, the increases in amniotic fluid proinflammatory cytokines and uterine natural killer (NK) cells found with LPS injection were abolished by treatment with the PDE4 inhibitor rolipram, though no changes in prostaglandin levels were detected. This novel finding is promising for the treatment of fetal brain injury, since tocolytics alone only prolong labour, while rolipram also decreases the generalized inflammatory response.

Since it has been proposed that inflammation causes the most damage in the absence of sufficient levels of developmental protectors (e.g. neurotrophins) usually present during development, some treatment proposals have focused on enhancement of these endogenous protectors and the modification of inflammatory mediators. These protectors or modifiers would enhance oligodendrocyte maturation or interfere with the pro-inflammatory cascade. Since oligodendrocytes develop from their precursor cells in a distinct pathway, it is known that the period of migration and proliferation, in addition to the period of myelination in the early third trimester, may be especially vulnerable. In addition, it is known that the proinflammatory cytokines proposed to be causing the white matter damage may be protective in some circumstances. For instance, IL-1β causes in vivo excitotoxic damage to the brain but appears to protect against neuronal cell death in vitro. TNF-α after glutamate challenge in rat embryo neuronal cultures is protective to hippocampal cells, but the same situation is damaging to human neuronal cultures. IL-6 is also known to have anti-inflammatory properties.
in some cases, and is capable of downregulating TNF-α levels, inhibiting demyelination, and protecting neurons. It is unclear at present what can be done to induce the protective effects of the proinflammatory cytokines, as they tend to act in a positive feedback cycle with respect to inflammatory effects.

III. Supplementation of endogenous protective agents

Activation of astrocytes in cases of reactive oxygen or nitrogen species may also be protective due to their ability to mediate antioxidant actions and moderate excessive glutamate presence. Alternatively, these astrocytes may also be detrimental due to their continued contribution to the oxidative pathway. It has been noted that antioxidant enzymes are downregulated in oligodendrocyte precursor cells, indicating a decrease in endogenous protection within these cells. It is possible that upregulation of antioxidant activity by astrocytes or exogenous treatment may be protective of these developing cells.

One endogenous molecule of recent research interest is IL-1ra, the receptor antagonist of the IL-1 cytokines. This antagonist binds to the IL-1 receptor but does not initiate a response. Exogenous administration of IL-1ra into the brain revealed its ability to diffuse rapidly into damaged areas, indicating its possible efficacy in treatment modalities. A lack of this antagonist leads to an increase in the levels of ischemic damage present. Neonatal and adult rats given exogenous doses of IL-1ra demonstrate less ischemic damage, even after an excitotoxic response is elicited. It has also been found that injection of IL-1ra decreases damage from other insults, such as traumatic brain injury and heat stroke, in addition to reducing ischemic damage up to 70%.

When an intracerebral injection of LPS is accompanied by a
subsequent injection of IL-1ra in neonatal rats, ventricle dilation and decrease in myelin caused by LPS alone is almost completely abolished. Soluble receptors of the proinflammatory cytokines also exist in the circulation (e.g. soluble TNF receptor), and effectively block the effects of cytokines by binding them without causing a cellular response. Because this data was obtained from rodent studies, efficacy of this therapeutic avenue could be further tested using the \textit{in vitro} human placental perfusion set-up before making conclusions about its use in human cases.

Antibodies to the proinflammatory cytokines may decrease the permeability of the BBB by inhibiting their antigen targets, thus preventing further cytokine migration across the BBB and resulting in less direct cytokine damage to the white matter. Antibodies to caspases, molecules involved in programmed cell death, may reduce neuronal apoptosis, and anti-inflammatory cytokines such as IL-10 may also be protective. Antibodies to the messenger molecules downstream of the cytokines, such as ICAM-1, may also be useful treatment pathways to investigate, though more widespread benefit may occur with regulation of the upstream molecules. In addition, supplementation of anti-inflammatory cytokines may also improve outcome via the inhibition of the proinflammatory response. Previous studies in lung disease have indicated that a lack of expression of anti-inflammatory cytokines may be due to underlying genetic factors, and that supplementation may decrease levels of damage.

It is known that developmentally protective entities (e.g. trophic factors) supplied to the fetus by the mother or placenta may be lacking in cases of fetal brain injury; supplementation of these endogenous protectors may be another possible modality of protection for the fetal brain. Factors that promote growth and development of the fetal brain while suppressing cell death may
be especially helpful to infants who are at risk of preterm birth. For instance, recent research has pointed to neuro- and oligotrophins as possibilities for the enhancement of survival and development of fetal CNS tissue. By promoting the survival of immature neurons and oligodendrocyte precursor cells, the immature fetal brain is given a greater chance of developing mature and healthy cells. These trophic factors are most effective when present in concert, as the individual factors often have transient or ineffective roles when present alone. Other agents necessary for normal brain development, such as thyroid hormones, may also confer effective treatment benefits in the future. The absence of these hormones is known to hamper neuronal maturation and oligodendrocyte differentiation, and thus supplementation with these hormones may confer a benefit to the CNS.

IV. Exogenous treatment modalities

Corticosteroids, usually administered to premature infants in order to reduce risk of respiratory distress syndrome, has also been linked with decreased risks of various forms of fetal brain injury. Several previous studies have indicated that maternal administration of a full course of corticosteroids during pregnancy can decrease the risks of brain injury to the fetus, particularly the risk of intraventricular hemorrhage in very preterm infants. Studies have found corticosteroid treatment to be particularly beneficial for infants subject to PROM. Follow-up from most studies indicate no associated adverse developmental effects in childhood, even in pregnancies that were complicated by factors such as high blood pressure. A study in Australia found that there was a 50% reduction in brain injury such as CP in infants who had
corticosteroid exposure in utero. However, a recent retrospective follow-up study has indicated a non-significant increase in incidence of CP diagnosis in children after multiple courses of steroids during gestation.

The proposed mechanism of corticosteroids is that of a CNS protector, in that it is proposed to down-regulate the cytokine response, enhance oligodendrocyte maturity, improve cerebral blood flow, and inhibit oxidative damage, thus counteracting the effects of the inflammatory response on brain tissues. Corticosteroids are also proposed to act via growth factors in order to inhibit astrogliosis, and thus decrease oxidative damage. In addition, it has been proposed that corticosteroids can increase the availability of ciliary neurotrophic factor (an oligotrophin), which acts to block the actions of TNF. Ciliary neurotrophic factor in the presence of brain-derived neurotrophic factor also increases oligodendrocyte precursor cell survival. As an added benefit, corticosteroid exposure is usually followed by a decreased time spent in hospital after birth, indicating decreased neonatal problems and an associated decrease in health care costs.

A certain amount of controversy surrounds the issue of corticosteroid use, however, in that universal criteria for indications of use, type of corticosteroid, and dosage schedule have not been established, and variation in these factors is known to influence benefits and risks to the infant. For instance, repeated doses of betamethasone in sheep leads to increased blood pressure and cerebral vascular resistance which, if sustained, may cause perfusion problems in the fetus. In addition, the early suppression of the inflammatory response by betamethasone is followed by an upregulation of inflammatory responses later in treatment. It has been suggested
that multiple courses of corticosteroids have no benefit over a single course, and may only have a detrimental effect on the developing fetus.

Several retrospective studies have indicated a decrease in the risk of CP (OR 0.11-0.14) in infants who were exposed to magnesium sulphate during gestation. It is proposed that the magnesium ion’s ability to regulate cerebral vasculature and minimize inflammatory processes is responsible for this decrease. However, many have charged that this decrease is confounded by co-existing conditions of early preterm labour or preeclampsia in most patients to whom magnesium sulphate is administered. In fact, a diagnosis of preeclampsia seems to protect the infant from CP (OR 0.08, 95% CI 0.02-0.67). Even in very preterm infants, the odds ratio of intraventricular hemorrhage was 0.71 (95% CI 0.58-0.87) when preeclampsia was present. It is known that preeclampsia is caused by first trimester placental complications in which spiral arteries are improperly remodelled, leading to reduced circulation to the fetus later in pregnancy. It is possible that this initial insult is somehow protective since the fetus has been exposed to nutritional challenges for an extended period by the time the insult causing fetal brain injury occurs. This preconditioning hypothesis has also been proposed in cases where the second or third trimester fetus is subjected to both hypoxic and infectious stimuli, and was discussed in Chapter 3. More data is needed in order to determine whether the protective effect to the infant is due to magnesium sulphate treatment or to the presence of preeclampsia itself, and how pre-existing diseases such as preeclampsia may protect the fetal brain.

Previous studies in which LPS was administered to rat microglial cultures indicated that levels of NO, IL-6 and TNF-α were decreased in lower temperature cultures within 6 hours of inoculation as compared to controls at body temperature. A trend towards delay in cytokine
response was also found in cultures of human blood mononuclear cells exposed to LPS under moderate hypothermia. In the rat microglia study, NO and IL-6 remained lowered until 48 hours post-inoculation, while TNF-\( \alpha \) levels increased after 6 hours. The study concluded that moderate hypothermia may possess efficacy in neuroprotection against LPS damage.

In human cases, it has been found that the effects of endotoxins on neuroendocrine processes can also be attenuated by estrogen supplementation in females. In addition, it is thought that there is at least some neural control over systemic inflammatory processes, as demonstrated by a study in which injection of the hormone alpha-MSH (known to have effects in neuroprotection in addition to appetite and pigmentation) was shown to decrease systemic inflammation when administered in the brain but not when administered systemically. Animal studies have indicated that antioxidant vitamin treatments may attenuate brain injury due to secondary energy failure. A recent study in rats also indicated pro-inflammatory cytokine attenuation in maternal serum and fetal amniotic fluid with treatment regimens of the antioxidant N-acetyl-cysteine. At present, vitamin supplementation studies in randomized clinical trials have focused primarily on the incidence of preeclampsia and long-term cardiovascular disease.

The hypothesis that cytokines gain direct access to the brain by permeating the blood brain barrier presents further complications in formulation of treatment modalities. Inflammatory events occurring before 27 weeks gestation will allow cytokines to freely cross into the fetal brain since the BBB, ependymal cells and endothelial cells are not yet developmentally mature. In addition, factors that increase angiogenesis, and thus blood flow and oxygen availability to the developing fetal brain, may be harmful in that they also increase the permeability of the new vessels, adding to the permeation of unwanted inflammatory
mediators into the fetal brain.

6-3: FUTURE DIRECTIONS: STUDY PROPOSALS

I. Preterm premature rupture of membranes (PPROM) and Fetal Brain Imaging

Brain ultrasound lesions occur in 7-26% of very low birth weight (<1500g) infants. Of those with lesions, 47-80% will develop cognitive difficulties, and 62-100% will develop cerebral palsy. Evidence supports a link between PPROM, chorioamnionitis, PVL and CP. Approximately 17% of infants born after PPROM develop PVL, while 22% develop PVL if PPROM was accompanied by intrauterine infection.

Echolucencies indicative of PVL on neonatal ultrasound are a key predictor of subsequent neurodevelopmental abnormalities. In cases of PPROM or preterm labour without PPROM, significantly more infants have abnormal cranial ultrasounds, particularly in the detection of major lesions. Previous research has noted an increase in the incidence of intracranial hemorrhage in infants who were born with an infection after a short duration PROM. In infants where PROM occurred within 2 hours of hospital admission, there was an increased risk of CP diagnosis (OR 2.60, 95% CI 1.1-6.2). The risk of CP after PROM is further increased by preterm birth, with the odds ratio for CP of low birthweight infants after prolonged rupture of membranes reaching 6.6 in one study.

Neonatal ultrasonography can be predictive of later neurodevelopmental outcome. In a study of preterm births, all the children found to have CP had demonstrated abnormal ultrasounds in the neonatal period. Ultrasound echodensities in the periventricular region are
diagnosed as intracranial hemorrhage, while echolucencies in the cerebral white matter are classified as PVL. White matter echolucencies and ventriculomegaly are the best ultrasound predictors of childhood cognitive and developmental deficits. When white matter lesions are classified as cystic periventricular leukomalacia due to lesions detected on early ultrasounds, motor impairment usually follows in childhood. The detection on early ultrasounds of persistent densities of white matter indicates a diagnosis of non-cystic periventricular leukomalacia, and outcome is less predictable with respect to neuromotor impairment. Previous studies have found that a diagnosis of echolucencies indicative of PVL lead to CP diagnosis in 62-100% of cases. It has been noted that early PVL damage may occur quickly after a fetal challenge, but ultrasound detection may not occur until the lesion is up to 6 weeks old. This indicates that cases where neonatal ultrasonography has found evidence of IVH or PVL, the damage occurred during gestation and not in the antenatal period. Gross lesions found on ultrasound are characteristic of necrotic foci, and are found in 7-26% of low birth weight infants (<1500g).

A previous study in preterm infants (born at 25-32 weeks gestation) used weekly cerebral ultrasound scans and electro-encephalogram recordings to diagnose cystic and non-cystic PVL in the neonate. A further study in the preterm population (<32 weeks at delivery) subjected neonates to multiple cerebral ultrasonography exams followed by transfontanellar MRI at 32-36 weeks postnatal, and found no association with diagnosis of clinical chorioamnionitis.

MRI is effective in identifying lesions in 70-90% of CP cases, and aids in classifying the disorder based on the size and location of the lesion. For instance, lesions found in the periventricular area (as in PVL) are characterized as pyramidal tract lesions, while those
identified in the thalamus or basal ganglia are characterized as extrapyramidal. The location of the lesion aids in characterizing the category of cerebral palsy of the patient and can be useful in identifying physiotherapy options. Ultrasound has been the traditional method of postnatal cerebral investigation until recently, when studies began to demonstrate underdiagnosis of neonatal stroke and diffuse white matter damage when compared to investigations using MRI.

Current and future research focus should continue to focus on MRI as a more useful tool of determining early evidence of fetal brain injury, such that appropriate interventions and treatments can be offered to the patient.

II. Investigation of processes at the molecular level

The work performed in this thesis brings us closer to elucidating the mechanisms by which inflammatory processes can influence developmental challenges to the fetal brain. There are still a great many unknowns associated with the proposed inflammatory pathways. Great strides have been made in the fields of molecular signalling and protein function and analysis, and these tools will have a great impact on further determining the entities that are of most danger in the development of healthy fetal tissues.

The interaction of inflammatory and ischemic pathways is intriguing, and research into the interactions between these pathways, as well as their systems of checks and balances. Of particular interest are the adhesion molecules and coagulation factors. These entities are stimulated by proinflammatory cytokines, and their stimulation in the central nervous system tissues may be a part of the pathway by which damage occurs, in addition to their functions as tissue repair mediators. For instance, ICAM-1 is known to facilitate neutrophil invasion in damaged tissues and self-inhibit through negative feedback cycles, but has also been implicated
in ischemia-reperfusion injury, especially after initial cerebral traumas. Research into the antibodies and inhibitors of ICAM-1 and an associated antigen, LFA-1, have provided evidence that inhibition may mitigate the damage caused by overstimulation of these entities.

Many pathologies of late pregnancy and neonatal life have their origins in early embryonic development and placentation. The inverse correlation between preeclampsia and cerebral palsy is a controversial result that should be studied further. If the association is valid, it is possible that the processes in early placentation that are responsible for the development of preeclampsia are responsible for protection of the fetal brain. Another possible explanation for this purported association could have to do with the treatment of preeclamptic mothers; associations between preeclampsia and fetal brain injury may be due to prescribed magnesium sulphate. An association between CP and intrauterine growth restriction (IUGR) has also been reported, and promising work into fetal circulation using Doppler monitoring is further investigating the adverse consequences of IUGR.

III. Additional work using animal models

The guinea pig model utilized in this study is a valuable model for investigating the causation of fetal challenges due to the similarities in placental structure and the brain growth spurt. Unfortunately, the animals are very sensitive to LPS and external stressors. Recent work in our lab has indicated that there is a threshold level of LPS that can be used in order to affect brain injury while maintaining health of the pregnant animal. This information will allow future research into the timing and methodology of fetal brain injury. In the current study, we were unable to obtain maternal blood in several cases; processing the fetuses rapidly necessitated
leaving the maternal blood collection for several minutes, in which time the blood had begun to coagulate. Optimization of blood or urine collection from the maternal and fetal animals would be useful in measuring maternally-circulating entities during the course of the inflammatory process. Further investigation of maternal uterine tissue and the fetal membranes may also allow investigation into the health of the fetus at the time of maternal euthanization. We were unable to determine with a high degree of certainty whether some fetuses were dead before maternal euthanization, as necrotic cerebral tissue could have been due to improper/delayed fixative perfusion, previous demise, or gross necrosis. Additional work using fast-frozen fetal tissues is also suggested for future use of the guinea pig model, and analysis of guinea pig sera and amniotic fluid for other inflammatory mediators or hypoxic markers may also be helpful.

IV. Further investigation of ovine study tissues

The ovine study detailed in this thesis (Chapter 3) yielded a great deal of data and tissues that can be analysed in further research into immunology and molecular signalling. While time did not permit me to do so, there are a number of immunostaining protocols that can be optimized for the ovine tissues. The main difficulty encountered in the analysis of ovine tissues is the lack of suitable antibodies in the commercial market. The ovine model poses a number of research problems in the modern animal facility, with animal size, cost, and airborne Q-fever disease being the main reasons preventing commercial pharmaceutical companies and research establishments from utilizing this highly valuable model of pregnancy. Further study of our tissues will require time to optimize available antibodies or develop new ones. Staining for molecular markers of oligodendrocyte maturation, astrocyte activation, and the abundance of leukocytes and glial cells in the fetal brain tissue is also recommended,
particularly because a neuroanatomist that was consulted (Dr. Michael Kawaja, Dept. of Anatomy and Cell Biology, Queen’s University) postulated that the many cells with an apparent halo in our slides may be infiltrating leukocytes. Fast-frozen tissues were also taken from the ovine fetuses, and protein and molecular analysis for inflammatory mediators and pathway regulators can be performed. Fetal lung tissue was also fast-frozen from our ovine experiments, which may be of research interest in the study of bronchopulmonary dysplasia, another neonatal complication associated with chorioamnionitis.
SUMMARY AND CONCLUSIONS

The research undertaken as detailed in this thesis has constituted an effort towards elucidating the role of infection and inflammation on the well-being of the developing fetal brain. Through the use of animal and human placental models, we have attempted to define the methods by which the inflammatory response, initiated by an adverse gestational event, may lead to lasting damage to fetal neurodevelopment. While further work is required in order to fully elucidate the exact pathways of injury, it is our belief that the work presented here substantially advances the knowledge base within this area of research. It is becoming increasingly evident from basic science and epidemiological studies that a host of factors may be involved in the pathogenesis of cerebral palsy. We were unable to control for differences in immune capability or for genetic disorders predisposing to white matter susceptibility in our animal models, but it is important to recognize that these confounding factors may also have an important role in the human case of cerebral palsy development.

To summarize, the main findings of our studies in this area have revealed the following;

1 Maternal endotoxin injection in the guinea pig causes increased inflammatory mediators in the amniotic fluid and an increase in fetal white matter injury.

2 The proinflammatory cytokine response after maternal endotoxin injection occurs in a shortened time frame and with a lower magnitude with respect to intracervical bacterial inoculations.

3 Ovine fetuses exposed to umbilical cord occlusions in utero had poor physiologic profiles compared to baseline parameters and to groups exposed to bacterial infection in utero.

4 Intra-amniotic bacterial inoculation in the ovine model causes a greater level of fetal
brain injury than those fetuses not exposed to bacteria in utero, but not a significantly greater level of fetal brain injury when compared to fetuses exposed to both bacterial inoculation and acute hypoxic episodes.

5 LPS administered to the human placenta in the maternal arterial line causes an increase in IL-1β on the fetal side. Injection of IL-1β on the maternal side is also associated with increased levels on the fetal side.

6 Proinflammatory cytokines IL-6 and TNF-α, when administered to the maternal arterial line of the in vitro human placenta, do not correspond with increased levels of cytokines on the fetal side.

7 The E.coli-derived endotoxin LPS may cross the placenta in small quantities.

8 Guinea pig pups exposed to chorioamnionitis in utero do not demonstrate deficiencies in learning abilities in the Morris Water Maze, and show non-significant and age-dependent differences in behavioural activity as measured by the Tri-Axis Motility Monitor.

While research continues into the complicated pathways of inflammation in the maternal system, placenta, and fetal compartment, we propose a renewed focus on the interactions between the fetus and inflammatory processes in order to determine the conditions under which defensive mechanisms may become detrimental. In particular, our research has indicated further need to investigate cyclicity in proinflammatory mediators in the etiology of fetal injury.

Inflammatory responses in the presence or absence of bacteria lead to increased white matter damage in the fetus. We have demonstrated that proinflammatory cytokines are found in the amniotic fluid after a maternal endotoxin administration in the guinea pig. Further, we have shown that a pre-existing bacterial infection can attenuate the levels of white matter damage.
incurred in the ovine fetus. Using these models and the knowledge we have gained, a great deal of information can be added to the known mechanisms involved in the pathway leading to fetal brain injury and resultant disorders such as CP. This knowledge base will be instrumental in the discovery of novel prevention and treatment modalities.


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Thorngren-Jerneck K, Herbst A. Perinatal factors associated with cerebral palsy in


APPENDIX I: Preparation of bacteria for inoculation in ovine experimental model using spectrophotometry, optical density (OD), and Klett measurements

To begin bacterial concentration calculations, we begin at the point where tryptic soy broth has been incubated overnight after brief exposure to frozen bacterial bead from *E.coli* culture. Bacterial broth is diluted 1:20 in sterile saline in a spectrophotometer tube (50µL broth with 950 µL saline).

Spectrophotometer reading of is taken at 600nm and is measured against control value (sterile saline).

Consider a sample that gives a reading of 0.180 optical density (OD) units.

We had diluted the broth at a concentration of 1:20 in saline for spectrophotometry, so the actual value for our broth is thus 0.180 (20)=3.6 OD units.

In order to get an OD value of 0.1 in 20mL of tryptic soy broth for our timed incubation, we perform an additional calculation:

\[ C_1V_1 = C_2V_2 \]

where \( C = \)concentration in OD units, and \( V = \)volume in mL

Let \( C_1 \) be the calculated OD value after overnight incubation, in this hypothetical case, 3.6 OD units

Let \( C_2 \) be the desired OD value for our 1 hour incubation, 0.1 OD units
Let V2 be the volume of TSB used for our 1 hour incubation

Solve for V1, the volume of overnight-incubated broth needed to begin our 1 hour incubation.

\[ V1 = \frac{(C2V2)}{C1} \]
\[ = \frac{(0.1)(20)}{3.6} \]
\[ = 0.279\text{mL}, \text{or } 279\mu\text{L} \]

After inoculating the side-arm flask with the desired volumes of TSB and bacterial broth, the initial Klett value is taken, and should be adjusted to zero. The Klett after a timed 1 hour incubation of our side-arm flask at 37°C is taken, and the change in Klett value is used to calculate the concentration of bacteria in our flask, given that

1 OD unit = \(10^8\) CFU bacteria = 147 Klett units

So if our resultant Klett value is 27, then our OD is \(\frac{27}{147}=0.102\) units, and therefore \(1.02\times10^7\) CFU of \(E.\text{coli}\)

Serial dilutions were then performed such that

Dilution 0 = \(1.02\times10^7\)
Dilution 1 (1:10) = \(1.02\times10^6\)
Dilution 2 (1:100) = \(1.02\times10^5\)
Dilution 3 (1:1,000) = \(1.02\times10^4\)
Dilution 4 (1:10,000) = \(1.02\times10^3\)
Dilution 5 (1:100,000) = \(1.02\times10^2\)
Dilution 6 (1:1,000,000) = \(1.02\times10^1\)
Dilution 7 (1:10,000,000) = \(1.02\times10^0\)

100\µL of dilutions 4-7 were plated on TSB to determine validity of calculations, and a calculated portion of dilution 4 was used for the experimental inoculation.
The TSE ActiMot / MoTil system is a flexible system for studying open field behavior, hole-board exploration and home-cage activity of small laboratory animals. It can be operated with multiple cage inserts enabling the operator to perform further experimental tasks, e.g. place preference or light / dark experiments. Square-shaped or home-cage shaped frames detect the animal's activity via infrared sensors. Several frames can be stacked on top of each other for advanced open field analysis in several planes. The ActiMot frame is a square-shaped frame (the so-called base unit). This frame features two pairs of light-beam strips, each pair consisting of 1 transmitter strip & 1 receiver strip. These basic light barrier strips are arranged at right angles to each other in the same plane. They are used to determine the X and Y coordinates of the animal and thus its location (XY frame). Up to 2 further pairs of unidimensional light-barrier strips (Z1 and Z2), whose height can be detected in addition to location (Rearing indicators). The light barrier levels are scanned with a frequency of 100Hz each on fast computer platforms. They can be operated at almost any light condition, even in complete darkness.
In the trial preparation phase all control and descriptive parameters are entered by the user. After the test preparation has finished, the animal is placed in the cage and data acquisition is started by pressing a key on the keyboard. During the trial a schematic diagram of the boxes connected is shown. The actual location of the test animal is represented by a square whose position changes as the animal moves. This square corresponds to the centre of gravity calculated from the interrupted light beams.

VIDEOMOT2 TRACKING SYSTEM

VideoMot2 is a versatile video tracking system for automatically recording and analyzing animal activity, movement and behavior. Whether you are working with an open field, an elevated plus maze or a radial maze - VideoMot2 is a flexible tool suitable for a variety of behavioral tests that can be adapted to meet your individual requirements. Animal identification is performed via contrast detection according to user-defined object filters (including an adjustable contrast threshold) and integrated automatic background correction. The trial design is featured by a search for objects in experimental regions drawn by the user with tools provided. Zones of interest with any desired shape and marked with a name and a color can be easily generated inside these areas to perform spatial analyses. Regions may also act to control data acquisition, e.g. terminate, pause or restart a trial depending on the
experimental paradigm. Experiments can be started by a remote control at a distance from the computer. In contrast to other systems the video source is displayed on the screen during the trial - no second monitor is therefore required. Coordinates are collected with up to 13Hz sampling rate during the "live" experiment or offline using videotaped material. Series of experiments may be performed one after the other and stored in a single file for comfortable group analysis. Evaluation is designed to be modular. Choose between a variety of analysis packages calculating specific results parameters. The track pattern can be plotted at variable speed for any time interval and bitmap files are generated by a mouse click. Track pattern shown for different hardware configurations: