THE ROLE OF ZINC IN PREVENTING FETAL DYSMORPHOLOGY AND BRAIN INJURY MEDIATED BY MATERNAL EXPOSURE TO INFECTION IN PREGNANCY

A Thesis Submitted For the Degree of Doctor of Philosophy

by

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ABSTRACT

Maternal exposure to viral and bacterial infection during pregnancy is associated with fetal dysmorphology and neurodevelopmental disorders including schizophrenia, cerebral palsy, autism and mental retardation. Previous studies in our laboratory using an established mouse model of endotoxin-induced fetal dysmorphology have led to the hypothesis that birth defects caused by infections during pregnancy are the result of fetal zinc deficiency resulting from the induction of a zinc-binding protein, metallothionein (MT) in the maternal liver as part of the maternal inflammatory response. Thus, we predicted that zinc deficiency would exacerbate the negative fetal outcomes caused by bacterial endotoxin lipopolysaccharide (LPS) and that zinc supplementation would protect against LPS-mediated teratogenicity. This premise was investigated herein and was extended to investigate underlying molecular mechanism, including the identification of markers of neurodevelopmental damage following LPS administration in early and late pregnancy, and to determine the influence of zinc treatment on any changes in expression of these markers.

In Chapter 2 it was demonstrated that prenatal exposure to LPS on gestational day (GD) 8 resulted in the development of physical birth defects including exencephaly, microcephaly, cleft lip and or palate, and micrognathia in GD 18 fetuses. Dietary zinc supplementation throughout pregnancy was found to prevent the LPS-related abnormalities. Furthermore, low dietary zinc and LPS exposure were found to be synergistic on teratogenicity. In addition, an inverse linear relationship was observed

between the concentration of zinc in the diet and teratogenicity with a reduction in the incidence of birth defects observed with increasing concentration of dietary zinc, a finding suggesting that even small increments of zinc above normal dietary intake are likely to have a beneficial impact on teratogenicity.

Maternal infection during late pregnancy has also been linked with prenatal brain damage. A major causal link underpinning this relationship is thought to be the cytokines released following a maternal inflammatory response to infection. In Chapter 3, the presence of cytokines released in response to LPS given on GD 16 was demonstrated by an increased number of tumour necrosis factor-alpha (TNF-α)-reactive cells and astrogliosis accompanied by extensive apoptotic cell death in GD 18 fetal brain. Recently our laboratory has reported that dietary zinc supplementation throughout pregnancy, prevented impairments in object recognition memory in offspring from dams exposed to prenatal LPS on GD 8. The question arises as to whether zinc is protective against LPS-exposure in late pregnancy. In Chapter 3, it is further demonstrated that LPS-induced brain injury was prevented by concurrent zinc treatment at the time of LPS exposure.

In Chapter 4, the expression of activity-dependent neuroprotective protein (ADNP) mRNA was identified as a marker of changes occurring in the fetus as a result of LPS exposure in early pregnancy. ADNP has been found to be essential for organogenesis and is a sensitive indicator of brain injury. Here it was demonstrated that LPS caused a rapid increase in embryonic ADNP expression, which was highly significant 24 hours after exposure. Whether the elevation in ADNP expression is in response to inflammatory damage or is induced by cytokines released by the maternal inflammatory

response is not clear. However, a major finding of the study is that concomitant zinc treatment prevented the LPS-induced increase in ADNP activity. The mechanism of protection by zinc is presumed to be centred on preventing the fall in plasma zinc and associated fetal zinc deficiency caused by LPS induction of MT, but may also include MT-independent actions of zinc including prevention of apoptosis and oxidative damage, or enhance tissue repair processes.

Taken together the findings in this thesis support earlier evidence that maternal MT-mediated transient fetal zinc deficiency in early pregnancy underpins LPS-induced teratogenicity. This is the first study to demonstrate that this mechanism may also apply to LPS-induced neurodevelopmental damage in early and late pregnancy. However, further studies are warranted to discriminate between the influence of MT and that of other inflammatory reactants (e.g. cytokines) on LPS-mediated damage late in pregnancy. The major finding of the thesis is that zinc treatment (either given subcutaneously with LPS or as dietary zinc supplementation throughout pregnancy) prevents the negative fetal outcomes including neurodevelopmental damage caused by prenatal exposure to LPS. This finding highlights the importance of zinc nutrition in pregnancy and the benefits that might be gained as a potential prophylactic treatment to minimise fetal damage caused by infections during pregnancy.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available in all forms of media, now or hereafter known.

Joanne Chua Sing Cheng

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Signature Date

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ABBREVIATIONS

ADNP	Activity Dependent Neuroprotective Protein
BBB	Blood-brain Barrier
BV	Bacterial Vaginosis
Cg	Cingulate Cortex
CMV	Cytomegalovirus
CNS	Central Nervous System
CP	Cerebral Palsy
CWM	Central White Matter
DG	Dentate Gyrus
GD	Gestational Day
GFAP	Glial Fibrillary Acidic Protein
H	Hippocampus
IL	Interleukin
LPS	Lipopolysaccharide
MT	Metallothionein
Poly I:C	Polyriboinosonic-polyribocytidilic Acid
PVL	Periventricular Leucomalacia
SCx	Subventricular Cortex
TNF-α	Tumour Necrosis Factor-alpha
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UTI	Urinary Tract Infection

CHAPTER 1

CHAPTER 1: INTRODUCTION

1.1 INFECTION & DEVELOPMENTAL OUTCOMES

For over 200 years, prenatal infection has been linked with adverse physical and developmental outcomes in children. In 1787 William Perfect suggested that a virulent influenza epidemic sweeping the country might be responsible for an increase in psychotic behaviour among those exposed *in utero*. Fifty-eight years later, a French psychiatrist noted that insanity was independent of moral causes and was more prevalent during times of epidemic. At the turn of the 20th century, the German psychiatrist, Emil Kraepelin, commented that infections during development might be the cause of dementia praecox, which was later renamed schizophrenia. It was not until the 1940s that studies began to focus on the relationship between viral infections and profound physical and neurological outcomes.

In 1941 Sir Norman Gregg, an Australian pediatric ophthalmologist discovered that children prenatally exposed to Rubella not only had severe eye cataracts, but also displayed an increased incidence of a range of other physical deformities including deafness, cardiac, pulmonary and renal defects and mental retardation (Hertzberg, 1985). In 1988, researchers turned their attention toward more subtle psychological and behavioural outcomes associated with prenatal infection. Mednick and colleagues (Mednick *et al.*, 1988) were the first to demonstrate an increased risk of schizophrenia in

a Finnish population after the influenza pandemic in 1957. Since then, there have been many studies showing positive associations between viral infections and schizophrenia.

1.1.1 Infection-mediated Neurodevelopmental Disorders

There is now a substantial body of epidemiological evidence linking maternal viral and bacterial infection with a range of adult motor and psychiatric disorders including cerebral palsy (CP), schizophrenia, autism and mental retardation (Mednick *et al.*, 1988; Ciaranello & Ciaranello, 1995; Brown *et al.*, 2001; Pearce, 2001; Berger *et al.*, 2002; Lazzarotto *et al.*, 2003; Gibson *et al.*, 2006). The incidence and ramifications of these disorders has major impacts not only on the costs involved in medical care but also on the welfare of the families involved.

Cerebral Palsy.

Cerebral palsy was first identified in 1988 and is characterized by the aberrant control of movement or posture originating early in childhood as a result of interference or defect of the developing brain (Bell & Hallenbeck, 2002; Yoon *et al.*, 2003). It was estimated that up to 12 % of spastic CP may be due to intrauterine infection (Schendel, 2001) including chorioamnionitis and herpes viruses such as cytomegalovirus (CMV) and vericella zoster. In 1978, it was reported that low birthweight infants born to mothers with chorioamnionitis had an increased risk of CP with 12 to 39 cases per 1000 live births (Nelson & Ellenberg, 1978). In another study, premature infants born before 32 weeks to mothers with chorioamnionitis also had an increased risk of developing cerebral palsy (3% to 17%) (Murphy *et al.*, 1995). In a population based case-control study of South Australia, prenatal exposure to CMV and herpes group B virus (i.e.

vericella zoster, human herpes virus-6 and -7) were found to be associated with a 4.5% increased risk of developing CP later in life (Gibson *et al.*, 2006).

Schizophrenia.

Prenatal infection with influenza virus is commonly associated with schizophrenia. Although other viral and bacterial infections including measles (Torrey *et al.*, 1988), rubella (Brown *et al.*, 2001), varicella zoster, polio (Suvisaari *et al.*, 1999), herpes simplex type 2 (Buka *et al.*, 2001), toxoplasmosis (Brown *et al.*, 2005) and respiratory tract infections (O'Callaghan *et al.*, 1994; Brown *et al.*, 2000; Sorensen *et al.*, 2008) have been associated in single case studies, these are yet to be replicated. The prevalence of schizophrenia in the United States is 4.5 per 1000 live births with an annual incidence of approximately 15 per 100,000 live births (Tandon *et al.*, 2008). In 1988, Mednick and colleagues found that individuals born to mothers who were in their second and third trimesters of pregnancy during the 1957 influenza endemic in Finland were more predisposed to developing schizophrenia (Mednick *et al.*, 1988). Since then, a host of studies have shown positive associations throughout Europe (O'Callaghan *et al.*, 1991; Adams *et al.*, 1993; Takei *et al.*, 1993), Japan (Kunugi *et al.*, 1995) and Australia (McGrath *et al.*, 1994). In a cohort study of over four decades, Brown and co-workers found that children from mothers infected with influenza virus during early-to-mid gestation had an increased risk (up to 21-fold) of developing schizophrenia as adults (Brown *et al.*, 2004). Although numerous studies have found positive associations between influenza infection and schizophrenia, there are studies that have demonstrated negative associations presumably due to difference in classifying the origin or timing of infection and limited access to records or biological samples. For more information, the reader is referred to the manuscript by Brown and colleagues (Brown *et al.*, 2000).

Autism.

In utero exposure to viral infection has been associated with an increased incidence of non-genetic autism. The characteristics of autism include deficits in social relatedness, communication and routines / interests (Ciaranello & Ciaranello, 1995). Autism occurs predominantly in boys with an incidence of 1 in 2000 live births. During the rubella pandemic in 1964, it was reported that 8-13% of children born during this time developed autism along with other malformations associated with congenital rubella syndrome such as skeletal deformities, cardiac dysgenesis and blindness (Desmond *et al.*, 1967; Chess, 1977). Deficits of the CNS including widespread focal neuronal cell death, gliosis and vasculitis were also observed (Desmond *et al.*, 1967). Although other infections have been associated with an increased risk of developing autism, most of those reported were isolated cases. These include prenatal toxoplasmosis, syphilis, varicella-zoster, CMV, mumps and herpes simplex infection (cited in Ciaranello & Ciaranello, 1995).

Mental Retardation.

Maternal CMV infection occurring during the first trimester (≤ week 16) has been shown to result in fetal morbidity (Stagno *et al.*, 1986). The incidence of CMV infection has been reported to occur in 1 of 100 live births with 10-15 % of these exhibiting clinical symptoms such as microcephaly, perivascular calcification, micropthalmia, hearing impairment and chorioretinitis at birth (Li & Tsutsui, 2000; Lazzarotto *et al.*, 2003). Infants that are asymptomatic at birth (85-90%) may develop delayed sequelae of brain disorders including mental retardation, progressive hearing loss, neuromuscular disorder and chorioretinitis by 4 years of age on average (Pass *et al.*, 1980). On the other

hand, some studies have shown that children who remain asymptomatic after 12 months of age are unlikely to be at risk of neurodevelopmental or intellectual impairments (Conboy *et al.*, 1986; Ivarsson *et al.*, 1997).

1.1.2 Infection-mediated Fetal Dysmorphology

Apart from neurodevelopmental disorders, a plethora of epidemiological studies have also associated prenatal infection with an increased risk of physical birth defects. Congenital abnormalities and central nervous system (CNS) damage was first linked to perinatal infection in the 1960s (Patrick, 1966; Ornoy & Altshuler, 1976). In a Collaborative Perinatal Study by the National Institute of Neurological Diseases and Stroke, pregnant women with fever and bacteriuria were found to give birth to a higher incidence of infants with craniofacial and neural tube defects (Niswander & Gordon, 1972). In studies where children were born to women who experienced fever within weeks 4 to 7 in the first trimester presented with neural tube defects, in particular anencephaly (Miller *et al.*, 1978; Shiota, 1982; Shaw *et al.*, 1998) and craniofacial abnormalities including cleft lip and/or palate, micropthalmia, midface hypoplasia, external ear abnormalities and impairments of distal limb development (Pleet *et al.*, 1981).

Prenatal urinary tract infections caused by gram-negative bacterium such as bacterial vaginosis (Lehnardt *et al.*) early in pregnancy have also been shown to result in spontaneous abortion, pre-term birth and physical defects involving the eye, brain, limb and neural tube defects (Kimberlin & Andrews, 1998; Lehnardt *et al.*, 2002; Nelson & Macones, 2002; Cottrell & Shannahan, 2004). BV is an infection of the lower genital

tract where the normal hydrogen peroxide-producing lactobacillus is replaced by anaerobic and gram-negative bacteria (Kimberlin & Andrews, 1998; Carmichael & Shaw, 2000; Hansen *et al.*, 2000; McGregor & French, 2000; Culhane *et al.*, 2001; Nelson & Macones, 2002). The prevalence of BV ranges from 10 to 35% in pregnant women (Nelson & Macones, 2002) and highly predisposes women to intrauterine complications including chorioamnionitis (Martius *et al.*, 1988), which is associated with an increased risk of cerebral palsy in children (Huleihel *et al.*, 2004).

1.1.3 Animal models and timing of exposure

Whilst epidemiological studies have provided the link between maternal infection and fetal morbidity, research into the mechanisms behind infection and teratogenicity has been greatly advanced with the use of animal models. Some of the advantages in using animal models include the ability to manipulate the timing of infection as well as the magnitude (dose concentration) and route of infection. For years, animal studies have demonstrated an association between the timing of prenatal infection and fetal morbidity. Most of these studies have been simplified by using the bacterial endotoxin lipopolysaccharide (LPS) or the viral mimic polyriboinosinic-polyribocytidilic acid (Poly I:C) rather than the pathogen itself. When high intravenous doses of LPS was administered to golden hamsters on GD 8, there was 100% resorption of implantation sites (Collins *et al.*, 1994) while lower dosing regimes led to development of fetal malformations involving the eye, brain (Ornoy & Altshuler, 1976), neural crest defects, cleft palate and limb anomalies (Collins *et al.*, 1994; Carey *et al.*, 2003a). On the other hand, when either *E. Coli*, LPS or Poly I:C was administered in mid-to-late gestation, brain lesions and behavioural deficits resembling that of cerebral palsy and

schizophrenia were observed in the offspring (Young *et al.*, 1983; Yoon *et al.*, 1997b; Debillon *et al.*, 2000; Borrell *et al.*, 2002; Meyer *et al.*, 2006b).

While maternal infections during the critical period of organogenesis (GD 6 to 14 in rodents (Junaid *et al.*, 1996); Week 3 to 8 in humans) are associated with a higher incidence of craniofacial birth defects, it has been argued that later in pregnancy neurodevelopmental abnormalities are more common as the brain goes through critical phases of oligodendrocyte precursor proliferation and differentiation (GD 15 onwards in rodents (Bell & Hallenbeck, 2002); Week 23 to 32 in humans (Back *et al.*, 2001). However, other studies where LPS was administered during early-to-mid gestation have also reported behavioural deficits in the adult offspring (Table 1). Our laboratory has shown that when LPS was administered to pregnant dams on GD 8, not only was there a high incidence of fetal craniofacial abnormalities (Carey *et al.*, 2003a; Chua *et al.*, 2006) but also deficits in object recognition memory in adult offspring (Coyle *et al.*, 2008). Therefore, whilst the timing of infection during pregnancy may in part determine the type of abnormalities displayed in the offspring, other factors including the magnitude and route of infection may also be important in the outcome of the fetus.

Table 1. Summary of neurodevelopmental outcome in animal models exposed to prenatal infection.

Reference	Method of Infection	Model / Species	Route of Infection	Timing of Insult	Neurodevelopmental Outcome	Associated Disorder
Yoon et al. 1997	*E. coli*	Rabbit: NZ White	Intrauterine	Mid gestation (GD20-21)	White matter lesions accompanied by apoptosis, focal rarefaction and disorganisation of cerebral white matter, and karyorrhexis and nuclear fragmentation of glial cells.	Cerebral Palsy
Li and Tsutsui, 2000	Murine CMV	Mouse: ICR (Japan)	Intraplacental	Mid gestation (GD12.5)	GD18.5 brain contained virus-antigen positive cells in ventricular zone, subventricular zone, cortical plate and pyramidal layer of hippocampus.	Congenital CMV Infection
					PD7 offspring developed microcephaly.	
Borrell et al. 2002	LPS - bacterial endotoxin	Rat: Wistar	Subcutaneous	Alternate days throughout pregnancy	Disrupted PPI of acoustic startle response in adult offspring.	Schizophrenia
					Increased astrogliosis and microglial reactive cells in white and grey matter; Expression of MHC class II molecules by microglia and leukocytes in white matter.	
Shi et al. 2003	Human Influenza Virus	Mouse: Balb/c	Intranasal	Early-to-mid gestation (GD9.5)	Hyperanxiety in novel situations and disrupted pre-pulse inhibition (PPI) of acoustic startle response.	Schizophrenia & autism
	Poly(I:C) - viral mimic	Mouse: C57BL/6J	Intraperitoneal			
Zuckerman et al. 2004	Poly(I:C) - viral mimic	Rat: Wistar	Intravenous	Mid-to-late gestation (GD15)	Decreased latent inhibition, abnormally rapid reversal learning (analogue of excessive cognitive switching in Schizophrenia) and impaired NMDA receptor function.	Schizophrenia
Fortier et al. 2004	LPS - bacterial endotoxin	Rat: Sprague-Dawley	Intraperitoneal	Mid gestation (GD18-19)	Enhanced amphetamine-induced locomotion and acoustic startle response in adult offspring.	Schizophrenia
Golan et al. 2005	LPS - bacterial endotoxin	Mouse: C57BL/6J	Intraperitoneal	Mid-to-late gestation (GD17)	Increased number of hippocampal pyramidal and granular cells and pyramidal cell shrinkage in PD7 and 14 pup brains.	Neurodevelopmental brain bamage
					Impaired associative learning, retention memory and object recognition in adult offspring.	
Meyer et al. 2006a, b	Poly(I:C) - viral mimic	Mouse: C57BL/6J	Intravenous	Early-to-mid gestation (GD9)	Behaviour - Decreased open-field exploration & latent inhibition.	Schizophrenia, mental retardation & autism
					Neuropathology - Reduced hippocampal Reelin-positive cells and impaired neurogenesis in dentate gyrus in PD24 brain.	
				Mid-to-late gestation (GD17)	Behaviour - Impaired reversal learning.	
					Neuropathology - Reduced neurogenesis in dentate gyrus accompanied by increased level of apoptosis in PD24 brain.	

1.2 POTENTIAL MECHANISMS OF INFECTION-MEDIATED FETAL AND NEURODEVELOPMENTAL DAMAGE

Although an epidemiological relationship has been established between maternal infection, birth defects and fetal brain damage, the mechanism(s) behind this damage are unclear. Multiple factors have been postulated, some involving a direct effect of the pathogen on the fetus while others have argued alternative mechanisms including maternal inflammatory response and impaired blood flow or nutrient delivery to the fetus may be responsible for the damage. The next section will discuss these topics in more detail.

1.2.1 Direct Effects of Infection on the Fetus

Some viruses (herpes viruses and enteroviruses) can cross the placenta and infect the fetus by passing through the immature blood-brain-barrier (BBB) due to increased permeability and infect the brain, directly damaging vulnerable neural tissues. For example, herpes virus is capable of establishing itself within the brain where it can remain latent indefinitely or become reactive and cause damage (Gibson *et al.*, 2006). In addition, Borna disease virus has also been shown to directly cause neuronal cell death in the brain (Weissenbock *et al.*, 2000).

1.2.2 Maternal Inflammatory Response

Other viral and bacterial agents (such as influenza, chorioamnionitis and gram negative bacteria from BV) that do not cross the placenta can also cause damage to the fetus. Studies with bacterial endotoxin and viral mimics such as LPS and PolyI:C, respectively, have demonstrated that these agents are capable of inducing

damage to the developing fetus even in the absence of a pathogen. While LPS has been reported to cross the placenta and was detectable in the fetus 1 h after maternal administration of ^{125}I-labelled LPS (Kohmura *et al.*, 2000), this finding was opposed by a similar study where ^{125}I-labelled LPS was detectable only in maternal tissues and placenta, but not in the fetuses, 2 – 8 h after treatment (Ashdown *et al.*, 2006). This indicates that the maternal inflammatory response may be the common underlying factor. The primary host response to infection is the recruitment of immune cells that in turn initiate the release of proinflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor-alpha (TNF-α). Both LPS and PolyI:C treatment has been shown to cause a rapid increase in these cytokines in the maternal serum of pregnant mice (Kohmura *et al.*, 2000; Leazer *et al.*, 2002; Meyer *et al.*, 2006a).

It is recognized that cytokines released by the maternal immune system can cross the placenta into the fetal circulation where they may induce fetal death and impede fetal growth. Moreover, these cytokines have been shown to be present in the maternal amniotic fluid where intrauterine infection was present (Yoon *et al.*, 1997a; Yoon *et al.*, 2003). Cytokines that cross the fetal BBB may alter the permeability of the BBB to other inflammatory factors, which may further induce the production of cytokines by fetal immune system and neural cells including microglia and astrocytes that in turn may cause oligodendrocyte progenitor cell death leading to the development of white matter lesions (Dammann & Leviton, 1997; Rezaie & Dean, 2002). Yoon and colleagues found that white matter lesions were observed in newborns from mothers with high levels of proinflammatory cytokines (IL-6 and TNF-α) in the amniotic fluid and in the umbilical cord plasma but not in newborns born to mothers with normal cytokine levels (Yoon *et al.*, 1997a; Yoon *et al.*, 2003). In addition, Cai and

co-workers reported that exposure to LPS at late in pregnancy (GD 18) not only induced increased expression of IL-1β and TNF-α mRNA in the fetal brain but also increased astrogliosis and decreased myelin basic protein (a marker of oligodendrocytes) (Cai *et al.*, 2000). Moreover, systemic infection with either LPS or Poly I:C from GD15 onwards have also been shown to result in impaired neurogenesis and enhanced apoptosis and astrogliosis in the white matter of fetal and pup brains (Borrell *et al.*, 2002; Meyer *et al.*, 2006a; Meyer *et al.*, 2006b).

1.2.3 Impaired Blood Flow and Nutrient Delivery to the Fetus

It has been argued that TNF-α released in response to inflammation causes vasoconstriction of the placental vasculature, which during the critical periods of gestation could be detrimental to the developing embryo by impairing placental blood flow and nutrient delivery to the fetus. In this regard, LPS has been shown to potentiate the degree of brain injury in neonatal rats following a brief period of hypoxia-ischemia (Eklind *et al.*, 2001; Yang *et al.*, 2004). Vasoconstrictions of blood vessels may also impair delivery of essential nutrients to the fetus during critical periods of organogenesis increase the risk of growth retardation and birth abnormalities in the offspring.

1.2.4 Induction of Acute Phase Reactant Following Maternal Inflammatory Response

Other reactants of the maternal inflammatory response may also be detrimental to fetal development. In response to proinflammatory cytokines released during infection, the maternal liver undergoes an acute phase response resulting in a change

in the composition of its proteins. Increased synthesis of C-reactive protein, complement factors, ferritin, serum amyloid A and ceruloplasmin are well recognized following an inflammatory challenge. One acute phase reactant is the intracellular zinc-binding protein metallothionein (MT), which has been postulated to play a major role in birth abnormalities caused by prenatal LPS and alcohol exposure (Daston *et al.*, 1991; Taubeneck *et al.*, 1994; Carey *et al.*, 2000b; Carey *et al.*, 2003a). MT has been found to be induced in the liver in a dose-dependent manner by various effectors including LPS, cytokines including IL-1β, IL-6 and TNF-α (Sobocinski & Canterbury, 1982; Liu *et al.*, 1991; Taubeneck *et al.*, 1995) and by a variety of chemicals such as urethane (Daston *et al.*, 1991; Taubeneck *et al.*, 1994), alpha-hederin (Daston *et al.*, 1994; Taubeneck *et al.*, 1994), ethanol, malphalan (Taubeneck *et al.*, 1994), corticosteroids (Bracken & Klaassen, 1987a; Jacob *et al.*, 1999) and metals (Bracken & Klaassen, 1987b; Klaassen & Liu, 1998a).

In 1994, Daston and co-workers hypothesized that developmental toxicity may, in part, be mediated by changes in zinc metabolism as a result of maternal hepatic MT induction (Daston *et al.*, 1994). In this benchmark study in rodents, they showed that exposure to alpha-hederin on GD 8 resulted in 11 to 15-fold increase in maternal hepatic MT, which corresponded with an increase in zinc retention in the liver, a 75% decrease in plasma zinc and a significant decrease in zinc uptake by the conceptus. Concomitant with this fetal zinc deficiency was a higher incidence of abnormal and low birth weight fetuses. Later, our group demonstrated that wildtype (MT +/+) dams retained double the concentration of ^{65}Zn in the liver compared to MT-knockout mice lacking the MT-gene (MT -/-) after alcohol (a strong hepatic MT inducer) exposure on GD 12 (Carey *et al.*, 2000a). In contrast, MT -/- fetuses and placentas retained significantly more ^{65}Zn compared to MT +/+ fetuses and placentas

following alcohol treatment. Recent findings in our laboratory have further supported the MT-mediated zinc redistribution as a causal factor in LPS-induced teratogenicity (Carey *et al.*, 2003a). In this study, LPS administration on GD8 produced a 30-fold induction of maternal hepatic MT coinciding with an 80% decrease in plasma zinc. Most importantly, this was associated with significantly more abnormal fetuses from MT +/+ dams compared to fetuses from MT -/- dams, which showed no more birth defects than saline treated controls. Altogether these findings suggest a major role of MT in LPS-mediated teratogenicity involving zinc redistribution into the maternal liver and fetal zinc deficiency as the major cause of teratogenicity.

An overview of how the maternal inflammatory response to prenatal infection may cause birth defects and neurodevelopmental damage is presented in Figure 1.

The importance of zinc for fetal growth and development will be discussed in the next section.

Figure 1. Proposed mechanism behind maternal infection and development of birth defects and neurodevelopmental damage.

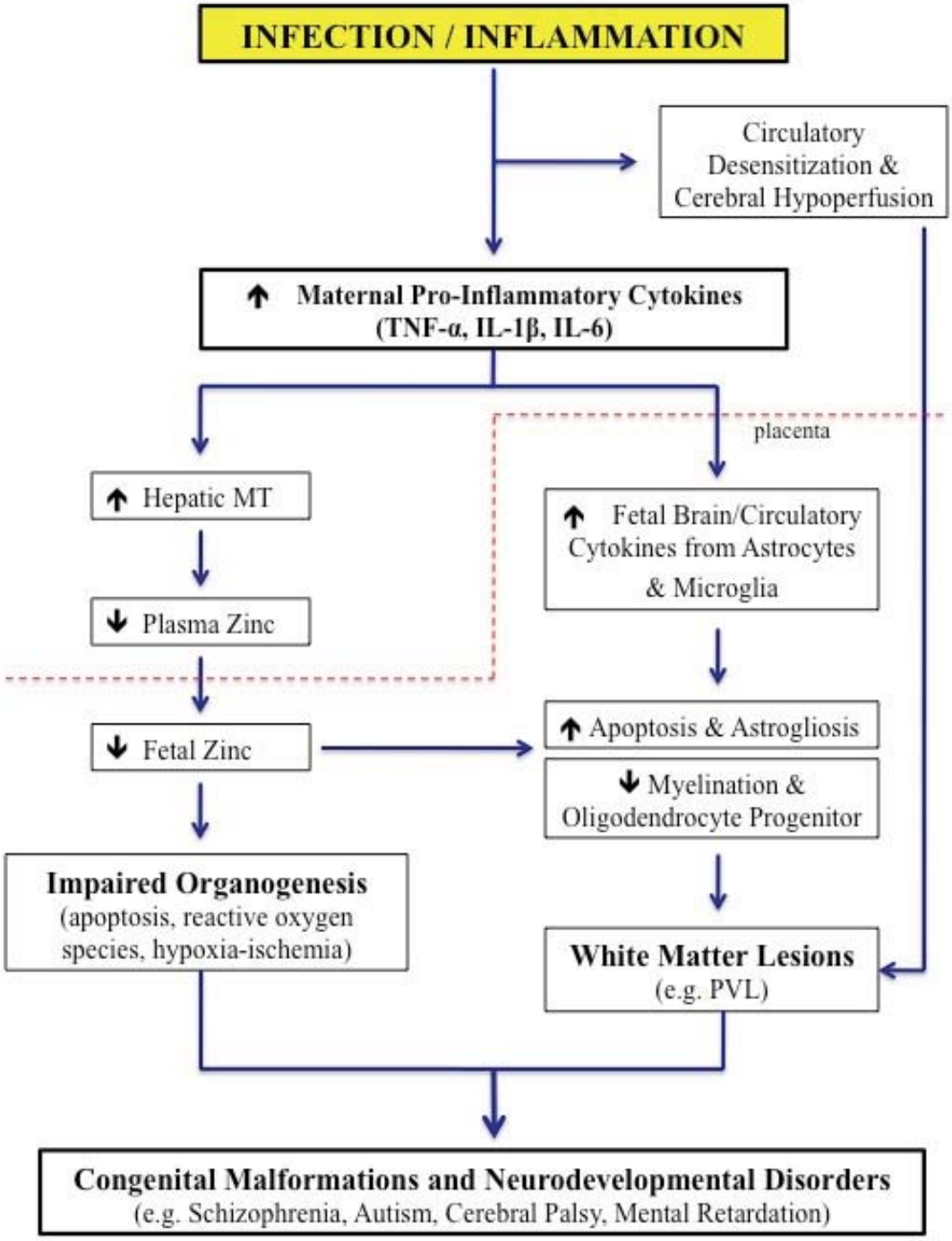


Figure 1: Early in infection, proinflammatory cytokines are released from maternal immune system. These cytokines may activate the acute phase response in the maternal liver, which includes inducing MT. The increase in hepatic MT leads to the sequestration of plasma zinc into the liver resulting in a fall in plasma zinc decreasing zinc supply to the fetus. As it has been shown that placental zinc uptake can not occur against a concentration gradient (Beer *et al.*, 1992), the nett result is that the fetus becomes deficient in zinc, which directly impairs fetal development. Alternatively, cytokines released from the maternal circulation may cross the placenta and permeate the immature fetal BBB. TNF-α is capable of increasing the permeability of the fetal BBB thus providing access to other inflammatory mediators. The fetal brain may further release cytokines from astrocytes and microglia resulting in astrocyte hypertrophy, oligodendrocyte progenitor cell death and impaired myelination leading to brain white matter lesions. These lesions can also be caused by hypoxic-ischemic cell death as a result of maternal circulatory desensitization and cerebral hypoperfusion. No one mechanism can be discounted at this time and direct and indirect effects of the infectious agent may act in combination to result in offspring with congenital malformations and neurodevelopmental disorders.

1.3 ZINC

It is well established that zinc plays an integral part in numerous physiological processes both in humans and animals. Zinc is involved in the regulation of gene expression, endocrine function, DNA and RNA synthesis, cell division and development of immune function, all of which are necessary for maintaining normal growth and development. The importance of zinc is recognized in its involvement in more than 300 catalytically active zinc metalloproteins and in more than 200 transcription factors involved in gene expression of various proteins required for normal biological functions (Prasad, 1993a, b) whereby zinc confers structural stability as well as catalytic and regulatory roles.

1.3.1 Zinc Biochemistry

In biological systems, zinc exists almost exclusively in the form of a divalent cation (Zn^{2+}). As free Zn^{2+} readily forms insoluble hydroxide at physiological pH there is very little free Zn^{2+} in the body (reviewed by Whitehead *et al.*, 1996). Consequently, Zn^{2+} is mainly associated with cellular proteins where most processes are carried out via zinc exchange from lower affinity to higher affinity Zn-binding sites. There are three distinguished features of zinc that are imperative to the biological function. Firstly, zinc is non-toxic when consumed within the reference dose (Maret & Sandstead, 2006), which was determined to be up to 17.5 mg per day for a 70 kg man and 15 mg per day for a 60 kg woman based on zinc supplements that might be 95 % absorbed. In rats, the oral LD_{50} is 237-623 mg/kg, which is significantly higher than the highest dose (100 mg/kg) of zinc used in the study, as discussed in Chapter 2. In

previous studies on mice fed 200 mg/kg dietary zinc throughout pregnancy have found no evidence of adverse effects on fetal resorptions, fetal dysmorphology, birth weight or cognition in adult offspring (Coyle *et al.*, 2009b; Summers *et al.*, 2009). Furthermore, previous studies with non-pregnant mice using levels up to 400 mg/kg zinc have also found no noticeable adverse effects (Tran *et al.*, 1998). The lack of zinc toxicity is largely due to the exquisite efficacy of homeostatic mechanisms that regulates its absorption, excretion and distribution between cells and tissues.

Secondly, zinc is redox inert. It does not undergo oxidation or reductions, which renders it stable in biological environments where there is a continual flux between the oxidised and reduced state. Thirdly, zinc is a small atom of high positive charge with an electronic configuration that allows it to assume five different coordination geometries, thus allowing versatility in regards to structural confirmation. Taken together, the dynamic physicochemical properties of zinc make it essential for the function of many proteins, enzymes and transcription factors involved in a plethora of biological processes. These will be discussed in the following sections.

1.3.2 Zinc-containing Enzymes

Zinc-containing enzymes have been identified across all six enzyme classification groups, including oxidoreductase, transferase, hydrolases, lyase, isomerase and ligases, since the 1940s (Vallee & Falchuk, 1993). For the vast majority, zinc plays a catalytic role where the zinc atom binds directly to specific ligands. Prominent examples of zinc-containing catalytic enzymes include alcohol dehydrogenase, angiotensin converting enzyme, carboxypeptidases, thermolysine, phospholipase C, alkaline phosphatase and collagenase (Vallee & Falchuk, 1993). Other enzymes that

fall into this category include gene-regulating enzymes such as RNA polymerase, reverse transcriptase, tRNA synthethase and DNA topoisomerase. Zinc also plays a co-active (or co-catalytic) function in zinc enzymes where zinc atoms either enhance or diminish the catalytic function. In other enzymes, zinc atoms are required solely for structural stability of quaternary structures. Enzymes included in this category include protein kinase C, aspartate transcarbamylase and alcohol dehydrogenase (which also contains catalytic zinc atoms).

1.3.3 Zinc-containing Proteins

Metal binding thioneins and gene regulatory proteins have also been recognised to contain functionally important zinc atoms. The first class of zinc proteins, metallothionein, appears to be involved in zinc homeostasis and will be discussed in detail in Section 1.4. Among the gene regulatory proteins, TFIIIA from *Xenopus laevis oocytes* was the first transcription factor to be identified as a zinc protein as it requires zinc to activate RNA transcription (Hanas *et al.*, 1983). Subsequent analysis of the structure have identified a tetrahedral DNA-binding motif in TFIIIA, now commonly known as a zinc finger (Miller *et al.*, 1985). The zinc finger motif consists of a loop structure DNA-binding site (in which a zinc atom binds to) between the pairs of conserved Cys and His residues (Miller *et al.*, 1985). Prime examples of zinc finger proteins are g32p in bacteriophage T4, Zif28 in mice (Vallee & Falchuk, 1993), Sp1 in humans (Kadonaga *et al.*, 1987) and Kruppel in Drosophila (Rosenberg *et al.*, 1986).

Two other classes of DNA-binding zinc proteins have been identified. These are the zinc twists and zinc clusters, which are distinguished by the difference in the

surrounding amino acid conformations and the interatomical distance between zinc atoms (Vallee *et al.*, 1991). The glucocorticoid and estrogen receptors are well-known examples of zinc twists, whereas the fungal transcription factor GAL4 is among the first zinc cluster to be identified. Structurally, both classes of proteins contain two zinc-binding sites that bind solely to cysteine residues, which are arranged in a "cluster" around the zinc atoms (Vallee *et al.*, 1991). It has been demonstrated that removal of zinc atoms either by genetic modification (Johnston, 1987) or chelation (Freedman *et al.*, 1988) from these proteins prevents DNA from binding and halts transcription.

For a more in-depth discussion on zinc biochemistry, the reader is directed to the review by Vallee and Falchuk (1993).

1.3.4 Dietary Zinc and Zinc Homeostasis

Dietary zinc is obtained mainly from red meat followed by pork, chicken, fish, milk, cheese, legumes and wholegrain products. The daily zinc requirements vary depending on age, growth stage and the extent of loss through excretions. In a human adult, the average recommended intake is 10 – 15 mg per day and up to 20 – 25 mg per day during pregnancy (Prasad, 1983; Falchuk, 1991). Zinc (approximately 2-3g per 70kg man) is distributed predominantly in the muscle, bone, skin and liver (in descending order) with only a fraction of the total body zinc found in the blood.

The regulation of zinc homeostasis occurs primarily in the small intestines where dietary zinc is absorbed (mainly in the jejunum and ileum) and is largely balanced by excretion of endogenous zinc through the pancreas and secretion across the intestinal

wall from the serosa to the lumen. Zinc absorption responds rapidly to changes in dietary zinc and its efficiency is increased at low zinc intakes and during pregnancy, but decreases when dietary zinc is excessive (Cousins, 1985). Although the homeostatic mechanisms are highly efficacious, the exact mechanisms underlying this process are not fully understood. Zinc absorption appears to occur via both passive diffusion and carrier-mediated processes (Davies, 1980; Hoadley *et al.*, 1987). In the past decade at least nine ZnT transporters and fifteen Zip transporters have been identified to be involved in transmembrane zinc movements (reviewed by (Liuzzi & Cousins, 2004).

Zinc excretion appears to be the major modulator of zinc balance under normal physiological conditions. Zinc is excreted mainly through pancreatic secretions and to a lesser extent via urinary losses and sweat (Vallee & Falchuk, 1993). The amount required to replenish the losses and to maintain zinc balance in the system is obtained through dietary intake. Zinc in the whole blood represents only 0.5% of total body zinc and only 0.1% is contained in the blood plasma (Cousins, 1989; Vallee & Falchuk, 1993), which acts as the main conduit of zinc to maternal tissues and to the fetus. Approximately 84 % of plasma zinc is bound to albumin and this represents part of a larger exchangeable zinc pool; the remaining 15 % is tightly bound to α2-macroglobulin with 1 % attached to other proteins and amino acids (Tapiero & Tew, 2003). Thus the plasma compartment is exquisitely sensitive to changes in zinc homeostasis. As the exchangeable zinc pool falls, its ability to donate zinc to other tissues is reduced. This is particularly important in pregnancy where zinc movement across the placenta is bidirectional and does not proceed against a concentration gradient (Beer *et al.*, 1992).

1.3.5 Zinc Deficiency in Pregnancy

It is important that zinc intake is adequate during pregnancy as zinc plays an integral role in fetal growth and development. It has been estimated that dietary zinc intake is below the recommended levels (15 mg per day) in 82% or pregnant women worldwide (Caulfield *et al.*, 1998). The consequences of maternal zinc deficiency in pregnancy are well documented and include spontaneous abortion, intrauterine growth retardation, premature rupture of membranes and congenital malformations. Long-term impairments in children born to zinc-deficient mothers are often associated with decreased immunity and cognitive deficits (Caulfield *et al.*, 1998; Salgueiro *et al.*, 2002).

Animal studies clearly implicate zinc deficiency during pregnancy with teratogenicity. In 1966, Hurley and Swenerton (1996) found that rats fed a zinc-deficient diet during pregnancy had an increased number of resorptions and produced fetuses with birth abnormalities involving the eye, brain, limbs, tail, vertebrae and urogenital system. Similarly, others have shown that zinc deficiency at varying times during pregnancy can result in fetuses with low birth weight as well as multiple skeletal defects, retarded growth of major organs and impaired immunological function (Hurley *et al.*, 1971; Hickory *et al.*, 1979; Beach *et al.*, 1982).

Gross abnormalities of the brain such as hydrocephaly, anencephaly and microcephaly are classic outcomes of maternal/fetal zinc deficiency. More recently, studies have indicated that more subtle neuronal damage, which may cause lifelong cognitive and behavioural problems, may also be caused by zinc deficiency. In one such study, pregnant dams that were fed a zinc deficient diet (7 μg Zn/g diet)

throughout pregnancy produced pups with reduced hippocampal and whole brain expression of the NMDA receptor and brain-derived neurotrophic factor (BDNF), both of which are critical for normal cognitive function. Moreover, these changes were found to persist into adulthood (Chowanadisai *et al.*, 2005). In addition, they also showed a reduction in polysialic acid neural cell adhesion molecule (PSA-NCAM) expression and PSA-NCAM positive cells in the granule cell layer of the dentate gyrus in post-natal pup brains that were exposed to prenatal zinc deficiency. These findings indicate that zinc is required for the proliferation or generation of neurons, and that zinc deficiency in pregnancy may have profound effects on neurodevelopment, which in the long-term could cause deficits in learning and memory in later life.

1.3.6 Teratogenic Similarities between Prenatal Zinc Deficiency and Infection

In animal studies, there are close similarities in teratogenic outcomes between zinc deficiency during pregnancy and the maternal inflammatory response to infection (Table 2). Increased incidence of physical birth defects and impairments in behaviour and cognition in offspring are common to both. When pregnant rats were fed a zinc deficient diet or exposed to endotoxin in early-to-mid gestation both resulted in fetuses of low birth weight as well as a high incidence of brain, limb and urogenital defects (Hurley *et al.*, 1971; Ornoy & Altshuler, 1976). These striking similarities are suggestive of a common zinc-mediated etiology. It is proposed that this common mechanism involves an infection-mediated induction of the zinc-binding protein MT and this protein will be discussed in more detail in the following section.

Table 2. Teratogenic similarities between prenatal zinc-deficiency and LPS exposure.

	PRENATAL LPS EXPOSURE			PRENATAL ZINC DEFICIENCY		
	Animal model / Reference	**Timing of Exposure**	**Fetal and Neurodevelopmental Outcome**	**Animal Model / Reference**	**Time of Deficiency**	**Fetal and Neurodevelopmental Outcome**
Fetal Birth Abnormalities	Rat (Ornoy & Altshuler, 1975)	Early to mid gestation	Microcephaly, micropthalmia, anopthalmia, hydrocephaly, severe brain displasia, impaired development of brain ventricles and urogenital ridge, and hydronephrosis.	Rat (Hurley & Swenerton, 1966)	Throughout pregnancy (GD0 - 21)	Low birth weight; Misshapen head, clubbed feet, fused / missing digits and short lower jaw; Skeletal defects include fused ribs, curved spinal cord, missing tail vertebrae and incomplete ossification of ribs and vertebrae; Soft tissue malformations include brain defects, micro- or anopthalmia, hernias, and heart, lung and urogenital abnormalities.
				Rat (Hurley et al. 1971)	Early to mid gestation (GD6-12)	
	Hamster (Collins et al. 1994)	Early gestation (GD8)	Neural crest defects, cleft palate and limb malformations.	Rat (Hickory et al. 1979)	Early to mid gestation (GD3-15)	Low birth weight and multiple skeletal defects involving the vertebrae, ribs and long bones.
				Mouse (Beach et al. 1982)	GD7 to parturition	Increased mortality, fetal growth retardation, low birth weight, retarded growth of major organs and impaired immunological function.
Fetal Brain Abnormalities	Rat (Cai et al. 2000)	Late gestation (GD18)	Increased GFAP-positive astrocytes, delayed myelination or decreased number of oligodendrocytes, and altered immunoreactivity of microglia.	Guinea Pig (Browning & O'Dell, 1995)	Weanlings	Decreased number of functional NMDA receptors in cortical synaptic membranes.
	Sheep (Duncan et al. 2002)	Mid-to-late gestation	Cerebral white matter injury ranging from diffuse subcortical damage to periventricular leukomalacia.	Rat (Chowanadisai et al. 2005)	Throughout pregnancy to PD20	Decreased expression of hippocampal and whole brain NMDA receptor, polysialic acid-neural cell adhesion molecule (PSA-NCAM), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in PD2 pups through to PD65.
	Rat (Bell & Hellenbeck, 2002)	Mid-to-late gestation (GD15)	Increased TUNEL+ nuclei in periventricular area, focal areas of TNF-alpha immunoreactive cells and decreased number of oligodendrocytes within the corpus callosum.			
	Rat (Rousset et al. 2006)	Late gestation (GD19-20)	Increased apoptosis, astrogliosis, and hypomyelination in multiple white matter sites.	Rat (Mackenzie et al. 2007)	PD0 to 21	Impaired cerrebelar development, dendritic differentiation and delayed maturation of Purkinje cells.

1.4 METALLOTHIONEIN (MT)

MT was first isolated from equine renal cortex by Margoshes and Vallee in 1957 and subsequently identified in the liver and kidneys of various animal species (Oh *et al.*, 1978). MT is an intracellular, low molecular weight (< 7000 Da) protein with a highly conserved 18 – 23 cysteine residue and a high affinity for zinc (Bracken & Klaassen, 1987b; Jacob *et al.*, 1999; Coyle *et al.*, 2002). There are four isoforms of MT arranged in tandem, 6 kb apart on mouse chromosome 8 (Palmiter, 1987). Of the four isoforms, MT-1 and -2 are most widely expressed in mammals whereas MT-3 is brain specific (Palmiter *et al.*, 1992) and MT-4 is expressed mainly in the squamous epithelium (Quaife *et al.*, 1994). As the latter two isoforms were only discovered more recently, their functions have not been elucidated and most studies have focused on MT-1 and -2, which are the only two inducible isoforms in the liver. For the remainder of this thesis the abbreviation MT will be used to describe MT-1 and -2 only.

1.4.1 Possible Roles of MT

Over 40 years, research into MT has yielded extensive information on the protein but a definitive role of MT is yet to be assigned. The zinc binding capacity of MT as well as its unique chemical structure and amino acid composition makes it a suitable candidate for a number of biological roles including zinc absorption and homeostasis, heavy metal detoxification and cellular free radical scavenger.

In 1993, Michalska and Choo produced an MT-knockout mouse of 129 OLA/C57BL6J genetic backgrounds where both the MT-1 and MT-2 genes were

nullified (MT -/-). This mouse as well as the MT -/- mouse of 129/SvCPJ genetic background (Kelly *et al.*, 1996)has since been used extensively to investigate Zn homeostasis in the absence of the inducible forms of MT. Although the MT -/- mouse has been found to be resilient to most challenges, the MT +/+ mice appears to have an advantage at times of zinc deficiency and zinc excess (Kelly *et al.*, 1996). In this regard, our group has demonstrated that in a zinc deficient state, MT +/+ mice absorb a greater proportion of zinc from solid food as well as from an orally administered ^{65}Zn solution compared to MT -/- mice (Coyle *et al.*, 1999, 2000b). This is thought to be due to a greater proportion of exchangeable zinc attached to MT in tissues of MT +/+ mice. Conversely at times of excess zinc, MT +/+ mice are more efficient in restricting zinc uptake and enhancing zinc excretion compared to MT -/- mice. In the zinc excess state, high levels of oral zinc can induce MT in the intestine leading to binding of zinc, thus restricting its absorption. Zinc-MT in the intestinal mucosa is later transported back to the lumen for excretion (Coyle *et al.*, 2002). Moreover, in MT +/+ mice pancreatic MT has been shown to protect against zinc deficiency and prevent the toxic effect of excess zinc on the pancreas (Dalton *et al.*, 1996; Kelly *et al.*, 1996).

MT has also been implicated in heavy metal detoxification due to its metal binding capacity, especially with respect to cadmium. Previous studies have demonstrated that while MT -/- mice are more susceptible to cadmium-induced hepatotoxicity compared to MT +/+ mice, MT -/- cells also exhibited enhanced cytotoxic effects following exposure to cadmium *in vitro,* which was protected by MT induction (Klaassen & Liu, 1998b; Coyle *et al.*, 2000a).

In addition, MT may also play a role as an antioxidant by facilitating the binding and neutralization of free radical molecules. Various studies have shown that a range of agents that generate free radicals (antineoplastic drugs, ultraviolet B radiation and gamma irradiation) are more damaging, both *in vivo* and *in vitro*, in the absence of MT (Kondo *et al.*, 1997; Liu *et al.*, 1998; Deng *et al.*, 1999; Hanada, 2000; Cai & Cherian, 2003; Wang *et al.*, 2004; Yang *et al.*, 2008).

1.4.2 MT and Inflammation

It is well established that MT is dramatically induced in response to tissue injury, infection, inflammation and disease. In this context, MT is an acute phase reactant that remains within the cell and is not secreted into the blood. In the liver, MT is induced as early as 2 – 4h after LPS injection and this is thought to be mediated primarily through the cytokine IL-6 (Liu *et al.*, 1991; Coyle *et al.*, 2002). This hepatic accumulation of MT in the early phase of inflammation consequently results in hypozincemia due to the movement of zinc from the plasma component to the liver, thus promoting a flux from zinc-donor tissues. Early experiments in our laboratory demonstrated that the hepatic accumulation of zinc is dependent on MT synthesis as LPS injection does not cause the accumulation of zinc in the liver of MT -/- mice (Philcox *et al.*, 1995).

Several potential benefits have been proposed for the whole body zinc redistribution that occurs in infection. These include:

1) Decreasing plasma zinc to enhance leukocyte function by stimulating cytokine production (Scuderi, 1990),

2) Sequestering zinc into the liver to provide sufficient zinc for maximal activity of zinc-dependent liver enzymes (Coyle *et al.*, 2002),
3) Providing a source of exchangeable zinc that can be released to allow repair processes to proceed once the inflammation stage of infection has abated (reviewed by Coyle *et al.*, 2002).

1.4.3 Induction of MT

MT-1 and MT-2 are induced in the liver by a variety of heavy metals, cytokines, glucocorticoids and xenobiotics. Although metals including Zn, Cu, Cd, Hg, Au and Bi have been found to induce MT, Zn is thought to be the primary physiological inducer of MT due to its relative availability in the cell compared with other metals, which with the exception of Cu, are only present in sufficient quantities in cells when the environment is contaminated by this metal.

An overview of hepatic MT gene regulation is presented in Figure 2.

Figure 2. Overview of hepatic MT gene induction during infection/inflammation.

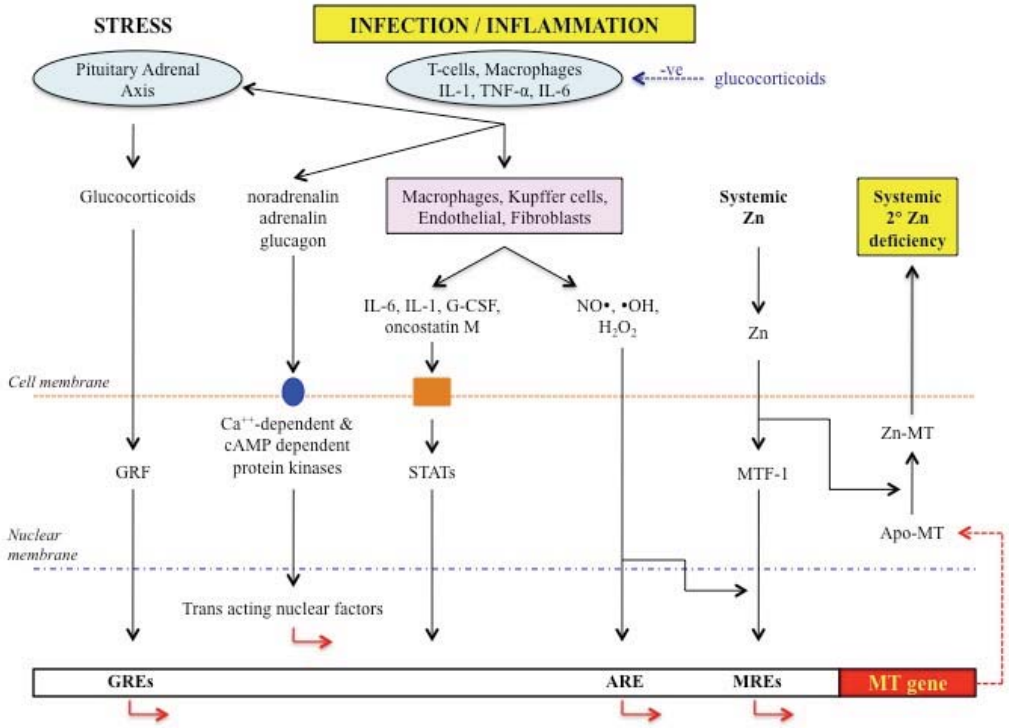


Figure 2: A plethora of factors act additively and synergistically on MT-gene transcription to regulate MT synthesis in response to infection and inflammation. Cytokines such as IL-1, IL-6 and TNF-α released from activated T-cells and macrophages activate the acute phase proteins by inducing tyrosine phosphorylation of signal transducers and activator proteins (STATs) that interact with sites on the promoter region of the MT gene to initiate transcription. Concomitantly, the pituitary/adrenal axis is stimulated by stress and the above cytokines leading to an increase in glucocorticoids that bind to the cytoplasmic glucocorticoid receptor complex (GRF), which in turn activates the GRE on the MT gene, thus increasing MT levels. As MT levels rise, zinc is sequestered into the liver thus increasing the MT-bound labile zinc pool. This in turn activates the MREs through the MTF-1 in a positive feedback loop. Cathecholamines and glucagons induced during infection also regulate the induction of MT via second-messenger systems that activate the trans-acting nuclear factors, which interact with yet to be identified control elements. Reactive oxygen species released during the inflammatory response may interact with the antioxidant response element (ARE) and MREs to further induce liver MT. It has also been proposed that the increase in glucocorticoids in the late stages of inflammation suppress the production of cytokines, which in turn inhibit further induction of MT synthesis thus restoring normal zinc homeostasis (Min *et al.*, 1992). Figure and data taken from the review by Coyle *et al.* (2002).

1.4.4 MT in pregnancy

MT induction occurs as a normal physiological response to pregnancy. Maternal elevations in hepatic MT occur in mid-to-late pregnancy peaking at GD 15 and decline rapidly towards parturition (Coyle *et al.*, 2009a). It is well established that fetal MT-gene expression is also coordinately programmed during pregnancy with fetal liver MT increasing rapidly late in pregnancy (from GD 17) and remain high early in postnatal life (Panemangalore *et al.*, 1983). It is argued that the change in MT expression is to maximize zinc availability for fetal growth and development (Panemangalore *et al.*, 1983).

1.4.5 Inappropriate MT induction

Inappropriate induction of maternal liver MT in early pregnancy can cause fetal zinc deficiency resulting in teratogenicity, particularly during the critical window of organogenesis (GD 6 to 14 in rodents (Junaid *et al.*, 1996), equivalent to Weeks 3 to 8 in humans) (Figure 3a, b, c).

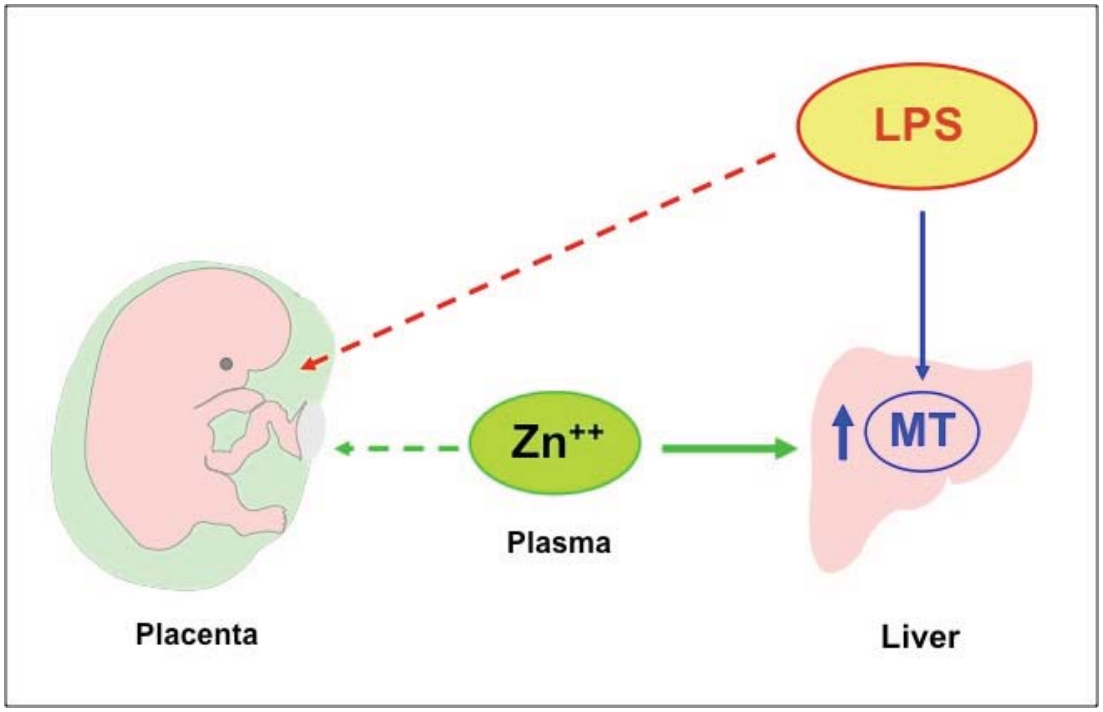
Various studies have shown that teratogens including urethane, α-hederin, 6-mercaptopurine, alcohol, TNF-α and valproic acid produce an increase MT and impair the transfer of zinc from the mother to the fetus when administered during the organogenic period (Amemiya *et al.*, 1989; Keen *et al.*, 1989; Daston *et al.*, 1991; Daston *et al.*, 1994; Taubeneck *et al.*, 1994; Taubeneck *et al.*, 1995; Carey *et al.*, 2000b). Studies in this laboratory using both MT +/+ and MT -/- mice have demonstrated that when pregnant dams were exposed to ethanol or LPS on GD 8, there was a significant increase in liver MT and a concomitant decrease in plasma

zinc in MT +/+ dams whereas plasma zinc was increased in MT -/- dams (Carey *et al.*, 2000a, b; Carey *et al.*, 2003a). In addition, not only was there a decreased transfer of zinc to fetuses in MT +/+ dams, but whole fetal Zn concentration were also significantly less compared to fetuses from MT -/- dams (Carey *et al.*, 2000a). This state of transient fetal zinc deficiency coincided with a higher incidence of abnormal fetuses from normal dams when examined at GD18 while MT -/- fetuses were not affected (Carey *et al.*, 2000b, 2003b). Most importantly, a single subcutaneous zinc injection at the time of exposure to LPS was found to prevent these birth abnormalities (Carey *et al.*, 2003a), presumably by providing sufficient zinc in the fetal blood to counteract that taken out of the blood as a result of zinc sequestration in the liver by MT. This was the first evidence that zinc treatment may ameliorate fetal dysmorphology caused by prenatal LPS administration and further supports the premise that LPS-induced teratogenicity is caused by zinc-deficiency secondary to the MT-mediated mechanism.

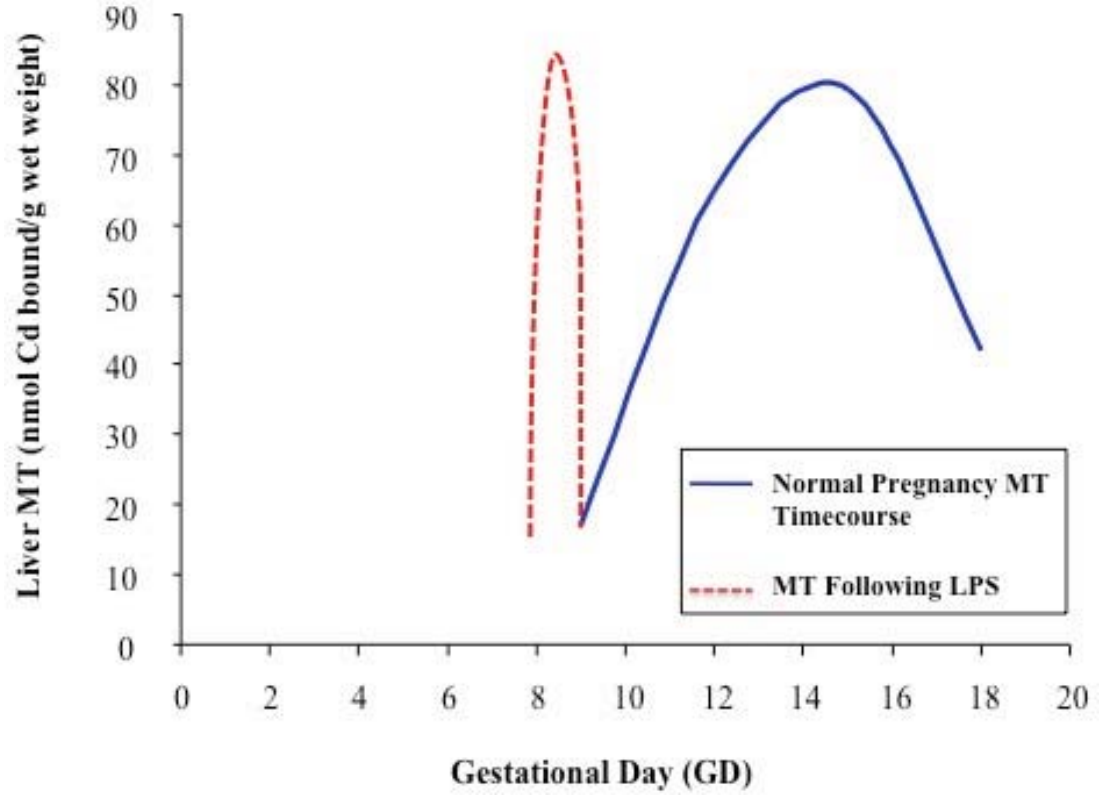
Thus, the question arises as to whether a more physiological approach to zinc treatment would be as effective in preventing infection-mediated teratogenicity. In this regard, dietary zinc supplementation has been safely used in pregnancy with an associated increase birth weight and reduced preterm labor, with variable results (King, 2000; Salgueiro *et al.*, 2002). Whether dietary zinc supplementation can prevent the negative fetal outcomes caused by prenatal exposure to LPS has not previously been investigated and is one of the aims of this thesis.

Figure 3. Inappropriate induction of liver MT during pregnancy.

a.



b.



c.

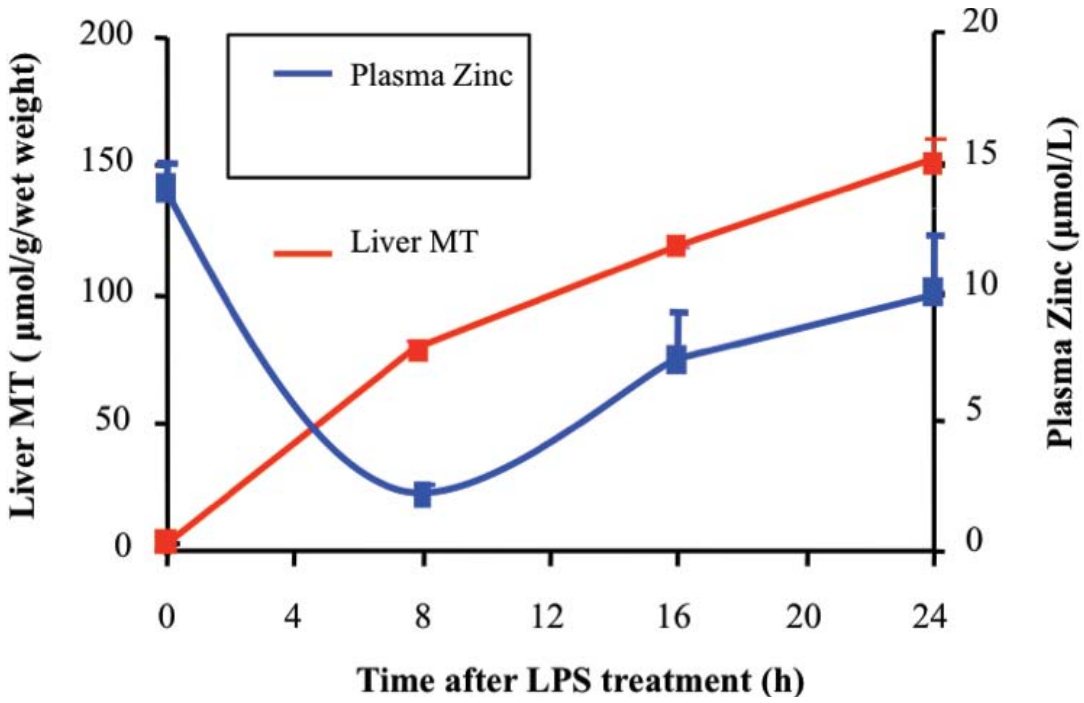


Figure 3 a, b and c. During pregnancy, exposure to LPS results in a rapid induction of maternal liver MT as a result of the maternal inflammatory response (b). This leads to a sequestration of zinc from the plasma compartment into the liver by MT, which in turn decreases zinc transfer to the fetus resulting in a transient fetal zinc deficiency (a). Previous work in our laboratory has shown that plasma zinc decreases by 4-fold as a result of maternal liver MT induction 8 h after LPS treatment (Carey *et al.*, 2003a) (c).

1.4.6 Timing of Inappropriate MT Induction

The timing of maternal inflammatory response to infection may also be critical in regard to the mechanism underlying LPS-mediated fetal damage. Our group has argued that when the maternal liver MT peaks during pregnancy (i.e. on GD 15) (Coyle *et al.*, 2009a), it is unlikely that MT can be induced further even in the presence of LPS. This is based upon preliminary evidence by Carey et al. (Carey *et al.*, 2000a) who found that MT synthesis was not increased further following ethanol administration on GD 12. However, recent findings indicate that MT induction in response to pregnancy falls rapidly after GD 15 (Coyle *et al.*, 2009a) suggesting that maternal insult after this time may cause induction of MT and therefore effect zinc homeostasis. While it is clear that fetal dysmorphology caused by LPS is associated with an MT-mediated mechanism in early pregnancy (Carey *et al.*, 2003a; Chua *et al.*, 2006), there is no such evidence of its involvement in late pregnancy. Nonetheless, several studies have demonstrated that brain damage may result from infections in both early and late pregnancy (Golan *et al.*, 2005; Meyer *et al.*, 2006b; Coyle *et al.*, 2008). Thus a second aim of this thesis is to identify markers of neurodevelopmental damage in early and late pregnancy and to investigate whether any infection-mediated damage could be moderated by zinc treatment.

1.5 HYPOTHESIS AND AIMS

The central hypothesis is that birth defects including abnormal neurodevelopment caused by infections in early and late pregnancy are the result of fetal zinc deficiency resulting from MT induction in response to the release of inflammatory cytokines as part of the maternal acute phase response. We predict that:

1) Zinc deficiency will exacerbate the negative fetal outcomes caused by LPS induction.
2) Zinc supplementation will protect against LPS-mediated teratogenicity.

The major aims are:

1) To determine whether a) zinc supplementation is protective against, and b) low dietary zinc intake exacerbates, teratogenicity caused by LPS administration early in pregnancy.
2) To identify markers of neurodevelopmental damage caused by LPS administration in early and late pregnancy and to determine the influence of zinc treatment on any changes in expression of these markers.

CHAPTER 2

STATEMENT OF AUTHORSHIP

DIETARY ZINC SUPPLEMENTATION AMELIORATES LPS-INDUCED TERATOGENICITY IN MICE

Pediatric Research 2006; Volume 59: Page 355-358.

CHUA, J.S.C. (Candidate)

Performed analysis on all samples, interpreted data and wrote manuscript.

I hereby certify that the statement of contribution is accurate.

Signed: _____ Date: _____

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Provided critical evaluation of manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

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COYLE, P.

Supervised in development of work, helped in data interpretation and manuscript evaluation.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis.

Signed: _____ Date: _____

CHAPTER 2

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DIETARY ZINC SUPPLEMENTATION AMELIORATES LPS-INDUCED TERATOGENICITY IN MICE

ABSTRACT

Maternal infection during the first trimester of pregnancy has been associated with pre-term birth, spontaneous abortion, growth retardation and congenital anomalies. Previously, our group have shown that subcutaneous injection of zinc prevents endotoxin (LPS)-induced teratogenicity (Carey *et al.*, 2003a). The purpose of this study was to investigate whether increasing or decreasing dietary zinc alters the teratogenic effects of LPS. Female C57BL6 mice were mated and placed on diets containing 5, 35 or 100 mg/kg zinc. On gestational day (GD) 8, pregnant dams were injected with either LPS (0.5 mg/kg sc) or saline and killed on GD18. LPS-treated fetuses from dams fed 5 & 35 mg/kg zinc diet had a significantly higher number of abnormalities per litter (2- & 1- fold saline controls, respectively) compared to those from LPS + zinc supplemented dams, which were not significantly different from the saline control groups. The beneficial effect and importance of zinc was also reflected in the larger size of fetuses (weight and crown-rump length) from the LPS + zinc supplemented treatment group. We have demonstrated that low dietary zinc during exposure to infection (i.e. LPS) in pregnancy augments the negative impact of LPS

alone, and that dietary zinc supplementation throughout pregnancy ameliorates LPS-induced teratogenicity.

INTRODUCTION

Gestational infections such as maternal fever and urinary tract infections, such as bacterial vaginosis (Lehnardt *et al.*) have been associated with an increased risk for pre-term birth, spontaneous abortion, growth retardation and birth defects in humans and animals (Edwards *et al.*, 1995; Chambers *et al.*, 1998; Kimberlin & Andrews, 1998; Hansen *et al.*, 2000; McGregor & French, 2000; Culhane *et al.*, 2001; Nelson & Macones, 2002; Cottrell & Shannahan, 2004). However, the link between maternal fever, BV and fetal morbidity remains largely unelucidated, with the exception of both being associated with the release of proinflammatory cytokines such as interleukin-1, -6 (IL-1, -6) and tumour necrosis factor (TNF) (Liu *et al.*, 1991; Leazer *et al.*, 2002). Endotoxin or bacterial lipopolysaccharide (LPS), a component of the gram-negative bacterial wall is potent inflammogen and is associated with the release of the above cytokines (Leazer *et al.*, 2002). Endotoxin was also found to occur in higher concentrations in women with BV (Platz-Christensen *et al.*, 1993). Previous studies in rodents have shown that administration of high intravenous doses (> 10μg/100g body weight) of LPS on GD8 caused 100% resorption of implantation sites (Collins *et al.*, 1994), while lower dosing regimes led to development of fetal malformations involving the eye, brain (Ornoy & Altshuler, 1976), neural crest defects, cleft palate and limb anomalies (Collins *et al.*, 1994; Carey *et al.*, 2003a). In contrast, when LPS was given to mice subcutaneously later in gestation (GD13), fetuses that were not resorbed were phenotypically normal

(Coid, 1976). This is perhaps not surprising since the time of exposure was outside the critical window of organogenesis (GD 6 – 14 in mice). Recently, work from our laboratory (Carey *et al.*, 2003a) has found a mechanistic explanation for LPS teratogenicity whereby metallothionein (MT) is the key mediator.

Metallothionein is an intracellular, low molecular weight, cysteine-rich binding protein with a high affinity for zinc (Bracken & Klaassen, 1987b; Jacob *et al.*, 1999; Coyle *et al.*, 2002). Previous studies employing various teratogens (including urethane, α-hederin, 6-mercaptopurine, tumour necrosis factor-α and valproic acid) suggested that metallothionein and its associated changes in maternal-fetal zinc distribution were the key mediators of teratogenicity (Amemiya *et al.*, 1989; Keen *et al.*, 1989; Daston *et al.*, 1991; Daston *et al.*, 1994; Taubeneck *et al.*, 1994; Taubeneck *et al.*, 1995). We have shown that alcohol (Carey *et al.*, 2000a) and LPS (Sobocinski *et al.*, 1978; Philcox *et al.*, 1995) are all potent inducers of MT in the liver resulting in sequestration of plasma zinc in the liver, with a consequent decrease in plasma zinc concentration.

Zinc is an important trace element that plays a critical role in growth and development, as it is a cofactor for over 300 enzymes involved in metabolism and gene regulation. Since only 0.1% of total body zinc is in the plasma, a sudden increase in zinc uptake by the liver can greatly depress plasma zinc concentration (Philcox *et al.*, 1995; Carey *et al.*, 2000b). Such a scenario in early pregnancy is highly unfavourable because the maternal plasma compartment acts as the conduit for fetal zinc supply. Therefore, this change in plasma zinc pool can be likened to a transient fetal zinc deficiency with deleterious effects on the fetus. The involvement of MT in this process can be seen in studies where MT -/- (MT-1 & -2 knockout)

mice exposed to alcohol or LPS on GD8 had fetuses that were morphologically unaffected by these teratogens (Carey *et al.*, 2000b; Carey *et al.*, 2003a). Moreover, our laboratory (Carey *et al.*, 2000a) also showed that when MT was induced by alcohol, not only was there a reduced transfer of zinc to the fetus but whole-fetus zinc concentration was also significantly less.

Previous studies have demonstrated that there are striking similarities in fetal outcome resulting from LPS or alcohol exposure and maternal zinc-deficiency in pregnancy. Pregnant mice treated with alcohol & LPS (Carey *et al.*, 2000b; Carey *et al.*, 2003a) presented with abnormalities similar to those observed in rodents fed zinc-deficient diet (≤ 1μg Zn/g diet) throughout pregnancy (Hurley & Swenerton, 1966; Hurley *et al.*, 1971; Andrews & Geiser, 1999). The observed abnormalities include craniofacial, neural tube and limb bud defects (Hurley & Swenerton, 1966; Hurley *et al.*, 1971; Andrews & Geiser, 1999; Carey *et al.*, 2000b; Carey *et al.*, 2003a). Previously, Keppen and co-workers (Keppen *et al.*, 1985) demonstrated that zinc-deficiency acts as a co-teratogen with alcohol and this was prevented in the chronic alcohol model by giving high zinc in the form of a liquid diet throughout pregnancy. Moreover, we showed that alcohol and LPS-induced teratogenicity can be prevented by subcutaneous zinc treatment (Carey *et al.*, 2003a; Carey *et al.*, 2003b). However, whether a diet low in zinc is co-teratogenic with LPS and whether dietary zinc supplementation throughout pregnancy can prevent the birth abnormalities caused by LPS are the focus of the present study. Here, we demonstrate that dietary zinc supplementation throughout pregnancy ameliorates teratogenicity associated with infection.

MATERIALS & METHODS

Animals and Mating Procedure

C57BL6 mice were purchased from the Institute of Medical & Veterinary Science (IMVS) Animal Care Facility (Adelaide, SA). Mice were maintained in an animal house at 22°C, subject to a 14h light/10h dark cycle and had access to water *ad libitum*. All mice were fed a commercial non-purified diet (Milling Industries, Adelaide, Australia) prior to and during the mating process.

Mating was carried out by pairing females (20-25g) with males and examining the females every morning for the presence of a vaginal plug (gestational day 1, GD1). Females were then removed from the males and housed individually throughout the duration of experimentation. On GD1, mice were randomly allocated to different treatment groups and fed specially formulated diets containing low zinc (5 ppm), normal zinc (35 ppm) or supplemented zinc (100 ppm) throughout their pregnancy. The minimum number of litters per group was estimated based on the frequency of pregnancy success and birth abnormalities per litter observed in previous studies (Carey *et al.*, 2003a).

All animal-related protocols followed the Australian Code of Practise for the care and Use of Animals for Scientific Purposes and were approved by the Animal Care and Ethics Committees of the Institute of Medical and Veterinary Science (IMVS), Adelaide, and the University of Adelaide.

Diet and Composition

All animals were fed a purified diet containing spray-dried egg white (ICN Biomedicals, Aurora, OH) as a protein source. The basic diet composed of AIN-93G mineral mix with no added zinc and AIN-93VX vitamin mix (ICN Biomedicals, Aurora, OH). The basic mixture without zinc had a zinc content of approximately 1 mg/kg. Appropriate amount of aqueous $ZnSO_4$ was added to the mixture to produce diets deficient, normal and supplemented with zinc. The zinc-deficient diet contained 5 ppm Zn, representing a moderate state of Zn deprivation, which at the same time can support pregnancy (Beach *et al.*, 1981). The control diet contained 35 ppm Zn whereas the supplemented diet contained 100 ppm Zn, which has been used in previous studies to enable maximal growth and reproduction (Beach *et al.*, 1980, 1981). In preliminary studies, we assessed serum copper and haemoglobin status following 200 ppm dietary zinc supplementation in mice and found no influence in these parameters (unpublished data).

Determination of Dietary Zinc Concentration

Zinc concentrations in all diets were determined using flame atomic absorption spectroscopy (Perkin Elmer Analyst 300, Überlingen, Germany). Briefly, two to three random aliquots (1 g) of each zinc-containing diet were dried at approximately 65°C for 48 hours and digested with concentrated nitric acid whilst heating to aid evaporation of the liquid. 1M HCl was then added to dissolve the remaining solid material and the resultant liquid was analysed for zinc content. The average concentration of zinc in the 5, 35 and 100 ppm zinc diet were 6.0 ± 0.0 (n = 2), 36.3 ± 0.4 (n = 2) and 94.3 ± 1.8 (n = 3) ppm, respectively. Food intake of all animals was measured every alternate day.

Dietary Zinc and GD8 LPS Treatment

Mice were randomly allocated into 6 different groups where they were either treated with LPS or saline (S), and fed diets containing deficient (LPS+Zn5 / S+Zn5), normal (LPS+Zn35 / S+Zn35) or supplemented zinc (LPS+Zn100 / S+Zn100) from GD1 throughout pregnancy. On GD8, mice in the LPS group were injected subcutaneously (sc) into the nape of the neck with 0.5 μg/g body weight lipopolysaccharide (LPS) in 0.85% saline. E. Coli LPS (serotype O111:B4) was purchased from Sigma Chemicals (St. Louis, MO). Control mice were treated with 0.85% saline in a similar fashion. Food was removed for 4h following treatment to control for any nutritional related effects. This dose and route of administration has been demonstrated in studies from this laboratory to initiate highly reproducible induction of hepatic MT and associated hypozincemia. Dams did not exhibit any behavioural abnormalities or obvious signs of toxicity such as diarrhoea following treatment.

LPS, Zinc and GD18 Fetal Morphology

Mice were euthanised on GD18 using halothane anaesthesia, bled by cardiac puncture followed by cervical luxation. Uteri were immediately excised, weighed and examined for number of resorption sites. Individual fetuses were separated from the placentas, weighed and crown-rump length (CRL) measured. Fetuses were examined under low power magnification to determine the extent of physical abnormalities such as micropthalmia, anopthalmia, cleft lip, micrognathia, microcephaly, exencephaly and other obvious malformations.

Statistical Analysis

All data were compared with repeated measures ANOVA using general linear model. Due to non-homogenous variances, data pertaining to fetal weight and crown-rump length were log transformed before using ANOVA. For presentation purposes, means were back-transformed into the usual units, which were reported in the tables. Non-parametric data such as resorption sites and percentage abnormalities were compared using Mann-Whitney. Significance was determined using Tukey's post hoc test and the Student T-Test.

All statistical analysis was performed using Minitab statistics software (Minitab Inc., State College, PA). Results are presented as mean ± SEM and differences considered significant at $p < 0.05$, unless otherwise stated in the text.

RESULTS

Pregnancy Success

LPS treated mice completed pregnancy 13 out of 27 times a plug was detected (48% success) while saline treated mice had 20 successful pregnancies out of 27 times a plug was observed (74% success). There were no differences in the percentage of successful pregnancy between LPS treated mice fed 5, 35 or 100 ppm Zn diet. However, saline treated mice fed the low zinc (5 ppm) diet had the lowest pregnancy success (55 %) compared to peers fed the normal (35 ppm) or supplemented (100 ppm) Zn diet (88% and 77% respectively). The normal success rate as assessed in our laboratory over the years is between 80 and 90%. Only the saline treated mice on normal Zn diet fell within this category**.**

LPS, Zinc and Teratogenicity

LPS combined with Zn deficiency had a more severe effect on the fetus than LPS or saline combined with normal or supplemented Zn. There were more resorption sites in LPS-treated dams than saline control dams regardless of dietary Zn consumption. LPS dams had 22 – 36% resorptions per litter compared to 7 – 22% in saline controls. Although LPS dams on the low and supplemented Zn diets had slightly more resorptions than those fed the normal Zn diet, this observation was not significantly different. However, LPS dams on the normal Zn diet had significantly more resorptions than the saline dams on the same diet (22% vs 7 %, respectively) (Table 1).

Fetal weights were lower in the LPS + Zn 5 and LPS + Zn 35 groups compared to LPS + Zn 100 group and saline + 35 Zn fetuses ($p < 0.001$), respectively (Table 1). However, there was no difference in fetal weights between saline control fetuses regardless of dietary Zn consumption. LPS-Zn supplemented dams had larger fetuses (in terms of crown-rump length) compared to those from LPS + Zn 35 group (Figure 3). There was no difference in size between LPS + Zn 5 and LPS + Zn 35 fetuses although fetuses from the latter group were significantly smaller when compared to saline control fetuses exposed to the same diet ($p < 0.001$) (Table 1).

External malformations were most profound in fetuses from LPS + low Zn group (96%) compared to all other treatment/dietary groups (Table 1). The most common abnormalities present were anopthalmia (80%), exencephaly (60%) and microencephaly (40%) (Figure 1). These abnormalities were consistent with those observed in a previous LPS study by our laboratory (Carey *et al.*, 2003a). Although

these abnormalities were also present in the LPS + Zn 35 group, they occurred at a significantly less frequency. Fetuses on the Zn-supplemented diet were least affected by LPS, mainly exhibiting eye abnormalities commonly found in the strain of mice used. Other abnormalities observed included cleft lip, micropthalmia, micrognathia, anagthia and spinal haemorrhage.

Table 1. GD18 fetal parameters following GD8 LPS treatment with 5, 35 & 100 ppm zinc diet throughout pregnancy.

	Treatment / Dietary Zinc (ppm)					
	LPS + 5Zn	LPS + 35Zn	LPS + 100Zn	Saline + 5Zn	Saline + 35Zn	Saline + 100Zn
Maternal data						
Litters (*n*)	4	5	4	4	8	7
Live Fetuses (*n*)	21	34	21	22	55	44
Litter Size	5.3 ± 1.0	6.8 ± 1.0	5.3 ± 1.3	5.5 ± 0.6	6.9 ± 0.8	6.3 ± 1.3
Resorptions (*n*)	12	9	11	6	5	6
Resorption sites/litter	3.0 ± 1.1	1.8 ± 0.5†	2.8 ± 1.6	1.5 ± 0.3	0.6 ± 0.2	0.9 ± 0.5
Fetal data						
Abnormal fetuses (*n*)	20	23	5	6	11	5
Litters with abnormal fetuses	4	5	4	3	4	1
Abnormal fetuses per litter	5.0 ± 0.9$^{\#}$	4.4 ± 1.0‡	1.3 ± 0.3*	1.5 ± 0.6	1.4 ± 0.9	0.7 ± 0.9
% Abnormalities	96 ± 3.6$^{\#}$	65 ± 8.7‡	30 ± 9.1*	30 ± 16.4	25 ± 11.8	14 ± 18.9
Weight (*g*)	0.58 ± 0.06§	0.61 ± 0.02†	0.68 ± 0.05	0.75 ± 0.08	0.74 ± 0.02	0.64 ± 0.05
Crown-rump length (*mm*)	17.16 ± 0.6	17.02 ± 0.2†,§	18.28 ± 0.5	18.84 ± 0.9	18.96 ± 0.3	17.8 ± 0.7

Values represent mean ± SEM where applicable. Data were analysed as three separate groups: 1) LPS vs saline, 2) LPS+5 vs LPS+35 vs LPS+100ppm Zn, and 3) saline+5 vs saline+35 vs saline+100ppm Zn. All data were tested via repeated measures ANOVA. Significance at $p < 0.05$ was determined using Tukey's post hoc test.

$^{\#, \ddagger}$ Significantly different from each other, and different between treatments (LPS or saline) and dietary groups.

* Significantly different between dietary groups within treatment.

$^{\dagger, \S}$ Significantly different between treatments within dietary groups.

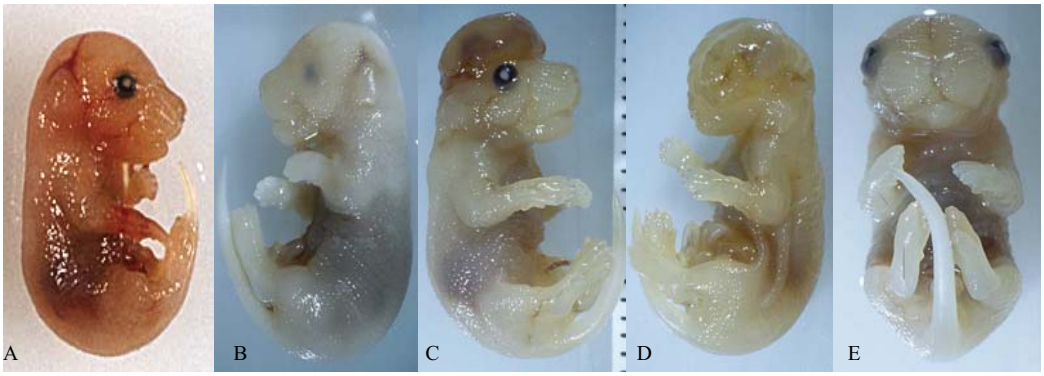

Figure 1. GD18 birth abnormalities in fetuses exposed to LPS. (A) Normal fetus. Fetuses from dams fed a low zinc diet combined with LPS exposure on GD8 presented with birth abnormalities such as micropthalmia (B), exencephaly (C), microencephaly (D), anopthalmia (D) and cleft lip (E) as observed on GD18.

DISCUSSION

Previous studies in our laboratory have shown that a single subcutaneous zinc injection at the time of lipopolysaccharide (LPS) exposure prevented the development of birth defects (Carey *et al.*, 2003a). In the present study we demonstrate that feeding mice a low zinc diet, which was sufficient to support pregnancy, nonetheless augmented the frequency of birth abnormalities when combined with exposure to LPS compared to that of LPS alone. We further demonstrated these abnormalities could be prevented through dietary zinc-supplementation throughout pregnancy. These findings lend support to the fact that adequate dietary zinc, especially during pregnancy plays an important role in fetal growth and development. Moreover, our findings are consistent with previous studies

from our laboratory with LPS or alcohol where zinc injection was given at the same time as the teratogens (Carey *et al.*, 2003a; Carey *et al.*, 2003b), thus supporting the proposed mechanism involving metallothionein (MT)-mediated zinc redistribution leading to impaired fetal zinc supply, hence jeopardising the fetus.

Gestational infection such as bacteria vaginosis (Lehnardt *et al.,* 2002), urinary tract infections (UTIs) and maternal fever has been associated with spontaneous abortion, stillbirth, growth retardation and birth defects. The occurrence of such infections during the first trimester of pregnancy, the critical window of organogenesis is particularly detrimental to the offspring resulting in physiological and neurological defects. Both BV and UTIs are commonly associated with the release of LPS or endotoxin, a component of the gram-negative bacterial cell wall (Ornoy & Altshuler, 1976; Platz-Christensen *et al.*, 1993) and a potent inducer of inflammatory cytokines such as IL-1, IL-6, TNF-α and acute phase proteins such as MT (Liu *et al.*, 1991; Dammann & Leviton, 1997; Leazer *et al.*, 2002). It is well documented that TNF-α plays a central role in mediating the pathophysiologic changes associated with LPS exposure by triggering the acute phase response leading to fetotoxicity and teratogenicity. Previously, Taubeneck & co-workers have shown that TNF-α is a strong inducer of liver MT when given as an intraperitoneal injection in mice (Taubeneck *et al.*, 1995). Moreover, it was demonstrated that maternal serum TNF-α levels increased rapidly for 1-1.5h after exposure to LPS (0.05 mg/kg), and when administered on GD9 approximately 50% embryolethality was observed within 24h (Leazer *et al.*, 2002). Furthermore, work from our laboratory (Carey *et al.*, 2000b) has shown that administration of 0.5 mg/kg LPS caused a rapid increase in liver MT levels within the first 5h peaking at 34-fold basal after 24h. This strongly supports the role of TNF-α as a central mediator of the acute phase response triggered by LPS.

However, as TNF-α was not found to directly induce MT in cultured hepatocytes, it most likely mediates its effect through the induction of other cytokines, in particular IL-6, which is a strong inducer of MT both *in vivo* and *in vitro* (Coyle *et al.*, 1993).

MT is a key zinc-binding protein, which plays a major role in zinc homeostasis. Findings from our laboratory has shown that an increase in MT due to either LPS or alcohol not only caused a marked depression in plasma zinc that lasts for 12-24h (Carey *et al.*, 2000b; Carey *et al.*, 2003a), but also decreased zinc transfer to the fetus (Carey *et al.*, 2000a). This impaired zinc supply to the fetus presents as a transient zinc deficiency, which has deleterious impacts on the fetus. Turk & colleagues (Turk *et al.*, 1959) were the first to observe the teratogenic effects of zinc deficiency in chicks hatched from hens fed zinc-deficient diet. Furthermore, rodents fed zinc-deficient diet (≤ 1μg zinc/g diet) throughout pregnancy had offspring with craniofacial, neural tube and limb bud defects (Hurley & Swenerton, 1966; Hurley *et al.*, 1971). However, whether or not a low zinc diet combined with exposure to infection or LPS during pregnancy is co-teratogenic has not been studied. Previously, Keppen & co-workers (Keppen *et al.*, 1985) showed that alcohol and zinc deficiency is co-teratogenic in a chronic alcohol model, which was prevented by high zinc throughout pregnancy.

In the present study, we found that mice fed a low zinc diet (5 mg/kg) exposed to LPS on GD8 had fetuses with the worst outcomes compared to those from dams fed normal zinc diet (35 ppm) and saline controls on the same diets. Fetuses from the LPS + low zinc group presented with gross neural tube defects and craniofacial anomalies, and exhibited delayed growth and development as evidenced by the lower weights and crown-rump length. More importantly, we demonstrated that dietary

zinc supplementation with exposure to LPS prevented the teratogenic effects to basal control levels, most of which consisted of spontaneous (i.e. non-teratogenic) malformations, in particular those relating to the eyes. Moreover, fetuses from the zinc-supplemented group regardless of treatment were heavier and had longer crown-rump length at birth compared to fetuses from other groups.

The abortigenic nature of LPS and importance of zinc was also demonstrated in this study. LPS-treated mice were less successful in completing their pregnancy compared to saline controls after detection of a vaginal plug (50% & 75%, respectively; normal 80 – 90%). Mice that were fed a low zinc diet were also unable to complete their pregnancy 55% of the time compared to mice on the normal and supplemented diet. This is indicative of complete resorption of fetal tissue. Furthermore, LPS + Zn-deficient mice that were successfully pregnant had the most resorption sites and fetuses that were grossly abnormal. Therefore, LPS exposure perhaps can be viewed as a double insult where directly, it causes intrauterine fetal death (IUFD) and indirectly, causing abnormal development of the fetus through the MT-mediated changes in maternal zinc homeostasis. This can be supported by previous studies in our laboratory where MT -/- (knockout) fetuses exposed to alcohol or LPS had survival advantage over their MT +/+ counterpart (Carey et al., 2000b; Carey et al., 2003a; Carey et al., 2003b). The possibility that high dietary zinc supplementation reduces the capacity of LPS to induce maternal liver MT is potentially negated by the findings of Summers et al. (2009) where ethanol induction of liver MT was not higher in mice fed 200 ppm zinc in their diet compared to those fed a control diet containing 35 ppm zinc. However, further studies are warranted to demonstrate that LPS induction of maternal liver MT is not compromised by high dietary zinc.

In conclusion, we have demonstrated that low dietary zinc combined with exposure to LPS in pregnancy augments the negative impact of LPS alone, and that zinc supplementation throughout pregnancy ameliorates LPS-induced teratogenicity and delayed development. This supports our hypothesis that LPS teratogenicity works through the MT-mediated zinc-redistribution resulting in a transient fetal zinc deficiency, leading to development of birth defects. Our findings further emphasise the importance of zinc during pregnancy in terms of fetal growth and development. Therefore, in the clinical setting, dietary zinc supplementation during pregnancy appears to be beneficial for ensuring proper growth and development of the fetus and in preventing the development of birth defects.

CHAPTER 3

CHAPTER 3

A manuscript submitted to the Journal of Paediatric Research.

ZINC PROTECTS AGAINST BRAIN INJURY CAUSED BY PRENATAL EXPOSURE TO LPS IN LATE PREGNANCY

ABSTRACT

Maternal infections in early and late pregnancy are associated with increased risk in offspring with neuropathological disorders including cerebral palsy, schizophrenia, autism and mental retardation. A causal link behind this relationship is the maternal cytokine-associated inflammatory response to infection. Since bacterial endotoxin lipopolysaccharide (LPS) is known to activate the cytokine network, this study examined the effect of LPS exposure in pregnancy on cellular markers of neurodevelopmental damage. As concomitant zinc treatment with LPS has been shown to protect against LPS-mediated fetal dysmorphology and cognitive anomalies in adult offspring the protective influence of zinc on cellular damage was investigated herein. Timed-pregnant C57BL6 dams were treated with either LPS or LPS + zinc on gestational day (GD) 16. Fetal brains were excised on GD 18 after LPS administration of GD 16. Maternal LPS administration induced astrogliosis accompanied by extensive cell death in the central white matter, hippocampus, and periventricular cortex. The increased astrogliosis and apoptosis were prevented by zinc treatment but not the LPS-induced increase in TNF-α reactive cells. These results demonstrate that zinc protects against infection-induced brain damage but not

against the cytokine response associated with inflammation during infection in late pregnancy.

INTRODUCTION

A substantial body of epidemiological evidence has associated maternal infection with prenatal brain damage leading to neurodevelopmental disorders such as cerebral palsy, schizophrenia, autism and mental retardation. Bacterial as well as viral infections have been implicated with the development of perinatal brain damage. Although it is unclear how a broad range of infectious agents might trigger neurodevelopmental anomalies, accumulating evidence suggests that the damage may be due to the maternal immune response rather than the infectious agent itself. In this regard, animal studies involving prenatal challenge with bacterial endotoxin lipopolysaccharide (LPS) (Cai *et al.*, 2000) or the viral mimic (polyriboinosinic-polyribocytidilic acid; PolyI:C) (Meyer *et al.*, 2006b) have demonstrated that neurodevelopmental abnormalities can occur in the absence of a pathogenic agent.

Previous studies have focused on maternal and fetal-derived cytokines that are released in response to infection as potential causative agents in neurodevelopmental damage. Proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-6 are known to cross the placenta into the fetal circulation and cause increased vascular permeability of the immature blood brain barrier (BBB) (Abbott, 2000), promoting leukocyte infiltration, microglial activation, astrogliosis and

apoptotic cell death via the release of cytotoxic mediators, nitric oxide and mitochondrial dysfunction (Rezaie & Dean, 2002; Kendall & Peebles, 2005).

Recent evidence indicates that the timing of the maternal immune challenge determines the specificity of inflammation-mediated brain pathology (Meyer *et al.*, 2006b). When PolyI:C, was used to challenge the mother's immune system early (GD 9) or late (GD 17) in pregnancy, administration on GD 9 resulted in decreased corticogenesis / neurogenesis, increased gliosis, and decreased Reelin expression (Meyer *et al.*, 2005; Meyer *et al.*, 2006b; Meyer *et al.*, 2006c) whereas challenge on GD 17 caused increased apoptosis which was associated with a different cytokine response and behavioural pathology including greater sensitivity to dopamine agonists and NMDA antagonists (Meyer *et al.*, 2006a).

The timing of the maternal inflammatory response may also be an important determinant of brain pathology to bacterial infection during pregnancy. Our group have demonstrated that challenge with LPS on GD 8 causes object recognition impairments in mice offspring that can be prevented by zinc treatment during pregnancy (Coyle *et al.*, 2008). Others have shown that LPS administration to dams late in pregnancy causes increased expression of TNF-α and IL1-β, which is associated with increased apoptosis and astrogliosis in the hippocampus, cortex (Cai *et al.*, 2000) and extensive cell death around the periventricular region of the perinatal brain, or periventricular leukomalacia (PVL) (Bell & Hallenbeck, 2002; Hagberg *et al.*, 2002; Bell *et al.*, 2004). In addition, brain damage in infants has been found to be commonly associated with prenatal exposure to bacterial infections such as chorioamnionitis, diphtheria and pneumonia (Dammann & Leviton, 1997, 2000; Gibson *et al.*, 2006).

Our findings that concomitant zinc treatment or dietary zinc supplementation throughout pregnancy can prevent fetal dysmorphology as well as cognitive anomalies associated with LPS exposure in early pregnancy (Carey *et al.*, 2003a; Chua *et al.*, 2006; Coyle *et al.*, 2008) pose the question as to whether zinc treatment could ameliorate the cellular damage caused by the maternal inflammatory response to infection in late pregnancy. Here we examine the effect of LPS exposure in late pregnancy on a range of cellular markers of brain injury and further determine whether concomitant zinc treatment has a protective effect.

MATERIALS AND METHODS:

Animals and mating procedure. C57BL6/J mice were maintained at 22 °C with a 12 h light /12 h dark cycle. Mice were purchased from the Animal Care Facility (Institute of Medical and Veterinary Science, Adelaide, Australia). All mice had *ad libitum* access to water and commercial non-purified diet (Milling Industries, Adelaide, Australia), both before and during the mating process.

Female mice (20 – 22g) were time-mated for 4 h from the start of the light cycle and were checked for signs of pregnancy at the same time each day. The presence of a vaginal plug indicated successful mating, and was designated GD 1. Females were then removed and housed individually throughout the duration of experimentation.

All animals were acquired and cared for in accordance with the NIH guidelines for the care and use of laboratory animals for research purposes. All animal-related procedures

followed the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Care and Ethics Committees of the Institute of Medical and Veterinary Science (IMVS) and the University of Adelaide.

Drug administration. Pregnant dams were randomly selected into three treatment groups containing six dams per group and administered with saline (control), or LPS or LPS + zinc on GD 16. LPS (*Escherichia coli* serotype O111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO) and formulated in 0.85% (w/v) sterile saline and was administered at a concentration of 0.5 μg/g body weight. Zinc was given as $ZnSO_4$ at 2 μg/g body weight. Saline, LPS and zinc were all administered subcutaneously at a volume of 10 μL/g body weight.

Fetal brain excision. On GD 18, pregnant dams were euthanised using isofluorane inhalation anaesthesia followed by cervical dislocation. Fetuses from each dam were removed from the uterus, decapitated and brains immediately immersed in 10 % neutral buffered formalin. Formalin was changed 24 h later and fetal brains preserved for 7 days before they were sectioned for paraffin embedding, followed by immunohistochemistry assessment. Three fetuses from each of six dams per treatment group were randomly selected for immunohistochemistry analyses.

Immunohistochemistry. GD 18 fetal brains were divided into four 2 mm thick representative sections using the Kopps mouse brain slicer (California, USA) and embedded in paraffin after formalin fixation. Brain sections containing the cortex, corpus callosum, white matter, hippocampus and dentate gyrus were chosen for immunohistochemistry analyses (see Figure 1 mapping the location of the sections investigated). Sections were taken from brain regions corresponding to coronal sections

9 and 14 from the atlas of the prenatal mouse brain (Schambra *et al.*, 1992). Coronal sections of 5 μm thick were sectioned using a Microm HM325 microtome (Microm, Germany) and mounted on Menzel Superfrost® Plus slides (HD Scientific, Australia). Sections were deparaffinized and hydrated with xylene and a graded series of alcohol solutions before staining with primary antibodies. Three consecutive brain sections from each fetus were assessed for astrocyte hypertrophy, cell death and inflammation using the astrocyte marker glial fibrillary acidic protein (GFAP), TUNEL and TNF-α, respectively. In order to determine which cell type was affected by LPS, double staining of TUNEL with the neuronal (PGP9.5), astrocytic (GFAP) and microglial (Iba1) markers were performed. Brain sections from 15 fetuses from 5 independent litters were analysed for the saline control group, while 18 fetuses from 6 litters were used for both the LPS and LPS + zinc treatment groups.

GFAP, PGP9.5 and Iba1 immunohistochemistry. After sections were deparaffinized and hydrated as described above, the endogenous peroxidase activity was quenched using 2 % (v/v) hydrogen peroxide in methanol for 30 min. The antigens were then retrieved by pressurized heating for 6 min then transferred to a convection microwave (Panasonic, Japan) for 10 min at maximal power in 10 mM citrate buffer at pH 6.0. Sections were then left to cool before incubation with 3 % (v/v) normal horse serum (NHS) for 30 min at room temperature to block endogenous immunoglobulins followed by overnight incubation with primary antibodies - polyclonal rabbit anti-GFAP primary antibody (1:30,000; DakoCytomation, Denmark), anti-mouse PGP9.5 antibody (1:20,000; Ultraclone, UK) and polyclonal rabbit anti-Iba1 antibody (1:5000; Wako Pure Chemicals, USA). The sections were then incubated with a biotinylated anti-rabbit IgG secondary antibody (Vector, USA) followed by a streptavidin-peroxidase conjugated tertiary antibody (Pierce, USA), developed in diaminobenzidine

(DAB) solution and counterstained with haematoxylin. Positive and negative controls were included in each protocol.

TUNEL. For visualisation of cell death, fetal brains were processed using the ApopTag® Plus peroxidase in situ apoptosis detection kit (Chemicon, CA) according to manufacturer's instructions. Briefly, deparaffinized sections were digested with 20 μg/mL Proteinase K for 15 min, washed with distilled water, then incubated with 3 % (v/v) H_2O_2 for 5 min to inhibit endogenous peroxidase. Sections were then rinsed in PBS and incubated at 37 °C for 1 h with terminal deoxynucleotidyl transferase (TdT) buffer. Immediately after incubation, sections were immersed in stop/wash buffer for 10 min, rinsed in PBS, followed by incubation at room temperature for 30 min with anti-digoxigenin conjugate. After washing with PBS, sections were developed in DAB peroxidase substrate and counterstained with haematoxylin. Positive control supplied in the kit was included in each assay.

TNF-α immunohistochemistry. Deparaffinized sections were immersed in 2 % hydrogen peroxide in methanol for 30 min followed by antigen retrieval using pressurized heating as described above in 1.3 mM EDTA buffer pH 8.0. Sections were then left to cool before incubating with 3 % NHS at room temperature for 30 min followed by overnight incubation with the primary anti-mouse TNF-α/TNFSF1A antibody (R&D Systems, USA) at a 1:30 dilution. The sections were then incubated with a biotinylated anti-rabbit IgG secondary antibody (Vector, USA), followed by a streptavidin-peroxidase conjugated tertiary antibody (Pierce, USA), developed in DAB solution and counterstained with haematoxylin. Positive and negative controls were included in each protocol.

TUNEL double labelling. For double labelling of TUNEL with immunohistochemical markers, sections were incubated overnight at room temperature with antibodies directed to PGP9.5, GFAP and the microglial marker Iba1 as per the method described above. On the following day, sections were incubated with the appropriate biotinylated secondary antibody for 30 min, followed by streptavidin-conjugated AlexaFluor 660 (1:500; Molecular Probes, Eugene, OR), for 1 h in a darkened environment. Sections were then rinsed twice for 5 min in PBS. Following this they were treated with proteinase K (20 μg/ml) for 15 min and washed twice in distilled water for 2 min. Endogenous peroxidases were inactivated by incubation in H_20_2 for 5 min, followed by rinsing in Tris buffer (10 mM Tris-HCl, pH 8.0) for 5 min. Sections were then equilibrated in TdT buffer (30 mM Tris-HCl, pH 7.2) for 10 min, prior to incubation in the same buffer containing TdT (0.15 U/μl; Promega, Madison, WI) and biotin-16-dUTP (10 μM; Roche, Castle Hill, Australia) for 60 min at 37 °C. The reaction was terminated by two washes in 30 mM sodium citrate solution containing 300 mM NaCl for 15 min and two washes in PBS for 5 min. Sections were then incubated with streptavidin-Alexafluor 488 conjugate (1:500; Molecular Probes, Eugene, OR) containing 2 % bovine serum albumin for 30 min at 37 °C. Sections were rinsed in PBS followed by distilled water, cover slipped using anti-fade mounting medium (ProLong Gold; Invitrogen, Carlsbad, CA), and the fluorescent signal visualised using a confocal laser-scanning microscope (PharosFX; Bio-Rad Laboratories, Hercules, CA).

Cell counting. For assessment of GFAP, TUNEL and TNF-α positive cells, tissue sections were quantitatively evaluated by a researcher blind to the treatment conditions and digital images collected using an Olympus DP12 Microscope with a digital camera system attached (Olympus, Japan). Brown-stained cells were counted in the

periventricular region, hippocampus, corpus callosum and cortex under 40X magnification. The number of positive cells was expressed as a number of cells per mm^2.

Statistical analyses. Quantitative data were expressed as mean ± SEM for each group and results were analysed by one-way analysis of variance (ANOVA) for multiple comparisons followed by Dunn's post hoc test using the Sigma Stat software. The significance level was set at $p < 0.05$.

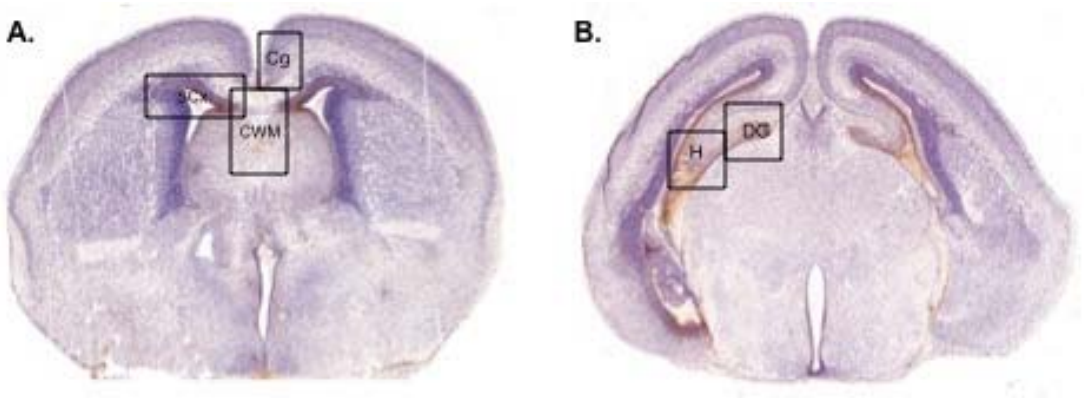

Figure 1. Coronal brain sections subjected to immunohistochemistry: Panel A. contains the subventricular cortex (SCx), cingulate cortex (Cg) and central white matter (CWM); and Panel B. contains the hippocampus (H) and dentate gyrus (DG). Respective areas (indicated by squares) are presented in larger magnification in Figures 2, 3 and 4. (Magnification = 5x).

RESULTS:

Assessment of GFAP-positive astrocytes

LPS alone caused a marked increase in GFAP-positive astrocytes in all the brain regions examined compared to the saline group ($p < 0.0001$) (Table 1). The highest frequency of GFAP-positive astrocytes were found in the hippocampus and subventricular cortex of fetal brains exposed to LPS as well as a significantly higher number in the corpus callosum and dentate gyrus compared to the saline controls ($p < 0.05$) (Figure 2A, B, D, E, G, H, J and K).

Zinc administration at the time of LPS exposure significantly reduced the number of GFAP-positive astrocytes in all brain regions examined to the level observed in the saline control brains ($p < 0.05$) (Table 1 and Figure 2C, F, I, L).

TUNEL cell death quantification

There was a significant increase in the number of TUNEL +ve cells in all the brain regions examined in the LPS-exposed fetal brain compared to the controls ($p < 0.01$). The highest frequency of apoptotic cell death was observed in the central white matter (CWM) area followed by the subventricular and cingulate cortex (Figure 3A, B, D, E, G and H). In these brain regions, there was an 80 – 85 % increase in TUNEL +ve cells in the LPS-exposed brains compared to the controls (Table 2).

When zinc was administered concomitantly with LPS, the number of TUNEL +ve cells was significantly decreased in all brain regions examined ($p < 0.01$) (Figure 3C, F and

I). The greatest inhibition of apoptosis was observed in the subventricular and cingulate cortex, 87 % and 93 %, respectively (Table 2).

Table 1: Number of GFAP-positive cells in GD18 fetal brains.

Treatment Group	Subventricular Cortex	Hippocampus	CWM	Dentate Gyrus
	GFAP (cells/mm^2)			
Saline	8 ± 1.9	30 ± 9.3	18 ± 2.3	18 ± 7.2
LPS	121 ± 13.9 *	129 ± 7.3 *	80 ± 10.4 ^	48 ± 7.3 ^
LPS + zinc	14 ± 6.4	28 ± 6.4	20 ± 6.9	13 ± 2.7

Values represent mean ± SEM. Data were analysed using all-pairwise one-way ANOVA followed by Dunn's post hoc analysis. Significance was set at $p < 0.05$.

* $p < 0.001$ versus saline control and LPS + zinc group in the same region.

^ $p < 0.05$ versus saline control and LPS + zinc group in the same region.

Expression of TNF- reactive cells

Maternal LPS administration on GD 16 resulted in a 2-fold increase in TNF- reactive cells in the CWM of GD 18 fetal brains compared to controls ($p < 0.05$) (Figure 4A, B and Table 2). A significantly higher number of TNF- reactive cells were also observed in the subventricular cortex and the cingulate cortex of fetal brains 48 h after LPS administration (Arai *et al.*) (Figure 4D, E, G and H). However, when zinc was administered concomitantly with LPS, there was no reduction in the number of TNF-reactive cells observed in any of the brain regions examined (Figure 4C, F and I).

Table 2: Number of TUNEL-positive and TNF-alpha reactive cells in GD18 fetal brains.

Treatment Group	Subventricular Cortex	Cingulate Cortex	CWM
	TUNEL (cells/mm^2)		
Saline	1 ± 0.2	3 ± 0.8	3 ± 0.8
LPS	15 ± 2.9 *	14 ± 1.4 *	20 ± 1.9 *
LPS + zinc	2 ± 0.7	1 ± 0.1	7 ± 1.1
	TNF-alpha (cells/mm^2)		
Saline	2 ± 0.7	1 ± 0.4	11 ± 2.5
LPS	9 ± 2.3 #	4 ± 1.2 #	22 ± 5.0 #
LPS + zinc	8 ± 0.8	2 ± 0.7	13 ± 1.9

Values represent mean ± SEM. Data were analysed using all-pairwise one-way ANOVA followed by Dunn's post hoc analysis. Significance was set at $p < 0.05$.

* $p < 0.01$ versus saline control and LPS + zinc group in the same region.

# $p < 0.05$ versus saline control group in the same region.

Identification of cell types affected by LPS

Double labelling of TUNEL with both PGP9.5 and GFAP showed no colocalization of TUNEL-positive nuclei (Figure 5 and 6). To determine whether TUNEL-positive nuclei may be microglial cells, the marker Iba1 was used. Only few microglia exhibited TUNEL-positive nuclei (Figure 7). Since there was no colocalization of TUNEL-positive nuclei with neurons or astrocytes, and only few microglial were positive for TUNEL, the majority of positive cells were thought to be oligodendroglial in origin.

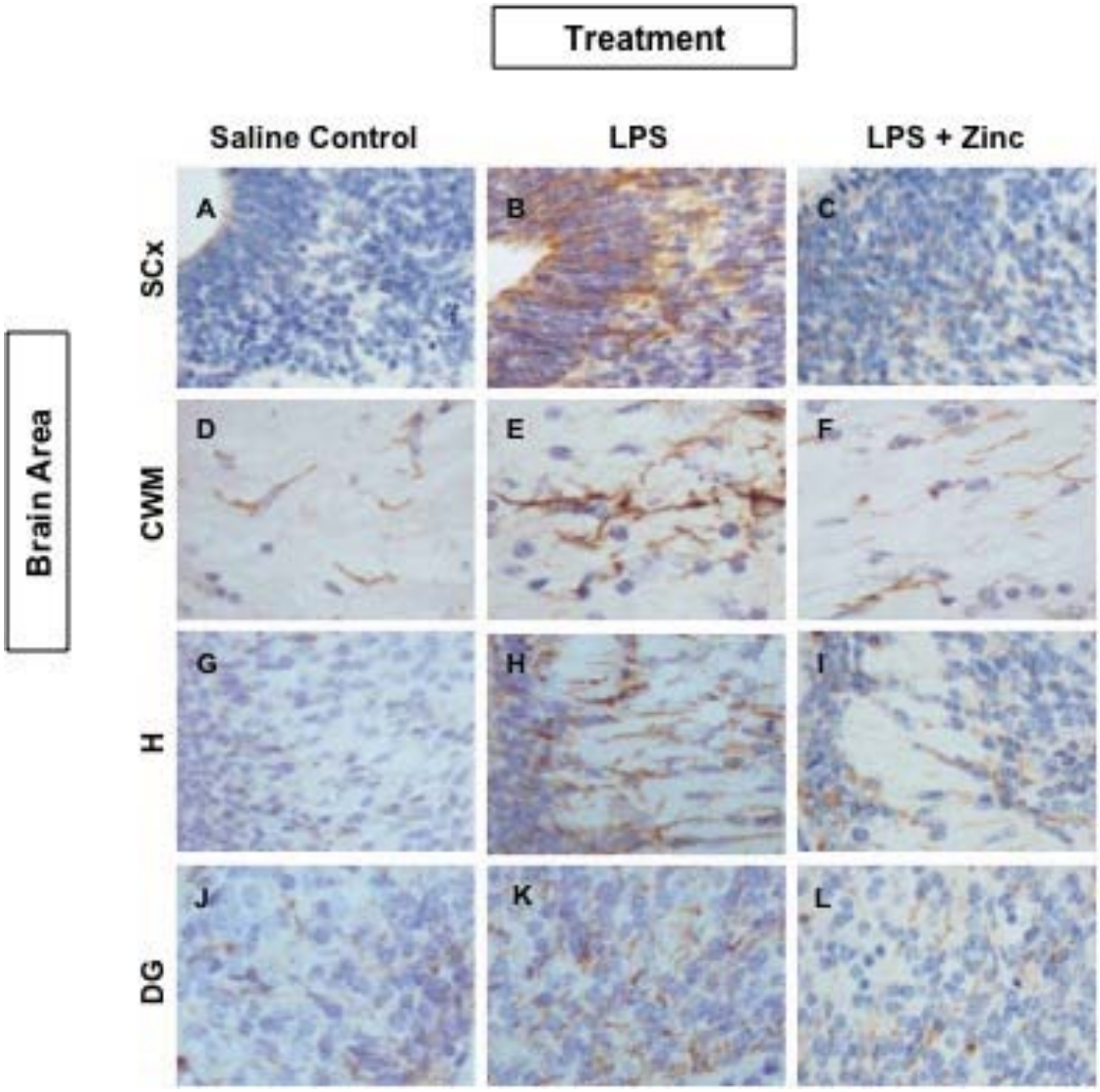

Figure 2. GFAP-positive astrocytes (in brown) in the saline – (A, D, G, J), LPS – (B, E, H, K) and LPS + zinc – (C, F, I, L) treated GD18 fetal brain. Brain sections from 15 fetuses from 5 independent litters were analysed for the saline control group, while 18 fetuses from 6 litters were used for both the LPS and LPS + Zn treatment groups. LPS induced an increase in GFAP expression in the hippocampus (H; A-C), subventricular cortex (SCx; D-F), central white matter (CWM; G-I) and dentate gyrus (DG; J-L) compared to the saline control, which were reduced by zinc treatment ($p < 0.05$). Magnification = 40x.

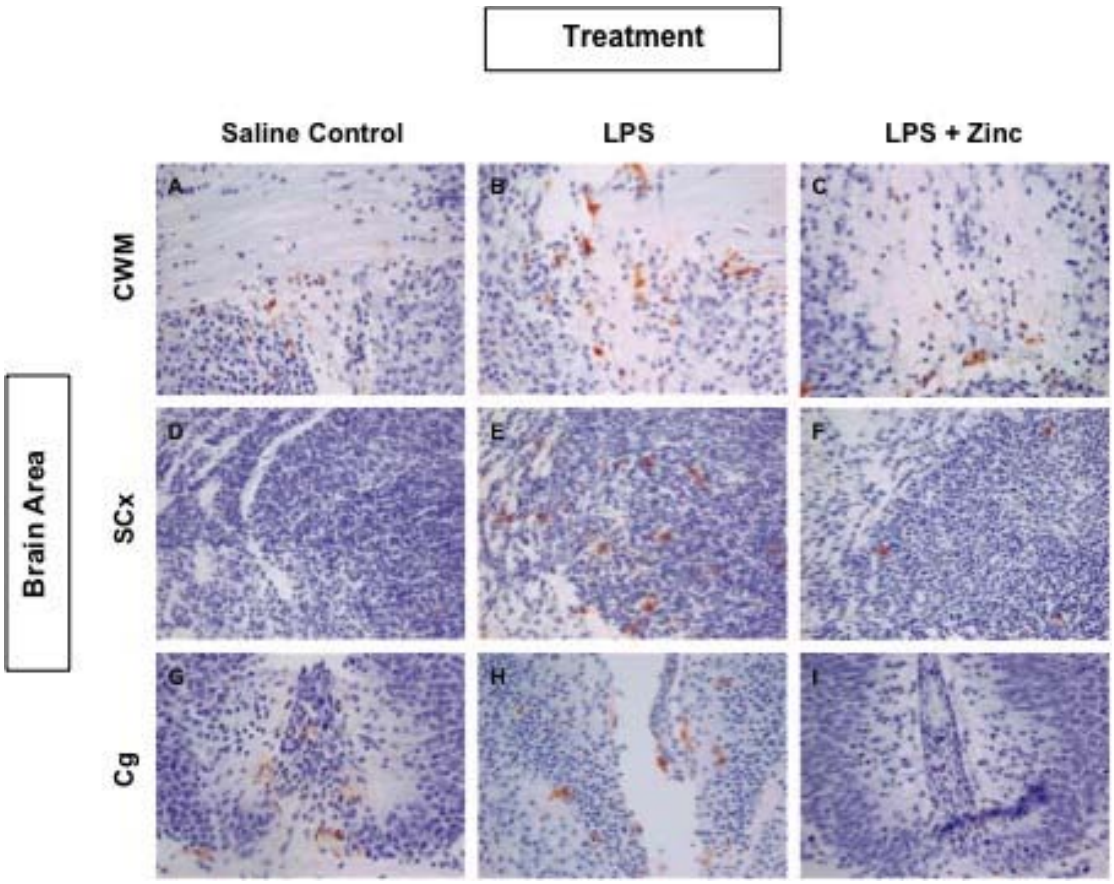

Figure 3. TUNEL +ve cells (in brown) in the saline – (A, D, G), LPS – (B, E, H) and LPS + zinc – (C, F, I) treated GD18 fetal brain. Brain sections from 15 fetuses from 5 independent litters were analysed for the saline control group, while 18 fetuses from 6 litters were used for both the LPS and LPS + Zn treatment groups. There was significantly more TUNEL +ve cells in the central white matter (CWM; A-C), subventricular cortex (SCx; D-F) and cingulate cortex (Cg; G-I) of the LPS-exposed brains compared to the LPS + zinc and saline control brains ($p < 0.05$). Magnification = 20x.

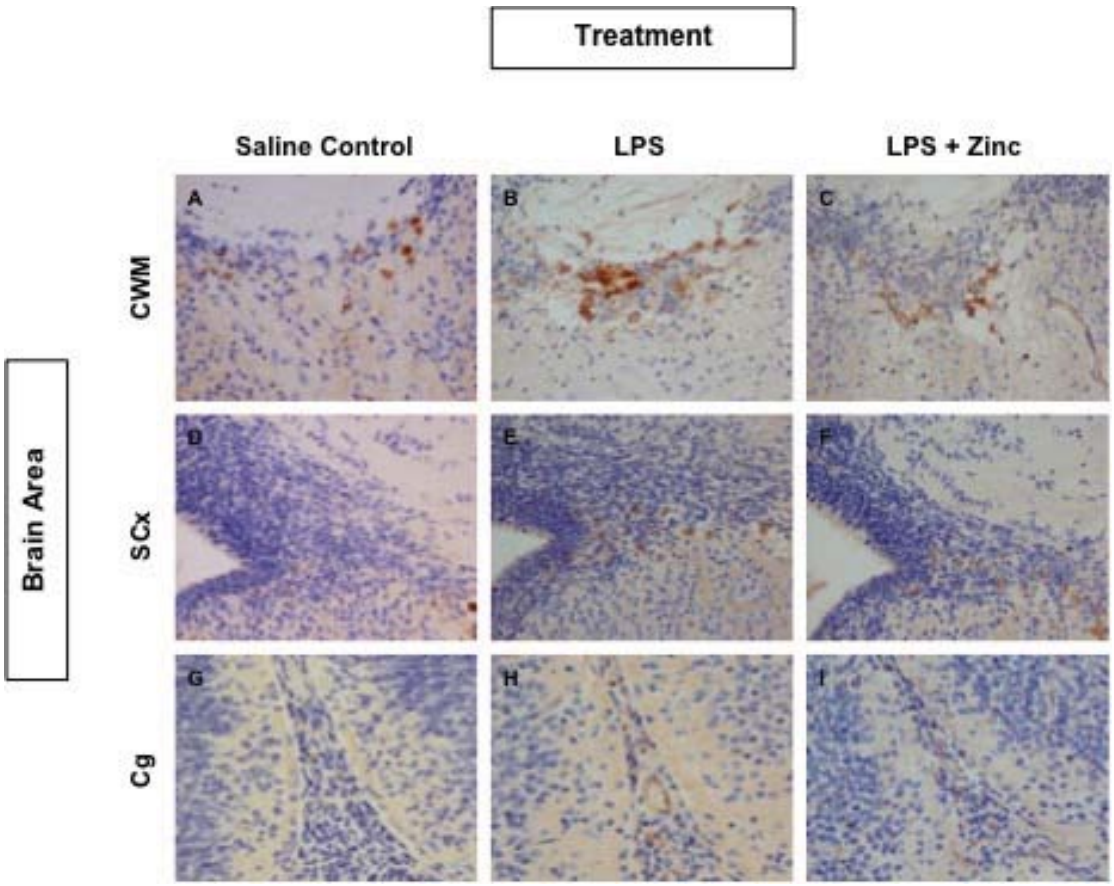

Figure 4. Expression of TNF-a reactive cells (in brown) in the saline – (A, D, G), LPS – (B, E, H) and LPS + zinc – (C, F, I) treated GD18 fetal brains. Brain sections from 15 fetuses from 5 independent litters were analysed for the saline control group, while 18 fetuses from 6 litters were used for both the LPS and LPS + Zn treatment groups. LPS significantly increased the number of TNF-a reactive cells in the central white matter (CWM; A-C), subventricular cortex (SCx; D-F) and the cingulate cortex (Cg; G-I) in the fetal brain compared to the saline controls ($p < 0.05$). Magnification = 20x.

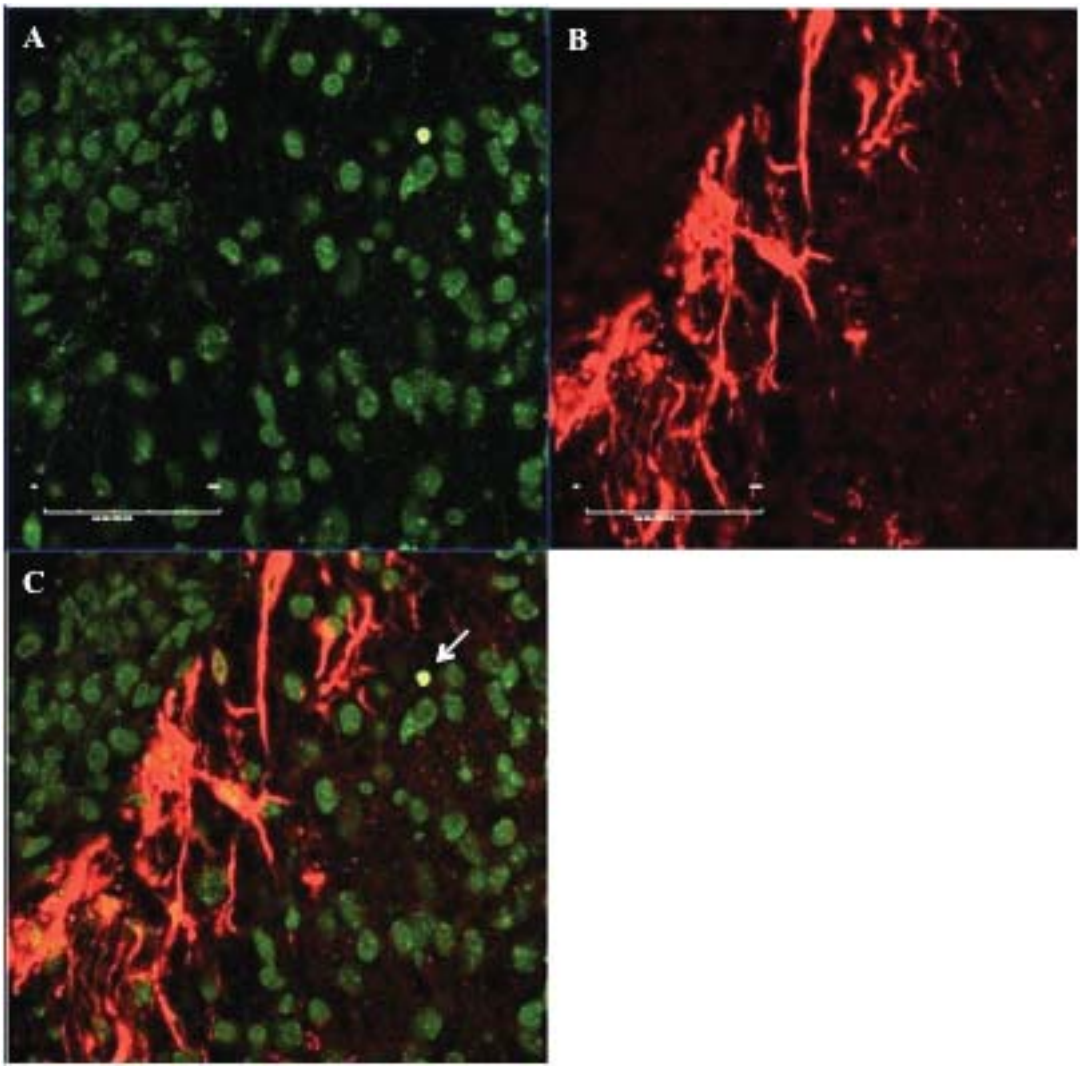

Figure 5. Characterization of LPS-induced apoptosis by double staining for TUNEL (green; A) and the astrocyte marker GFAP (red; B). Colocalization of TUNEL+ nuclei to astrocytes was not observed in the subventricular cortex of LPS-treated fetal brain (C). TUNEL staining labelled several non-astrocytic cells (white arrow (C)). Magnification = 40x. Bar graphs are 50 μm.

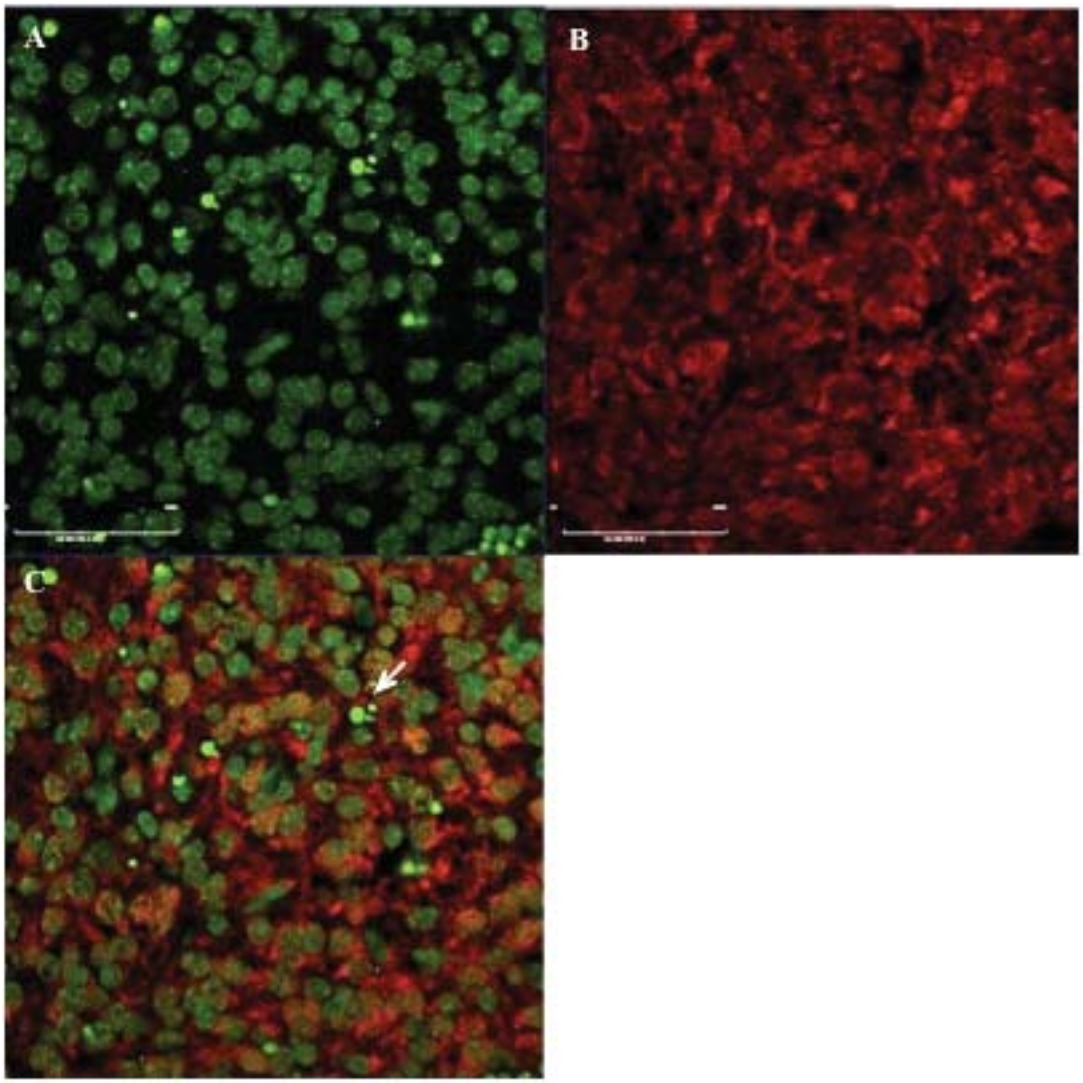

Figure 6. Characterization of LPS-induced apoptosis by double staining for TUNEL (green; A) and the neuronal marker PGP9.5 (red; B). Apoptotic neurons were not detected in the subventricular cortex of LPS-treated fetal brain (C). TUNEL staining labelled several non-neuronal cells (white arrow (C)). Magnification = 20x. Bar graphs are 50 μm.

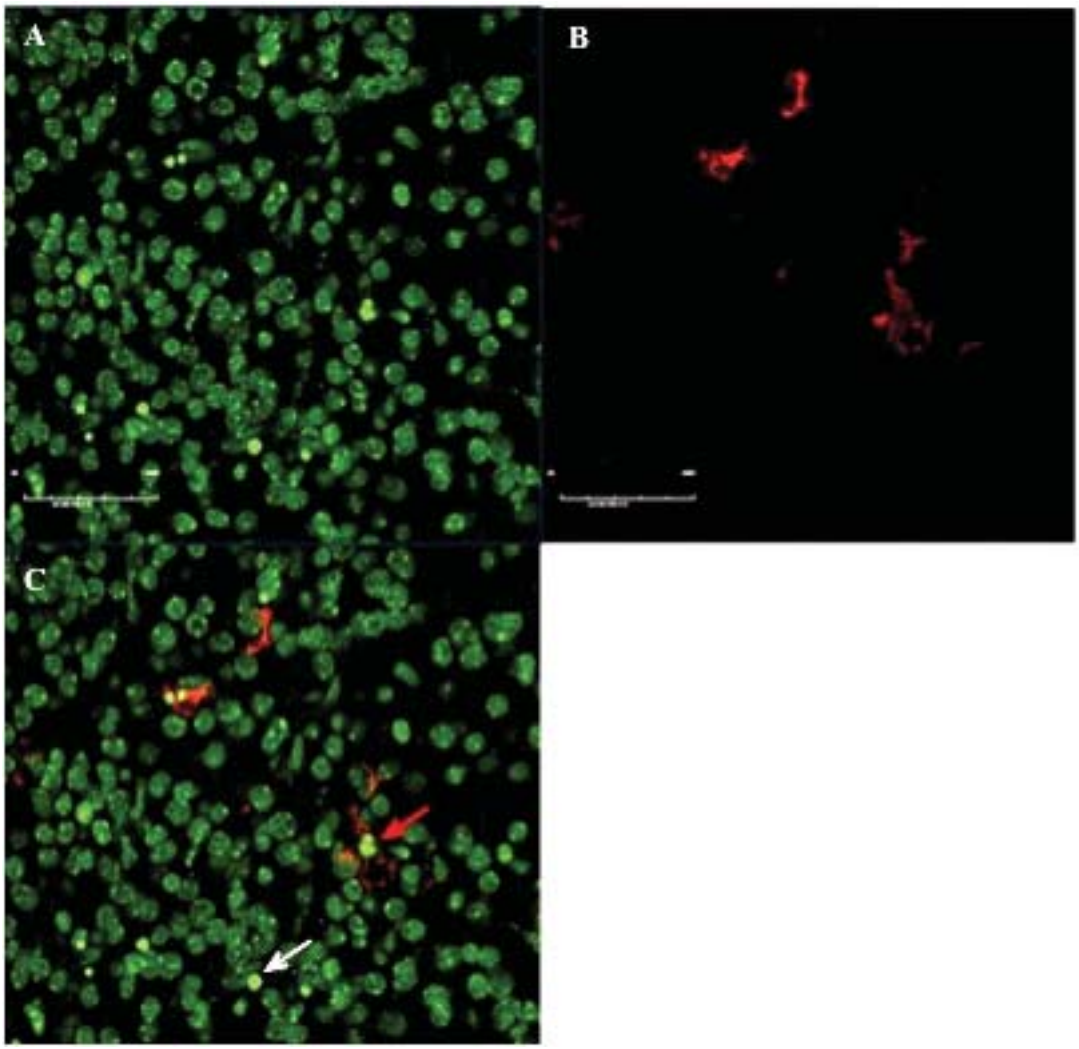

Figure 7. Characterization of LPS-induced apoptosis by double staining for TUNEL (green; A) and the microglial marker Iba1 (red; B). Colocalization of TUNEL+ nuclei was detected in some microglia cells (red arrow) in the LPS-treated fetal brain (C). TUNEL staining labeled several non-microglial cells (white arrow (C)). Magnification = 20x. Bar graphs are 50 μm.

DISCUSSION:

The susceptibility of the immature fetal brain to maternal infection and the subsequent neuropathology has been shown to be dependent on the timing of insult and the regional status of critical developmental processes (Meyer *et al.*, 2006b). Behavioural anomalies in offspring and molecular and cellular changes in fetal brain have been reported in rodents whose mothers were challenged with the viral mimic, PolyI:C early in pregnancy. Less is known about the potential for neurodevelopmental damage from bacterial endotoxin, which may cause different neuropathology as it works through a different Toll-like receptor than its viral counterpart (Takeuchi *et al.*, 1999; Matsumoto *et al.*, 2004) and therefore may produce differences in both cytokine response and its duration.

The window between GD 16 to GD 18 in mice, which equates to the second-to-third trimesters of human pregnancy, has been found to be a time where the fetal brain is vulnerable to maternal infection as neuronal proliferation in the hippocampus is most active (Meyer *et al.*, 2006b) and oligodendrocyte precursors are sensitive to pro-inflammatory cytokines particularly during the pre-myelination stage of maturation (Rezaie & Dean, 2002; Kendall & Peebles, 2005). Here we found that maternal exposure to LPS on GD 16 induced an increase in GFAP-positive astrocytes in GD 18 fetal brains. Higher numbers of GFAP-positive astrocytes were observed in the dentate gyrus, hippocampus, subventricular cortex and corpus callosum. This finding is consistent with previous studies of infection models in rabbits (Young *et al.*, 1983; Debillon *et al.*, 2000) and neonatal rodent models (Cai *et al.*, 2000; Yu *et al.*, 2004; Rousset *et al.*, 2006) and supports the premise that PVL consists of diffuse as well as

focal areas of white matter injury. It has been argued that the underlying mediator of infection-associated brain injury is the maternal cytokine release in response to inflammation. IL-1β and IL-6 can cross the placenta and increase the permeability of the BBB to inflammatory cells and mediators. This in turn can stimulate microglia and astrocyte in the fetal brain to release more inflammatory cytokines leading to damage and decrease the number of oligodendrocyte progenitors (Bell & Hallenbeck, 2002; Berger *et al.*, 2002; Huleihel *et al.*, 2004) resulting in white matter damage characteristic of PVL.

An increase in the number of TNF-α-reactive cells and in TUNEL-positive was further observed in the central white matter (CWM), subventricular cortex and the cingulate cortex of the fetal brain exposed to LPS *in utero*. The lack of colocalization of TUNEL-positive nuclei with both neurons and astrocytes, along with the limited TUNEL-positive microglia would indicate that the majority of TUNEL-positive cells were oligodendrocytes. This finding supports the premise that oligodendrocyte cell death resulting from pro-inflammatory cytokines released from astrocytes and microglia may contribute to the pathogenesis of PVL. Previously, Berger and co-workers (Berger *et al.*, 2002) have demonstrated that guinea pig hippocampal cultures exposed to LPS induced the release of TNF-α over 12 h. Others have also found increased expression of TNF-α and IL-6 in brains of neonates with PVL (Deguchi *et al.*, 1997; Yoon *et al.*, 1997c), and developing fetuses exposed to prenatal LPS (Cai *et al.*, 2000). TNF-α has also been found to decrease the number of oligodendrocytes as well as cause apoptosis of oligodendrocytes in the corpus callosum of adolescent rat pups (Bell & Hallenbeck, 2002).

A major finding of the present study was that concurrent zinc administration with LPS protected against astrogliosis and apoptosis but not against the increase in TNF-α-reactive cells in the fetal brain. This finding indicates that zinc does not prevent the activation of the cytokine response to LPS but prevents the damage caused by this response. We have previously argued that zinc treatment counteracts a fetal zinc deficiency caused by maternal zinc redistribution, which occurs as part of an acute inflammatory response to infection (Carey *et al.*, 2003b; Chua *et al.*, 2006; Coyle *et al.*, 2008). It is well recognised that the zinc-binding protein, metallothionein, which is induced in the mother's liver by inflammatory cytokines causes a fall in the plasma zinc during an acute phase response (Cousins, 1989). In early pregnancy, this hypozincaemia mediated by LPS has been shown to impair zinc uptake and cause a fetal zinc deficiency (Carey *et al.*, 2000b). However, whether the MT-mediated mechanism applies late in pregnancy is currently unknown. Maternal hepatic MT has been shown to be induced as a normal physiological response to pregnancy peaking in mid-gestation (GD 15) (Coyle *et al.*, 2009a). It is thought that the high levels of MT at this time might be unresponsive to further induction by the maternal inflammatory response. However, recent studies indicate that after GD15 maternal MT levels fall rapidly towards parturition, suggesting that the MT-mediated mechanism may still apply in late pregnancy. On the other hand, it cannot be excluded that zinc has other recognised roles in neuronal differentiation, maturation and synaptogenesis, and in repair processes, such as its role as an antioxidant or as an anti-apoptotic agent, which may offer alternative explanations for the neuroprotection observed herein (Dvergsten *et al.*, 1984; Cousins, 1989; Vallee & Falchuk, 1993; Frederickson *et al.*, 2000; Mackenzie *et al.*, 2007).

In conclusion, we have demonstrated that maternal LPS administration in the third trimester of pregnancy led to extensive astrocyte hypertrophy, which was accompanied by apoptosis in the hippocampus, subventricular cortex and corpus callosum in the fetal brain. The presence of TNF-α-reactive cells supports the involvement of pro-inflammatory cytokines as causative agents of PVL following maternal infection. Most importantly, we have shown that zinc administration at the time of LPS exposure can help protect against infection-mediated neural damage in the developing brain. This indicates that zinc supplementation may offer protection in late pregnancy from infection-mediated neurodevelopmental damage that is associated with disorders including cerebral palsy, autism, schizophrenia and mental retardation.

CHAPTER 4

CHAPTER 4

LPS-INDUCED ACTIVITY-DEPENDENT NEUROPROTECTIVE PROTEIN (ADNP) IS DECREASED BY ZINC TREATMENT IN WHOLE EMBRYOS

INTRODUCTION

Activity-dependent neuroprotective protein (ADNP) was first identified in 1999 by Bassan and colleagues as a peptide regulated by vasoactive intestinal peptide (VIP), an important mediator of post-implantation mouse embryonic growth and development (Bassan *et al.*, 1999; Poggi *et al.*, 2002; Pinhasov *et al.*, 2003; Poggi *et al.*, 2003). The protein structure of ADNP contains 9 zinc fingers, a proline-rich region, a nuclear bipartite localization signal, a homeobox domain as well as cellular export and import signals, suggesting that ADNP plays multiple roles in cellular functions including transcription factor, cytoplasmic and extracellular activities (Gozes, 2007). In the mouse, ADNP mRNA was found to be expressed in the maternal decidua and in the embryo during pregnancy. While maternal decidual ADNP mRNA is highly expressed from GD 6 to 9 and is present through to GD 18 (Poggi *et al.*, 2002), whole embryo ADNP mRNA was detectable from GD 7.5, with increased expression on GD 9 concomitant with neural tube closure, and remaining high throughout gestation albeit to a lesser extent from GD 14 onwards (Poggi *et al.*, 2002; Pinhasov *et al.*, 2003). This temporal pattern of ADNP expression throughout

gestation, in particular during the period of neural tube closure, suggests an important role of ADNP in organogenesis and fetal development. In addition, pronounced ADNP expression was detected by *in situ* hybridisation in the embryonic brain at GD 9. (Pinhasov *et al.*, 2003). The abundance of ADNP expression in the cerebral cortex, hippocampus, midbrain and cerebellum (Bassan *et al.*, 1999) further implicates a role of ADNP in brain function/development.

In a previous study, Pinhasov and colleagues reported that ADNP-knockout embryos died *in uteri* between GD 8.5 and 9.5 and exhibited defects in neural tube closure, which were associated with gross abnormalities (Pinhasov *et al.*, 2003). In turn, a peptide derived from ADNP, NAP, was shown to enhance neural tube closure in cultured GD8 embryos exposed to alcohol (Chen *et al.*, 2005) and also in fetuses exposed to prenatal alcohol (Sari & Gozes, 2006). ADNP has also been reported to possess growth promoting, anti-apoptotic and anti-oxidative properties (Spong *et al.*, 2001) and enhance the survival of neuronal cells by decreasing the accumulation of TNF-α in microglia (Beni-Adani *et al.*, 2001). In a closed-head injury model, the increase in ADNP mRNA levels following injury was localised to microglia and astrocyte-like cells in the injured side of the brain (Gozes *et al.*, 2005). Animal studies have shown that ADNP activity can prevent brain injury associated with growth restriction (Poggi *et al.*, 2003; Chen *et al.*, 2005) and behavioural deficits associated with fetal alcohol syndrome (FAS) (Sari & Gozes, 2006). Furthermore, ADNP has been shown to confer neuroprotection *in vitro* against toxic substances including beta-amyloid peptide, N-methyl-D-aspartate, dopamine, electrical blockade, AIDS virus envelope protein, hydrogen peroxide, nutrient starvation, zinc overload (Gozes, 2001, 2007) and *in vivo* studies to ethanol toxicity (Chen *et al.*, 2005). It has been postulated that increased ADNP activity following brain injury is

part of an endogenous compensatory mechanism to provide neuroprotection (Beni-Adani *et al.*, 2001). Thus it has been argued that ADNP expression is a sensitive marker for brain injury or alterations in CNS status.

Previous work in our laboratory and by others have demonstrated that maternal exposure to LPS during early pregnancy led to the development of craniofacial abnormalities (Carey *et al.*, 2003a; Chua *et al.*, 2006) as well as impairments in cognition in adult offsprings (Dammann & Leviton, 1997; Yoon *et al.*, 1997b; Debillon *et al.*, 2000; Bell & Hallenbeck, 2002; Coyle *et al.*, 2009a). Most importantly, our group has demonstrated that concomitant treatment with zinc or dietary zinc supplementation throughout pregnancy can prevent LPS-mediated birth abnormalities and cognitive impairments (Carey *et al.*, 2003a; Carey *et al.*, 2003b; Chua *et al.*, 2006; Summers *et al.*, 2006; Coyle *et al.*, 2008; Summers *et al.*, 2008; Coyle *et al.*, 2009a). In addition, we have also shown that zinc can protect against similar negative fetal outcomes caused by exposure to alcohol in early pregnancy (Summers *et al.*, 2006; Summers *et al.*, 2008). Since embryonic ADNP expression has been found to be induced by exposure to alcohol in early pregnancy (Poggi *et al.*, 2003), in this study we examine whether LPS will have a similar effect on ADNP expression and further examine whether concomitant zinc treatment can reverse any changes in ADNP expression caused by LPS exposure.

MATERIALS AND METHODS

Animals and mating procedure. See Page 57.

Drug administration and tissue collection. Pregnant dams were randomly selected into three treatment groups containing six dams per group and administered with saline (control), LPS or LPS + zinc on GD 8. LPS (*Escherichia coli* serotype O111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO), formulated in 0.85% (w/v) sterile saline and was administered at a concentration of 0.5 μg/g body weight. Zinc was given as $ZnSO_4$ at 2 μg/g body weight. Saline, LPS and zinc were all administered subcutaneously at a volume of 10 μL/g body weight.

At 6 and 24 hours after initial treatment dams were sacrificed via fluothane anaesthesia followed by cervical luxation. Uteri were immediately excised and embryos harvested into RNALater buffer (Qiagen, Australia), which were stored at -20ºC until required for analysis.

RNA extraction. Individual litters from each dam were pooled for RNA extraction. Pooled samples were homogenised using disposable micropestles to avoid cross-contamination and processed using the RNAEasy Mini Kit (Qiagen, Australia) according to the manufacturer's instructions. The quality of total RNA was determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop, USA). Total RNA samples were snap frozen in liquid nitrogen and stored at -80°C.

RT-QPCR. Total RNA samples and oligonucleotide primer pairs designed for targeting ADNP were amplified by real-time polymerase chain reaction (RT-PCR) with the SYBR® Green One-Step PCR Kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions. RNA sample was run in triplicates with each reaction containing 2x SYBR® Green PCR mix, 50x iScript reverse transcriptase, 10mM of forward and reverse primer pairs, 100ng of total RNA and nuclease-free water. The PCR reaction conditions were cDNA synthesis at 50°C for 10 min, iScript reverse transcriptase inactivation at 95°C for 5 min, PCR cycling at 95°C for 1 min and detection at 55°C for 30 sec per cycle for 40 cycles, followed by melt curve analysis which consisted of 80 cycles, 95°C for 1 min, 55°C for 1 min and 55°C for 10 sec per cycle. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard and was included in each run. The iCycler IQ:5 instrument (Bio-Rad Laboratories, Hercules, CA) was used with its internal quantitative software, which uses melting point analysis to assess the specificity of the amplified genes.

Oligonucleotide primer pairs were synthesised by Geneworks (Adelaide, SA, Australia). The ADNP primer pair was 5'-ACC TGC AGC AAA ACA ACT AT (sense) and 5'-GCT CGT TAC AGA TTG TAC (anti-sense). The primer pair used for GAPDH was 5'-ACC ACA GTC CAT GCC ATC AC (sense) and 5'-TCC ACC ACC CTG TTG CTG TA (anti-sense). Optimisation for ADNP and GAPDH specific primers resulted in annealing temperatures for ADNP of 59.5°C and GAPDH of 63°C. Relative quantification was calculated using normalized data with efficiency correction and presented as a normalised ratio of ADNP:GAPDH expression.

Statistical analyses. Quantitative data were expressed as mean ± SEM for each group and results were analysed by two-way analysis of variance (ANOVA) for multiple

comparisons followed by Tukey's post hoc test using the Sigma Stat software. The significance level was set at $p < 0.05$.

RESULTS

ADNP mRNA level was significantly increased in the whole embryo 24 h after LPS administration compared to the controls ($p < 0.05$) (Figure 1). Although there was a similar trend in ADNP mRNA level 6 h after prenatal exposure to LPS, this did not reach significance. When zinc was administered in conjunction with LPS on GD9, ADNP mRNA level was the same as that of the controls 24h later and was significantly different from the LPS alone group ($p < 0.05$) (Figure 1). Although there was a trend in the reduction of ADNP mRNA level from 6 h to 24 h following LPS + zinc administration this finding was not significantly different compared to LPS alone.

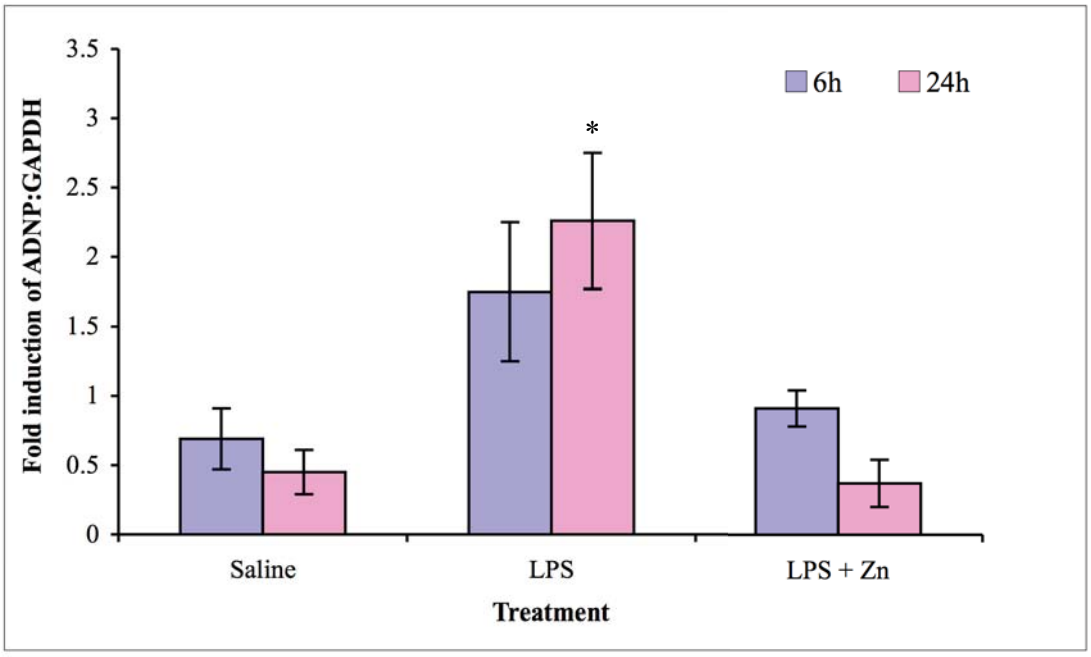

Figure 1: Expression of embryonic ADNP at 6 h and 24 h following saline, LPS or LPS + zinc administration (n = 6 litters per group). Values represents mean ± SEM. Data were analysed using two-way ANOVA for multiple comparisons. Significance at $p < 0.05$ was determined using Tukey's post-hoc test.

* Indicates significant difference between each group at the 24 h timepoint.

DISCUSSION

In this study, the expression of ADNP was up-regulated in embryos 24 h after exposure to LPS on GD 8. Although a similar trend was observed at 6 h this did not reach significance. This finding is consistent with that of Poggi and colleagues (Poggi *et al.*, 2003) who showed that mouse embryos exposed to prenatal alcohol early in pregnancy (GD 8) had increased embryonic ADNP expression after 24 h, and this persisted for 10 days. Although ADNP expression is reported to be highly expressed between GD 6 to 9 in pregnancy (Poggi *et al.*, 2002) the levels in the saline treated controls between GD 8.5 and 9 would indicate that these levels are relatively low compared to those in response to LPS. The basal levels of ADNP expression in the saline control embryos is thought to be required for neural tube closure since Pinhasov and colleagues (2003) demonstrated that embryos from ADNP knock-out mouse (ADNP -/-) exhibited neural tube defects and die *in uteri* by GD 9 – 9.5 whereas ADNP heterozygous (ADNP +/-) embryos showed delayed neural tube closure compared to wildtype (ADNP +/+) embryos.

It is currently unclear as to the mechanism behind the increase in ADNP expression caused by LPS. It is possible that ADNP is induced as a direct response to LPS or to the cytokines released by the maternal inflammatory response or to factors released in response to cellular damage. LPS might have a direct effect on ADNP expression, however whether it can cross the placenta is unclear at present. Whilst it has been reported that LPS can be detected in the placenta and fetuses 1 h after maternal administration of ^{125}I-labelled LPS (Kohmura *et al.*, 2000), this finding was opposed by a similar study where ^{125}I-labelled LPS was detectable only in maternal tissues and

placenta, but not in the fetuses, 2 – 8 h after treatment (Ashdown *et al.*, 2006). Alternatively, cytokines that are released by the maternal immune system can cross the placenta and enter the fetal circulation (Depino, 2006). It has been demonstrated that cytokines including TNF-α, IL-1β and IL-6 are elevated in the maternal serum and plasma as early as 1.5 – 2 h (Leazer *et al.*, 2002; Ashdown *et al.*, 2006), 4 h and 2 – 4 h, respectively, following LPS administration (Ashdown *et al.*, 2006). In addition, increased TNF-α concentration was found in the fetal brain from 2 h and peaking at 6 h after LPS treatment (Bell *et al.*, 2004). Thus, the induction of ADNP expression in the whole embryo from 6 h after LPS treatment falls within the same period when maternal and fetal inflammatory cytokines are increasing, making this a likely cause of increased ADNP expression. Nonetheless following head injury, ADNP activity has been found to reduce the accumulation of TNF-α in neurons and astrocytes thus preventing TNF-α-mediated cell death (Beni-Adani *et al.*, 2001). Thus there is a possibility that the LPS-mediated induction of ADNP expression in the embryo may also alter TNF-α accumulation in fetal cells. However, further studies are warranted to determine the mechanism of this increased ADNP expression. As the size of the mouse embryo on GD 9 limits separation of specific tissues, this might be accomplished by similar studies on GD 12 onwards where the brain can be isolated from the embryo for tissue-specific analyses.

While it can only be inferred that the increase in ADNP mRNA levels in whole embryo reflects the presence of cytokines that cause CNS damage in the developing fetus, the finding is nonetheless interesting particularly in relation to the effect of zinc treatment. In this study, concomitant zinc treatment was found to significantly reduce ADNP mRNA levels after 24 h. This is the first indication that zinc may protect against more subtle cellular damage caused by LPS exposure in early pregnancy, and this builds on

our previous observations demonstrating that zinc protects against fetal dysmorphology (Carey *et al.*, 2003a; Carey *et al.*, 2003b) and cognitive anomalies in offspring (Summers *et al.*, 2006; Coyle *et al.*, 2009a) exposed to LPS or alcohol on GD 8. In previous studies, we found that dietary zinc throughout pregnancy also prevented LPS-mediated fetal dysmorphology and that the number of abnormalities was inversely proportional to the zinc concentration in the diet (Chua *et al.*, 2006). These studies were based on the central hypothesis that LPS administration leads to transient fetal zinc deficiency as a result of maternal plasma zinc redistribution following the induction of maternal hepatic zinc-binding protein, metallothionein (MT; discussed in Chapter 1). Therefore, the early changes in ADNP mRNA levels in response to zinc treatment might indicate that zinc acts by preventing the MT-mediated fetal zinc deficiency thus allowing normal developmental processes to proceed. However, it cannot be excluded that zinc may modulate the inflammatory response to LPS either by limiting the damage or by increasing the efficacy of repair mechanisms possibly via the antioxidant or anti-apoptotic roles of zinc.

In conclusion, this study has shown that ADNP expression is increased following LPS administration in early pregnancy most likely as a direct response to cytokines released by the maternal inflammatory response. Future studies are required to examine how long ADNP expression persists after an inflammatory insult and to determine the cell types that express ADNP activity in response to LPS in the embryo. The finding that concomitant zinc administration with LPS prevented this rise in ADNP expression supports the premise that zinc treatment works at a very early stage possibly before damage occurs. This study thus highlights the importance of zinc nutrition in pregnancy in protecting against inflammatory-mediated fetal damage.

CHAPTER 5

CHAPTER 5

SUMMARY

Infection during pregnancy has been implicated with a range of birth abnormalities including craniofacial defects as well as neurodevelopmental disorders in the offspring. This association has been demonstrated by a plethora of epidemiological studies and has been confirmed in animal studies where it was found that the timing of exposure plays a major part in determining the phenotype and severity of fetal abnormalities. The fetus appears to be most vulnerable to maternal insults during the critical period of organogenesis between GD 6 to 14 in rodents (Junaid *et al.*, 1996) which corresponds to Weeks 3 to 8 in humans, a time even before a woman is aware of her pregnancy. Our group and others have postulated that infection-mediated induction of the zinc-binding protein MT is a major cause of fetal damage as it results in fetal zinc deficiency, which is a well-recognized cause of teratogenicity (Daston *et al.*, 1994; Taubeneck *et al.*, 1994; Carey *et al.*, 2000a).

In Chapter 2, it was demonstrated that LPS induction of MT on GD 8 led to fetal dysmorphology including exencephaly, microcephaly, micrognathia, cleft lip and limb anomalies when fetuses were examined on GD 18. More importantly, the teratogenic effect observed with low dietary zinc and LPS was synergistic. In addition, an inverse linear relationship was apparent between the concentration of zinc in the diet and teratogenicity where a lower incidence of birth defects was

observed with increasing amount of dietary zinc ($y = -0.672x + 94.6$, $R^2 = 0.742$ where y = % abnormalities and x = dietary zinc concentration). This finding suggests that even small increments of zinc above normal dietary levels will have a positive impact on fetal outcome during maternal challenge with LPS. Thus, this finding supports the premise that zinc treatment is protective by limiting the fall in plasma zinc caused by the LPS-mediated MT induction presumably by increasing the overall amount of zinc in the plasma compartment and hence that available to the fetus. In this regard, we have previously shown that a single injection of zinc solution increased plasma zinc concentration by 5-fold within 2 hours post-treatment (Carey *et al.*, 2003b) whereas dietary zinc supplementation throughout pregnancy was shown to maintain a higher level of maternal plasma zinc such that zinc concentration on GD 18 was twice as high in the zinc supplemented group (21.5 μmol/L) compared to that in the saline control dams (10.5 μmol/L) (Summers *et al.*, 2009). Moreover, our group has recently shown that dietary zinc supplementation can prevent the birth defects and neurodevelopmental damage caused by prenatal alcohol exposure, which is another potent inducer of MT and where it has been shown that dietary zinc increases the plasma zinc pool which in turn buffers the negative effect of MT induction on plasma zinc (Summers *et al.*, 2008). Nonetheless, it cannot be discounted that other processes may be involved in preventing fetal damage as the available zinc may prevent damage from reactive oxygen species, apoptosis or enhance repair processes via MT-independent mechanisms.

Epidemiological evidence has associated maternal viral and bacterial infection in pregnancy with prenatal brain damage leading to a range of neurodevelopmental disorders. In the mouse, a significant time of neural networking begins around GD 15 in the fetal brain and continues into the post-natal period, corresponding to the late-

second-to-third trimester in the human pregnancy (Week 23 to 32) (Back *et al.*, 2001; Clancy *et al.*, 2001; Bell & Hallenbeck, 2002). A major causal link underpinning the infection-induced fetal brain damage is thought to be the cytokines released following a maternal inflammatory response to infection. However, in light of the evidence reported in Chapter 2, it was of interest to determine whether zinc-deficiency played a role in the damage caused by LPS later in pregnancy particularly as we have recently shown that dietary zinc supplementation prevents impairments in object recognition memory after LPS challenge early in pregnancy (Coyle *et al.*, 2008).

In Chapter 3, maternal LPS administration on GD 16 was found to induce astrogliosis accompanied by extensive apoptotic cell death in the periventricular cortex, central white matter, hippocampus and dentate gyrus of the GD18 fetal brain. An increase in TNF-α-reactive cells in the subventricular cortex and corpus callosum of LPS-exposed fetal brain suggests that pro-inflammatory cytokines may contribute to the pathogenesis of brain damage. This finding is consistent with that of others where LPS administration in late pregnancy (GD 15 to 19) induced astrogliosis, as indicated by an increase in GFAP staining and TNF-α expression in the rat pup brain (Cai *et al.*, 2000; Yu *et al.*, 2004). In the present study, the absence of co-localisation of TUNEL+ nuclei in neurons or microglia suggests that the majority of apoptotic cells in the GD 18 fetal brain may be oligodendrocytes. This conclusion was made by exclusion, as there is currently no reliable histological marker for oligodendrocytes in the prenatal mouse brain at the gestational age examined. Nonetheless, this finding is in agreement with previous studies where LPS treatment was associated with decreased oligodendrocyte staining in the rat pup brain (Bell & Hallenbeck, 2002; Cai *et al.*, 2003). Moreover, *in vitro* studies have found that TNF-α can decrease the

number of oligodendrocyte progenitors as well as cause apoptosis of oligodendrocytes (Ladiwala *et al.*, 1999; Cammer, 2000).

Interestingly, whilst concurrent zinc treatment prevented LPS-induced astrogliosis and apoptosis, zinc did not prevent the increase in TNF-α-reactive cells in the fetal brain indicating that zinc does not prevent the cytokine response but prevents the damage caused by these inflammatory agents. This finding is consistent with our hypothesis that the primary cause of fetal brain damage is fetal zinc deficiency presumably as a result of maternal liver MT induction rather than a direct effect of TNF-α or other inflammatory cytokines. It is possible that zinc may be preventing oxidative damage caused by TNF-α and further studies are required to discriminate between the various potential protective roles of zinc. In this regard, studies with the MT-knockout mice would ascertain whether zinc protects against LPS-mediated damage by an MT-independent or -dependent mechanism.

In Chapter 4, we investigated whether changes caused by LPS on the fetus could be detected at an early stage in pregnancy and whether such changes could be prevented by zinc treatment. Activity-dependent neuroprotective peptide (ADNP) is essential for organogenesis and neurodevelopment (Pinhasov *et al.*, 2003) and was identified as a likely marker for neural injury as ADNP mRNA levels have been found to be elevated after brain damage caused by closed head injury and prenatal alcohol exposure (Beni-Adani *et al.*, 2001; Poggi *et al.*, 2003). Here, we found that embryonic ADNP expression was elevated from 6 hours with the increase persisting for at least 24 hours. Interestingly, this increase in ADNP expression occurred within approximately the same time frame as when pro-inflammatory cytokines were detected in the fetal brain (Bell *et al.*, 2004). A major finding in

Chapter 4 was that concomitant zinc treatment prevented the LPS-induced increase in ADNP expression. The fact that this was evident within 24 h of zinc treatment would indicate that zinc works very early in the inflammatory response, possibly to buffer the negative effects of LPS-induced liver MT-induction caused by the release of pro-inflammatory cytokines, which is presumed to precede fetal zinc deficiency. Thus the findings in Chapter 3 and 4 support the premise that fetal zinc deficiency is a major contributor to fetal damage. However, the mechanism by which zinc is protective needs to be elucidated. In this regard, studies with the MT-knockout mouse are warranted to differentiate the distinct roles of MT and zinc deficiency in LPS-mediated damage.

CLINICAL SIGNIFICANCE

There are many viral and bacterial infections that pose a significant risk to the fetus when contracted by mothers during pregnancy. Thus, dietary zinc taken prophylactically during pregnancy to help prevent fetal damage has significant appeal particularly as it is an essential metal with minimal toxic properties. In the human setting, zinc might be used to prevent fetal damage from prenatal infections in a similar way to which folic acid is currently used to prevent neural tube defects. Further studies are required to determine a safe level of zinc that could prevent birth defects without adverse side effects. In this regard, short-term supplementation of up to 50 mg per day has been shown to improve growth and body composition in infants and children (Friis *et al.*, 1997; Iannotti *et al.*, 2008). However, prolonged zinc supplementation at very high levels (660 mg per day) has been shown to affect the

absorption of other essential trace elements, in particular copper, resulting in anaemia (Porter *et al.*, 1977; Prasad *et al.*, 1978). Therefore, other factors that may influence zinc absorption including solubility and pH, as well as its bioavailability must also be taken into account when considering zinc supplementation as a possible therapeutic strategy.

The timing of zinc treatment may also be crucial in preventing fetal damage following prenatal exposure to infection. In this regard, studies are required to determine the timing of zinc treatment relative to the onset of infection. The MT-knockout mouse would be useful in this regard as it could be used to discriminate between MT-dependent and independent mechanisms. Zinc therapy at the onset of inflammatory response would only be beneficial if fetal damage works through the MT-mediated zinc deficiency mechanism. However, if zinc works through an MT-independent mechanism (such as enhancing repair mechanisms by preventing apoptosis and anti-oxidation) then zinc therapy may be beneficial even after the insult.

FUTURE DIRECTIONS

In light of the findings of this thesis, several studies are warranted to further elucidate the importance of zinc in fetal protection.

As mentioned previously, it is important to determine at which stage during or after infection zinc treatment is protective against fetal damage. The efficacy of novel zinc compounds should also be investigated to find those that can be absorbed more efficiently as well as determine factors that may enhance zinc absorption. Studies in larger animal models are required in order to more closely mimic human gestation, fetal development and zinc absorption. In this regard, the pig may be a useful model as it resembles the human in many ways including reproduction, anatomy, infancy, growth and neurodevelopment (Miller & Ullrey, 1987; Lind *et al.*, 2007).

Many other infectious agents are capable of activating the maternal cytokine response and may therefore impede fetal development by inducing hepatic MT and alter the maternal-fetal zinc homeostasis. In this thesis, we have only concentrated on the maternal response to bacterial endotoxin and future studies are warranted to determine whether viral agents would also work through the same MT-mediated mechanism. As a plethora of viral infections are associated with fetal birth defects and adult neuropsychiatric disorders, it is envisaged that future studies could examine the changes in MT and zinc homeostasis after administration of the viral mimic, PolyI:C. As LPS and PolyI:C are thought to work through different Toll-like receptors (Lehnardt *et al.*, 2002; Matsumoto *et al.*, 2004) they may elicit a different cytokine response and may thereby have different effects on fetal zinc homeostasis.

Again, the MT-knockout mice can be used to determine the involvement of MT in these processes.

As reported in Chapter 3, the increase in astrogliosis and apoptosis in fetal brains 48 h after LPS treatment was protected by zinc treatment. However, it is possible that the first sign of brain damage may have occurred within a few hours after the initial insult with the effect peaking before or after the time point examined in this thesis. Therefore, by following the progression of brain damage from initial onset up to adulthood, one could: 1) identify whether the damage observed in fetal brains persist or improve over time and 2) correlate the magnitude of behavioural deficits with the degree of damage. Studies in this direction would undoubtedly contribute to the significance of zinc supplementation or therapy in human pregnancies.

In Chapter 4, the expression of ADNP was assessed as a marker of neural injury following LPS exposure in early pregnancy. Interestingly, concomitant zinc treatment with LPS limited the expression of ADNP within 24 hours, which indicate that zinc may act early during the maternal inflammatory response to offset the inflammatory-induced damage. To date, this is the first report of such an effect. In this regard, studies examining ADNP activity in the MT-knockout mouse following LPS induction with or without zinc treatment would differentiate the mechanism by which zinc is protective. As the size of the embryo on GD 9 limits separation of specific tissues, future studies would be conducted later in pregnancy (e.g. GD 12 onwards) when the size of the fetus would allow one to identify tissues or cells that produce ADNP during normal pregnancy and at times of maternal insult. Moreover, gene microarray studies could be performed to identify changes in the expression of a

range of genes after LPS-challenge to determine possible pathways through which zinc is protective.

CONCLUDING STATEMENT

Taken together, the findings of this thesis provide compelling evidence that infection-induced fetal dysmorphology is associated with an MT-mediated transient fetal zinc deficiency caused by the maternal inflammatory response. In addition, there is a strong indication that LPS-induced fetal brain damage is also mediated in part through fetal zinc deficiency. However, the role that MT plays in this mechanism needs to be elucidated. The fact that zinc treatment prevented infection-induced fetal dysmorphology and brain injury indicates that zinc supplementation may potentially be used as a prophylactic therapy to prevent the negative fetal outcomes associated with prenatal exposure to infection.

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