ETHANOL-RELATED TERATOGENICITY AND NEUROBEHAVIOURAL IMPAIRMENTS: INFLUENCE OF DIETARY ZINC SUPPLEMENTATION DURING PREGNANCY

A thesis submitted for the degree of Doctor of Philosophy

by

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October 2008

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ABBREVIATIONS

°C	Degrees Celsius
µg	microgram
µl	microlitre
µmol	micromole
AE	Acrodermatitis Enteropathica
Ag	Silver
ARBD	Alcohol Related Birth Defects
ARND	Alcohol Related Neurdevelopmental Disorder
Au	Gold
BAC	Blood Alcohol Concentration
Bi	Bismuth
Cd	Cadmium
cm	centimetre
CNS	Central Nervous System
CRL	Crown Rump Length
Cu	Copper
d	day
dL	decilitre
DNA	Deoxyribonucleic Acid
EDC	Ethanol Derived Calories
EP	Escape Platform
FAS	Fetal Alcohol Syndrome

FASD	Fetal Alcohol Spectrum Disorder
g	gram
GD	Gestational Day
GLM	General Linear Model
GRE	Glucocorticoid Response Element
h	hour
HCl	Hydrochloric acid
Hg	Mercury
hZTL1	human ZnT-like transporter 1
IMVS	Institute of Medical and Vetinary Science
kg	kilogram
L	Litre
LPS	Lipopolysaccharide
LSD	Least Significant Difference
mg	milligram
Mg	Magnesium
min	minute
ml	millilitre
mm	millimetre
MRE	Metal Response Element
MT	Metallothionein
MTF	Metal Transcription Factor
n	number

NaCl	Sodium Chloride
NAD	Nicotinamide Adenine Dinucleotide
NTD	Neural Tube Defect
ORT	Object Recognition Task
PD	Postnatal Day
ppm	parts per million
REML	Restricted Maximal Likelihood
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
s	seconds
SEM	Standard Error of Mean
TF IIIA	Transcription Factor III A
TNF-α	Tumor Necrosis Factor alpha
v	volume
VSD	Ventricular Septal Defect
w	weight
Zn	Zinc
$ZnSO_4$	Zinc Sulphate

ABSTRACT

Ethanol consumption during pregnancy can result in wide range of negative outcomes, including pre- and post-natal mortality, growth retardation, physical abnormalities and brain deficits, manifested as behavioural impairments. These outcomes can result from "binge-drinking" (generally defined as >5 standard drinks on a single occasion) or chronic ethanol consumption. Ethanol-induced zinc (Zn) deficiency is one of the mechanisms proposed as a cause of ethanol teratogenicity. We have previously demonstrated in mice that ethanol exposure on gestational day (GD)8 (during organogenesis) can alter Zn homeostasis by inducing the Zn-binding protein metallothionein (MT) in the maternal liver. This causes plasma Zn concentrations to decrease as Zn redistributes into the liver, and consequently decreases the fetal Zn supply and increases the risk of teratogenicity. Subcutaneous Zn treatment with ethanol on GD8 can prevent the deleterious effects of ethanol on the fetus (i.e. physical abnormalities and spatial memory impairments). The main objective of this thesis was to investigate whether a less invasive approach of giving dietary Zn supplementation throughout pregnancy could provide similar protective benefits against a range of adverse outcomes caused by prenatal binge or chronic ethanol exposure.

Binge ethanol exposure in early pregnancy (i.e. where mice are injected with 25% ethanol (0.015 ml/g) intraperitoneally at 0 and 4 hours on GD8) significantly increased the incidence of birth abnormalities measured on GD18. These included craniofacial abnormalities (microphthalmia, anophthalmia) and limb defects. Ethanol

also increased postnatal mortality between birth and postnatal day (PD)60. In a separate study, offspring from dams given ethanol on GD8 were subjected to a physical and behavioural screening protocol (including tests for vision, olfactory, exploratory, anxiety and motor impairments) and subsequently a cohort of phenotypically-normal offspring were randomly selected for testing in a cross-maze escape task (for spatial learning and memory) and an object recognition test (for short-term non-spatial memory). While ethanol did not affect behaviour measured during screening, it resulted in spatial memory and object recognition memory impairments in adult offspring. The most important finding was that dietary Zn supplementation throughout pregnancy significantly increased plasma Zn concentrations at the time of ethanol exposure (avoiding the "typical" ethanol-induced decrease in plasma Zn) and prevented all negative outcomes resulting from early ethanol exposure (birth abnormalities, mortality, spatial and object recognition memory impairments). In the chronic ethanol mouse model (i.e. where mice were fed a liquid diet containing 27 % v/v ethanol-derived calories from GD6-18), ethanol did not affect offspring growth between birth and PD21 or spatial memory in adult offspring, thus, the influence of Zn supplementation could not be examined for these parameters. While ethanol decreased offspring weight at PD50 and increased mortality between birth and PD40, they were not prevented by Zn supplementation throughout pregnancy.

The findings from this thesis emphasise that organogenesis is a particularly vulnerable period to ethanol exposure and even a binge of ethanol during this time

can result in dysmorphology, mortality and spatial and object memory impairments in adulthood. In addition, dietary Zn supplementation is protective against the deleterious effects of binge ethanol exposure in early 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.

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- **Summers BL,** Henry C, Rofe AM, Coyle (2008) Dietary Zn supplementation throughout pregnancy prevents spatial and object recognition memory impairments caused by early prenatal ethanol exposure. Behavioural Brain Research, 186, pg 230-238.

Brooke Lee Summers

Signature…… Date……..

ACKNOWLEDGMENTS

I would like to thank my supervisors Dr Peter Coyle and Dr Allan Rofe, for their guidance and support over the duration of my PhD. In particular Peter who always made himself available for when I needed assistance (which was quite often at certain times of our study!!) and I certainly could not have asked for better supervisors.

Thanks also to a number of people I have had associations with in the Department of Clinical Biochemistry and the Institute of Medical and Veterinary Science. In particular, members of our metals research group, Joanne Chua, Jenny Fung, Nancy Tran, Matthew Rees, as well as Lisa LaVence and Catherine Henry (for their technical assistance with our behavioural studies), Michael Haywood (for passing on his expertise on atomic absorptions spectroscopy for tissue and diet Zn analysis) and Paul Anderson (for his general guidance during my PhD). A special mention must also go to Kate Dowling (Biometrics SA), Thomas Sullivan and Sue Lester, who helped with analysing our results at various stages during the study.

Thankyou to the foundations that financially supported the research undertaken in this thesis; The Channel 7 Children‟s Research Foundation (Binge drinking studies) and The Australian Brewers Association (Chronic alcohol study).

I would like to thank my parents, grandma and sister for their support throughout my study (in particular grandma who prayed so hard for me to hurry up and complete my

thesis that her rosary beads wore down and broke!). It took a little longer than expected to complete my PhD but I appreciated all the support throughout my student life to help me get through all the difficult times.

I would also like to thank my husband, Paul, for his love and support over the past 4 years and for providing me with a well deserved break from thesis-writing (our wedding day and honeymoon). Being married to a PhD student is not always fun when there is research and writing to be done, but you always helped and supported me during the stressful times and made things as easy as possible for me to get my work done. FINALLY!!!

PUBLICATIONS ARISING FROM THIS THESIS

Summers BL, Henry C, Rofe AM, Coyle P (2008) Dietary zinc supplementation throughout pregnancy prevents spatial and object recognition memory impairments caused by early prenatal ethanol exposure. Behavioural Brain Research, 186, 230-238. (Research from Chapter 3 of this thesis)

Summers BL, Rofe AM, Coyle P (2008) Dietary zinc supplementation throughout pregnancy protects against fetal dysmorphology and improves postnatal survival after prenatal ethanol exposure in mice, submitted in Alcoholism: Clinical and Experimental Research, [ACER-D-08-1929R2] (Research from Chapter 2 of this thesis)

PUBLICATIONS FROM ASSOCIATED RESEARCH

Summers BL, Rofe AM, Coyle P (2006). Prenatal zinc treatment prevents spatial learning and memory impairments caused by ethanol exposure in mice. Pediatric Research, 59 (1), 6-70.

Coyle P, Tran N, Fung JN, **Summers BL**, Rofe AM (2008) Maternal dietary zinc supplementation prevents aberrant behaviour in an object recognition task in mice offspring exposed to LPS in early pregnancy. Behavioural Brain Research, Aug 28 [Epub ahead of print]

Coyle P, Carey L, Martin S, **Summers BL**, Rofe A (2008) Ethanol mediated dysmorphology and its relationship to the ontogeny of maternal liver metallothionein. Submitted in Alcoholism: Clinical and Experimental Research, [ACER-D-08-2223].

CHAPTER 1

1.1 ETHANOL TERATOGENICITY

Alcohol (ethanol) is the most widely consumed and socially acceptable drug in western society. The growing presence of alcohol use as a part of our culture is supported by studies demonstrating a significant increase in binge drinking in American adults from 1993-2001 (Naimi et al., 2003). The term „binge drinking" generally has been defined as more than 5 standard alcoholic drinks consumed on a single occasion. In the UK, binge drinking accounts for 22 % of drinking occasions in women, with 27 % of women aged 16-24 binge drinking at least once a week (Cabinet Office Strategy Unit Alcohol Harm Reduction Project, 2003). In Australia, more than 750,000 women regularly binge drink and consume 9-30 drinks in one evening (Salvation Army, 2005).

Despite an increase in public health warnings emphasising the harmful effects of drinking alcohol during pregnancy, a recent survey in Australia found that more than 3 million women are still not convinced that it is necessary to abstain from alcohol while pregnant (Salvation Army, 2005). It is well established that the consumption of alcohol during pregnancy can have severe adverse effects on fetal development. Ethanol teratogenicity is the most common preventable cause of birth defects and brain damage in children, and produces by far the most serious neurobehavioural

effects in the fetus when compared to other drugs, including heroin, cocaine and marijuana (National Institute of Alcohol Abuse and Alcoholism and Institute Of Medicine, 1996). However, women across the world are continuing to put their babies at risk by consuming alcohol during pregnancy.

In the United States, more than 500,000 fetuses (approximately 13 % of all births) are exposed to alcohol each year (Centers for Disease Control and Prevention, 2002a). In Sweden, a study found that 30 % of subjects continued regular alcohol use during pregnancy (Goransson et al., 2003) and in Denmark, 40 % of pregnant women reported at least one binge episode since conception (25 % exceeded the recommended maximum daily intake of one drink in the second trimester on at least one occasion) (Kesmodel et al., 2003). Findings from the 2004 National Drug Strategy Household Survey demonstrate that prenatal alcohol exposure is also prevalent in Australia, with almost half of women (aged 14-49) continuing to drink alcohol during pregnancy. A recent study in Western Australia found 59 % of pregnant women drank in at least one trimester of pregnancy, with 15 % drinking more than the current Australian guidelines for alcohol consumption during pregnancy. Furthermore, 47 % of pregnancies were found to be unplanned, suggesting that many fetuses may inadvertently be exposed to alcohol before pregnancy is confirmed (Colvin et al., 2007).

1.1.1 Fetal Alcohol Syndrome (FAS)

Fetal alcohol syndrome (FAS) was first observed by Lemoine and colleagues in 1968, who reported in a series of case studies that offspring born to alcoholic mothers displayed remarkably similar physical and mental abnormalities (Lemoine et al., 1968). Jones and Smith later characterised a specific pattern of malformations, growth deficiencies and central nervous system (CNS) impairments observed in children exposed to ethanol during pregnancy and introduced the term FAS to identify individuals with these features (Jones and Smith, 1973). Since this time there have been numerous clinical and animal studies published, demonstrating the teratogenic nature of ethanol (Jones et al., 1973; Jones, 1975; Clarren and Smith, 1978; Randall and Taylor, 1979; Streissguth et al., 1980; Sulik et al., 1981; Streissguth et al., 1990; Becker et al., 1996; Mattson and Riley, 1998; Mattson et al., 2001). Furthermore, it is now clear that it is not exclusively the offspring of alcoholic mothers who are affected. It has been shown that binge drinking episodes or even moderate alcohol intake can have equally severe consequences for the fetus as chronic ethanol exposure (Padmanabhan et al., 1984; Becker et al., 1996; Eckardt et al., 1998).

1.1.2 Manifestations of fetal alcohol exposure

The consumption of ethanol during pregnancy in severe cases can result in spontaneous abortion or postnatal mortality (Kline et al., 1980; Faden et al., 1997). In surviving individuals, the type and severity of abnormalities associated with prenatal

ethanol exposure are highly varied, ranging from severe morphological defects at one extreme to more subtle behavioural problems at the other. Fetal alcohol spectrum disorder (FASD) is a term recently used to encompass this continuum of defects caused by fetal ethanol exposure. However, FAS, the most severe form of FASD, is defined by 3 distinct characteristics that patients must display to be diagnosed with FAS. These features are most evident between 4 and 14 years of age.

1) Pre and/or postnatal growth deficiency
2) Characteristic craniofacial abnormalities
3) Central nervous system deficits (behavioural impairments)

1) One of the major symptoms of fetal alcohol exposure is low birth weight (<2.5 kg, where 3.5 kg is normal), which is indicative of intra-uterine growth restriction. Infants are also often less than the 3rd percentile in length and/or gestational age. Postnatal „catch-up growth" that is often associated with low birth weight infants, does not tend to occur in babies with FAS (Tenbrinck and Buchin, 1975; Kyllerman et al., 1985).

2) Craniofacial features of FAS are shown in Figure 1.1. Three features that need to be present for diagnosis of FAS are an indistinct philtrum (the ridges between the nose and the mouth), thin upper lip and small palpebral fissures (eye slits). Other

Figure 1.1. Craniofacial dysmorphology associated with FAS (altered diagram from Warren and Foudin., 2001, available from National Institutes of Health website)

facial features include small head circumference, low nasal bridge, short nose, small chin and flat mid-face.

Other physical symptoms that are commonly observed in individuals with FAS, but are not necessary for diagnosis include; heart defects (heart murmurs, ventricular and atrial sepatal defects), limb abnormalities (of joints, hands, feet, fingers and toes), vision or hearing problems, speech and language issues, genital malformations, kidney and urinary tract defects.

3) While the physical abnormalities are the most obvious feature of FAS, brain-related behavioural impairments are the most serious and permanent consequence of maternal alcohol intake. The brain is one of the first organ systems to begin and the last to complete development. Therefore there is a large window of opportunity for ethanol to interfere with brain development during pregnancy, which may explains the broad variation in brain structures and cognitive processes affected. In humans, magnetic resonance imaging of individuals with FAS, often reveal underdevelopment of the brain (microcephaly; small brain weight and/or size). Some areas of the brain are more affected by ethanol than others, with the neocortex, hippocampus, cerebellum and basal ganglia especially susceptible to damage (Mattson et al., 1996; Swayze et al., 1997; Riikonen et al., 1999; Sowell et al., 2001; Autti-Ramo et al., 2002). These brain structures are involved in specific functions, such as motor and cognitive processes (cerebellum), perception and behavioural reactions (neocortex),

movement and control (basal ganglia) and memory (hippocampus) (National Institute of Alcohol Abuse and Alcoholism, 2000). Many of these functions are altered in children with FAS, with affected individuals displaying behavioural problems including; attention deficit/hyperactivity disorder, learning problems, memory deficits, difficulties with abstract concepts, poor problem solving skills, immature social behaviour, poor impulse control, poor reasoning and judgement, lack of control over emotions, impaired motor development and heightened stress reactivity (Mattson et al., 2001; Mattson and Riley, 1998: Sampson et al., 1997). Furthermore, the average reported IQ of patients with FAS from two separate studies was approximately 65-68, which is classified as mildly mentally retarded (IQ < 75) (Streissguth et al., 1978; Streissguth et al., 1991).

Not all individuals affected by prenatal ethanol exposure display all 3 features necessary for the diagnosis of FAS. Some may be less severely affected and display only some of the components and hence are classified as suffering from alcohol-related birth defects (ARBD). It is common for affected individuals to display behavioural impairments without other aspects of FAS and this is known as alcohol-related neurodevelopmental disorder (ARND).

1.1.3 Factors that influence the type and severity of symptoms

There are a number of risk factors that contribute to the type and severity of abnormalities resulting from prenatal ethanol exposure or are commonly associated

with alcohol abuse. These include the dosage and duration of ethanol exposure, timing of ethanol exposure during pregnancy, genetic susceptibility, nutritional status, smoking and drug use.

1.1.3.1 Dosage and duration of ethanol exposure

The dosage and duration of ethanol consumption required to impair fetal development is not known, therefore there is no safe („no-effect") level or time to consume ethanol during pregnancy. Acute ethanol exposure (i.e. duration of 1 or 2 days) typically results in partial expression of FASD, most often emerging as functional deficits. Prolonged and repeated ethanol consumption during pregnancy (i.e. chronic alcoholics) often results in a wide spectrum of defects or full blown FAS. However, several studies have shown that the incidence of mortality, malformations and behavioural impairments increase in a dose-dependant manner (Randall and Taylor, 1979; Streissguth et al., 1994; Sood et al., 2001). This supports the view that the number of drinks per occasion or peak blood alcohol concentrations (BAC), rather than solely the exposure to ethanol in utero, is the critical factor in producing defective embryos. The higher the BAC, the more ethanol is transferred to the fetus and more likely to reach the threshold range for FASD expression. Binge drinking has been demonstrated to be particularly harmful during pregnancy, as the consumption of more than 5 standard drinks in a short period results in high BAC"s. Children who were exposed to binge drinking were found to be 1.7 times more likely

to have IQ scores in the mentally retarded range and 2.5 times more likely to have clinically significant levels of delinquent behaviour (Bailey et al., 2004).

1.1.3.2 Timing of ethanol exposure

The timing of ethanol exposure during pregnancy is extremely critical in determining the „type" of fetal outcome. Embryonic development occurs in a very structured and regulated pattern, with particular growth and developmental processes occurring at specific periods during pregnancy. However, these processes and stages of pregnancy are not equally vulnerable to the damaging effects of ethanol. The pre-organogenic stage, which occurs from fertilization to implantation (humans: first two weeks of pregnancy, mice: gestational day (GD) 1-6), has often been seen as the period least sensitive to abnormalities, as ethanol exposure typically results in spontaneous abortion or resorptions to the embryo (Becker et al., 1996). The organogenesis period (humans: weeks 3-8, mice: GD 7-14) is marked by the progressive subdivision of the germinal layers, resulting in organ differentiation, a particularly sensitive stage of pregnancy for teratogenic insults. FAS usually occurs after high levels of ethanol consumption early in this period. Ethanol exposure typically results in craniofacial, ocular, cardiovascular and skeletal defects and deficiencies in a number of brain structures (incomplete/abnormal development). The postorganogenic period (humans: weeks 9-26, mice: GD 15-19) involves the growth of organ systems, maturation of organ function and a period of intense central nervous system development. Most women have completely abstained from ethanol during this stage

of pregnancy, however, ethanol exposure generally causes growth retardation and brain structural abnormalities (manifested as behavioural problems).

1.1.3.3 Genetics

Not all women who consume ethanol during pregnancy have children with impairments. Evidence that different mouse genotypes have different sensitivity to ethanol (Crabbe and Harris, 1991) and that some human genotypes confer increased adverse effects of alcohol on the developing fetuses while others provide protection (Jacobson et al., 2006), indicates that genetic factors can also influence the vulnerability of individuals to the effects of ethanol. Various polymorphic genes for enzymes involved in the production and/or removal of alcohol and its toxic metabolites have been discovered, and their different appearance in various ethnic populations could contribute to the difference in sensitivity to ethanol (Agarwal, 2001). Differences in the maternal metabolic capacity (i.e. rates of ethanol metabolism) could explain the variations in the peak alcohol exposure of the fetus between individuals and have been implicated in the pathogenesis of ethanol-related birth defects (Sanchis and Guerri, 1986; Boleda et al., 1992; Guerrini et al., 2005; Warren and Li, 2005; Gemma et al., 2007). Embryos are virtually incapable of metabolizing ethanol, therefore maternal metabolism of ethanol is critical in its elimination from the fetal circulation and amniotic fluid.

1.1.3.4 Nutrition

Maternal undernutrition is a risk factor commonly associated with chronic ethanol abuse (Guerrini et al., 2007). When the nutrients required to support fetal growth and development (e.g. folate, thiamine, Zinc, Iron, Vitamins A, C and D) and to maintain maternal health are less than optimal, it can increase the risk of abortion, growth retardation and brain abnormalities (Leis-Marquez and Guzman-Huerta, 1999; Black, 2001; Lozoff and Georgieff, 2006; Antony, 2007). Undernutrition is coteratogenic when combined with alcohol abuse, further potentiating any negative outcome resulting from ethanol exposure (Ruth and Goldsmith, 1981; Keppen et al., 1985; Wainwright et al., 1985).

1.1.3.5 Smoking and drug abuse

Smoking and drug abuse are also commonly observed in women who consume ethanol during pregnancy. In fact, a higher risk of prenatal ethanol abuse has been shown to be predicted by smoking status (Flynn et al., 2003). Smoking can contribute to adverse pregnancy outcomes, particularly low birth weight (Nakamura et al., 2004).

1.1.4 Incidence of FAS and other alcohol-related disorders

Studies in the US suggest that the incidence of FAS is approximately 0.5-5 per 1000 births (Sampson et al., 1997; May and Gossage, 2001; Centers for Disease Control

and Prevention, 2002b). In the general obstetric population, this incidence is estimated at 0.97 per 1000 births, and among heavy drinkers, 40.3 per 1000 births (4.3%) (Abel, 1995). These figures, however, ignore individuals that are born with partial expression of FAS (i.e. ARND, ARBD). Reviews of the literature conclude that about 9.1-10 in 1000 births (1%) have adverse effects due to maternal ethanol exposure (i.e. FAS and ARND combined) (Sampson et al., 1997; May and Gossage, 2001). There are marked differences in incidence rates between communities and different sub-populations, with indigenous, low socioeconomic and minority groups overrepresented (Burd and Moffatt, 1994; Abel, 1995) Recent studies demonstrate that in Italy (a setting with daily alcohol consumption with meals), the combined incidence of FAS and ARND is 20-40.5 per 1000 births (May et al., 2006). The highest incidence of FAS, however, has been reported in South African, where 65.2-74.2 per 1000 children were affected in a school first grade population (Viljoen et al., 2005).

In Australia, the issue of FAS has not been the subject of extensive research. Therefore there is very limited data on the incidence of FAS in Australia. According to the South Australian Birth Defects Register, there were only 9 cases of FAS diagnosed in 216,938 births between 1986 and 1998. A recent study estimated that the prevalence of FAS in Victoria is 0.006-0.014 per 1000 live births (Allen et al., 2007). In the Northern Territory, where the per capita adult ethanol consumption is higher than any other state in Australia, the prevalence of FAS is 0.68 per 1000 births

and in indigenous children is estimated at 1.87 per 1000 live births (Harris and Bucens, 2003). While this prevalence reported in indigenous children is relatively comparable to the high rates in indigenous populations worldwide, the prevalence in other populations of Australia are far lower compared with rates in other countries with similar cultures and drinking patterns. Therefore, it may be hypothesized that FAS is highly underreported in Australia. Many affected individuals have not been recognized due to a lack of general awareness and the inability of doctors to diagnose a child with FAS. Only 12 % of health professionals in a recent study in Western Australia could actually identify all of the essential diagnostic features of FAS and only 2 % felt prepared to deal with the disorder (Payne et al., 2005). The wide spectrum of abnormalities make definitive diagnosis problematic and determining whether a mother has consumed alcohol during gestation is also difficult, as women are often reluctant to discuss drinking habits with health authorities. In addition, some features of FAS can be caused by other contributing factors, such as smoking and other drug usage, thus it can be difficult to single out ethanol as the harmful teratogen.

1.1.5 Cost to the community

Due to the limited research on FAS in Australia, it is very difficult to estimate just how much this disorder and other alcohol-related impairments costs the Australian community. Studies primarily conducted in the American community report that the estimated annual cost for dealing with FAS alone in the US, ranged from $75 million

in 1984 to $4.0 billion in 1998. The estimated lifetime cost to care for an individual with FAS is $2.9 million (Lupton et al., 2004). The majority of this amount is associated with costs for medical treatment (e.g. caring for infants with low birth weight, surgically repairing alcohol-related defects), health care, developmental disabilities services, special education and residential care for individuals with mental retardation. However, these figures do not include the costs associated with ARBD or ARND, nor do they include costs required to aid secondary disabilities resulting from ethanol exposure during pregnancy. A study by Streissguth and colleagues found that more than 90 % of affected individuals have mental health problems, 60 % have disrupted school experience, 60 % have trouble with the law, 50 % have been confined in correctional facility, 35 % have alcohol and drug problems and 80 % have problems with unemployment and remained in dependant living status (Streissguth et al., 1996). These all impair the individuals quality of life and are costly to society (i.e. juvenile and criminal justice, substance abuse treatment and vocational services). It is therefore expected that the total costs of dealing with all alcohol-related problems are far in excess of the estimates quoted.

1.1.6 Animal Studies

The teratogenic nature of ethanol has been extensively investigated using rodent models of FAS (Streissguth et al., 1980; Sulik et al., 1981; Becker et al., 1996). Rodents exhibit the full range of ethanol-related effects seen in humans, ranging from physical dysmorphology to behavioural impairments. Furthermore, these deleterious

effects of ethanol have been identified following BAC‟s that approximate typical levels of exposure reported in alcohol-consuming women (Driscoll et al., 1990). Chronic ethanol models, designed to mimic alcoholism in humans, typically involve rodents being maintained on a liquid diet containing ethanol during pregnancy. This results in moderate BAC‟s over a prolonged time period (DeCarli and Lieber, 1967). In comparison, acute or binge-type models of ethanol exposure usually involve intraperitoneal (ip) injections of ethanol on a specific day of gestation (Webster et al., 1980; Dumas and Rabe, 1994; Carey et al., 2000b; Summers et al., 2006), resulting in significantly higher BAC‟s. Animal studies are important for increasing our understanding of the relationship between fetal ethanol exposure and teratogenic consequences, as they allow the control of numerous confounding factors that cannot be accounted for in human studies. This includes the dosage, duration and timing of ethanol exposure and nutrition, as well as elimination of factors such as smoking, drug use and disease associated with alcohol abuse in humans. Furthermore, manipulating the genetics of FAS mouse models can be exploited to provide information about the molecular mechanisms that mediate the adverse effects of ethanol on the fetus (e.g. (Carey et al., 2000b; Bonthius et al., 2002).

1.1.7 Aetiolgy of ethanol teratogenicity

Despite extensive research to determine the mechanisms underling ethanol teratogenicity, the molecular pathways leading to FASD are still poorly understood. Experimental evidence demonstrates that ethanol interferes with numerous

molecular, neurochemical and cellular events which occur during normal fetal development. Furthermore, the effect of ethanol varies depending on the duration and timing of ethanol use, the species or organ investigated or whether in vivo or in vitro models are used, making it extremely difficult to establish the major pathways involved. It is therefore likely that multiple factors are involved in mediating the teratogenic effects of ethanol at different stages of development, rather than a single dominant factor. These include direct effects of ethanol on the fetus, effects of acetaldehyde (the primary metabolite of ethanol) and indirect effects mediated by changes in maternal-fetal homeostasis (see reviews (Randall et al., 1990; Schenker et al., 1990; Goodlett et al., 2005). Due to the vast range of molecular pathways associated with ethanol teratogenicity, only the more prominent general hypotheses are briefly discussed as examples below.

1.1.7.1 Direct effects of ethanol on the fetus

Several lines of evidence support that ethanol, has a direct teratogenic effect on the fetus. (1) Ethanol crosses the placenta and equilibrates rapidly between the mother and the fetus (Ng et al., 1982; Wilkening et al., 1982; Clarke et al., 1986); (2) in vitro studies demonstrate that rat embryos grown in culture medium containing ethanol have dose-dependant embryonic growth retardation and impaired differentiation (Brown et al., 1979); (3) tert-butanol (another form of alcohol), which is not metabolized to acetaldehyde, causes some of the teratogenic effects of ethanol in animals (Grant and Samson, 1982), and (4) ethanol teratogenicity occurs even in the

presence of products which inhibit ethanol metabolism to acetaldehyde (Blakley and Scott, 1984). The cellular effects of ethanol include; impaired DNA or protein synthesis (by inhibition of DNA methylation and thymidine incorporation (Dreosti et al., 1981a; Garro et al., 1991), impaired RNA transport (Henderson and Schenker, 1977; Henderson et al., 1979) or transcription by depressing ribosomal function (Horbach et al., 1989)), alterations in cell membrane fluidity and composition leading to changes in transport and receptor dynamics, growth signaling (Schenker et al., 1990; Shibley and Pennington, 1997) and apoptosis of specific cell populations (Dunty et al., 2001). These effects consequently could impair fetal development.

1.1.7.2 Effects of Acetaldehyde

Ethanol metabolism occurs almost exclusively within the liver, and is predominantly catalysed by alcohol dehdrogenase.

$$\text{Ethanol} + \text{NAD} \xrightarrow{\text{Alcohol dehydrogenase}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$

$$\text{Acetaldehyde} + \text{NAD} \xrightarrow{\text{Aldehyde dehydrogenase}} \text{Acetic Acid} + \text{NADH} + \text{H}^+$$

Considering that ethanol is metabolized to acetaldehyde and that the toxicity of acetaldehyde is well-documented, it has been proposed that acetaldehyde may be one of the underlying mediators of ethanol teratogenicity (Dreosti et al., 1981a). Findings from experimental studies investigating this idea, however, have been inconsistent

(O'Shea and Kaufman, 1979; Webster et al., 1983; Blakley and Scott, 1984; Priscott, 1985). The perfused human term placenta can form small amounts of acetaldehyde from ethanol and can transfer acetaldehyde to the fetus where it reaches about 50% of the maternal perfusate concentration (Karl et al., 1988). While acetaldehyde has demonstrated teratogenic effects when administered as an independent agent (O'Shea and Kaufman, 1979; Sreenathan et al., 1982), it has been considered unlikely that the concentrations of acetaldehyde in a physiological setting would reach a level comparable to these studies. It has been reported that peak concentrations of acetaldehyde in the maternal and fetal blood following ethanol administration are considerably less (approximately 1000-fold) than corresponding ethanol concentrations (Brien and Loomis, 1983). Furthermore, a study utilizing the acetaldehyde dehydrogenase inhibitor disulfiram (in an attempt to raise blood acetaldehyde levels after administration of ethanol) failed to demonstrate any increase in teratology following ethanol treatment (Webster et al., 1983).

1.1.7.3 Excess formation of prostaglandins

Prostaglandins are critically important in all stages of pregnancy from implantation through the initiation of labour (Goldberg and Ramwell, 1975; Keirse, 1978), as well as normal fetal growth and development (Persaud et al., 1973; Persaud, 1975; Persaud, 1978; Challis and Patrick, 1980). However, in excess they have been shown in several species to be teratogenic (Persaud et al., 1973; Persaud, 1975; Hilbelink and Persaud, 1981; Collins and Mahoney, 1983). Data from human pregnancies

suggest that maternal ethanol exposure increases prostanoid release (Ylikorkala et al., 1988). In addition, administration of agents which inhibit prostaglandin synthesis (e.g. aspirin), reduce ethanol-induced birth defects and growth abnormalities in animals (Randall and Anton, 1984; Pennington et al., 1985). Evidence suggests that the increase in prostaglandins following ethanol exposure could be due to increased synthesis (Anton et al., 1983) and/or a decrease of their degradation by a lower prostaglandin dehydrogenase activity (Pennington et al., 1983). One suggested effect of an increase in prostaglandins is inhibition of fetal blood flow (discussed below).

1.1.7.4 Oxidative stress (excess reactive oxygen species)

Enhanced production of reactive oxygen species (ROS) have also been suggested to be involved in mediating the teratogenic effects of ethanol on the fetus (Kotch et al., 1995; Chen and Sulik, 1996; Henderson et al., 1999; Kay et al., 2000). Under normal conditions, the level of ROS and other free radicals formed during various biochemical reactions in the cell are controlled by antioxidants which eliminate ROS and prevent damage. However, if ROS levels exceed the cells ability to eliminate them, or if the normal antioxidant levels in a cell are reduced, then oxidative stress can occur. This can cause damage to cellular components, such as membranes, DNA and proteins. Evidence suggest that ethanol exposure does result in the generation of oxidative stress (including increased fetal and maternal levels of oxidative markers such as lipid peroxides and isoprostanes and reduces antioxidant levels (e.g. GSH), possibly contributing to cell damage and death in the fetus (Henderson et al., 1995;

Kotch et al., 1995; Devi et al., 1996; Guerri, 1998). Furthermore, treatment with antioxidants in animal models and embryonic cells appear to ameliorate ethanol-induced oxidative stress and fetal abnormalities (Mitchell et al., 1999; Heaton et al., 2000; Cano et al., 2001; Wentzel et al., 2006).

1.1.7.5 Altered blood supply/ placental transport

The fetus is dependant on maternal blood for the delivery of oxygen and essential nutrients (e.g. amino acids, fatty acids, glucose, trace metals and vitamins). Any interference in their delivery is likely to have a detrimental effect on fetal growth and development. Several studies have demonstrated impaired nutrient transport in pregnant animals following ethanol exposure, including amino acids (Henderson et al., 1982; Fisher et al., 1983), glucose (Snyder et al., 1986) and folate (Lin and Lester, 1985). Ethanol exposure has also been associated with fetal hypoxaemia (Mukherjee and Hodgen, 1982). In addition, fetal hypoxia and hypoglycemia have been implicated in the etiology of ethanol-induced growth retardation (Nitzan, 1981; Abel, 1985). Impairments of placental nutrient transfer could be accounted for by ethanol-induced placental dysmorphology and dysfunction, supported by the fact that placental size and histopathology is abnormal following chronic ethanol consumption (Kennedy, 1984). Alternatively, ethanol could decrease fetoplacental blood flow, as studies have demonstrated a decrease in blood flow in ethanol-treated pregnant rats (Jones et al., 1981), decreased luminar diameter in the umbilical vessels in monkeys infused with ethanol (Mukherjee and Hodgen, 1982) and human umbilical artery

strips have been shown to spasm following exposure to ethanol (Altura et al., 1982; Savoy-Moore et al., 1989). It should be noted that studies using human placental perfusion models did not find any effect of acute ethanol infusion on transport of amino acids, glucose or folate (Schenker et al., 1989), suggesting that placental transport may only be altered primarily after chronic alcohol consumption.

It is important to note that none of the mechanisms discussed have been proven or disqualified as a major cause of ethanol teratogenicity. In addition, new evidence is continuously being found supporting other molecular mechanisms which are associated with only specific aspects of fetal alcohol damage (e.g. brain). Determining a common mechanism that is involved in the aetiology of a range of ethanol-related abnormalities (physical and behavioural) is therefore important to enable the development of intervention strategies to prevent FASD.

This thesis focuses on a potential mechanism which is currently being investigated in our laboratory, Metallothionein-induced fetal Zn deficiency, and the possible intervention of this pathway by Zn treatment. In order to demonstrate how maternal ethanol consumption can decrease Zn availability to the fetus and why this transient deficiency of Zn is a potential factor underlying teratogenicity, it is necessary to first explain the functional importance of Zn in growth and development (in particular fetal development) and of the Zn-binding protein Metallothionein (MT). Therefore, the remainder of the

introduction will review the properties, functions, homeostatic regulation of Zn and effects of Zn deficiency in adults and fetal development, the role of MT and finally the evidence supporting MT-induced fetal Zn deficiency as a potential mechanism of ethanol teratogenicity.

1.2 ZINC

Zn is the second most abundant trace element in the body (behind iron) and is essential for a wide range of fundamental biological processes in both animals and humans. It is currently estimated that more than 1000 enzymes and proteins require Zn for their function, where Zn is critical for their catalytic activity or structural stability (see reviews: (Vallee and Falchuk, 1993; Auld, 2001; Laity et al., 2001). These Zn-binding proteins play a critical role, not only in general metabolic processes (e.g. reproduction, vision, taste, immune function and cognitive behaviour), but also in the regulation of growth and development. Thus, at the cellular level, Zn participates in protein, nucleic acid, carbohydrate and lipid metabolism, the control of gene transcription, regulation of cell proliferation, differentiation and apoptosis (Vallee and Falchuk, 1993; Zalewski et al., 1994).

1.2.1 Zinc biochemistry

In biological systems, very little Zn is free in solution. Zn carries out its biological roles as a divalent cation, primarily when bound to enzymes and other proteins. There

are several physical and chemical properties of Zn which make it highly adaptable to meeting the demands of enzymes and proteins, in order to carry out their function. Firstly, Zn does not undergo either oxidation or reduction under physiological conditions, and therefore is stable in a biological medium subject to a continual flux between the oxidized and reduced state. Secondly, it is amphoteric and therefore exists as the aquo and hydroxo metal complex at pH levels near neutrality. Thirdly, Zn ion complexes can participate in strong but rapid exchange of ligands (an essential characteristic for catalycity). Fourthly, Zn has a variable coordination sphere and can assume multiple coordination geometries, ranging from the most common tetrahedral complexes, to trigonal bipyramidal, square pyramdical and octahedral complexes. These features contribute to the versatility of Zn to interact with varying ligand types and are an important means for translating the chemical structure of enzymes and proteins into multiple biological functions (Vallee and Falchuk, 1993).

1.2.2 Zinc enzymes

Currently, there are thousands of Zn enzymes identified representing 50 different types, with examples from all 6 enzyme classes (i.e. oxidoreductases, transferases, hydrolases, lysases, isomerases and ligases). Zn has three primary functions in Zn enzymes: structural, catalytic or coactive (or cocatalytic) (Vallee and Falchuk, 1993; Auld, 2001). A structural Zn atom is required for the structural stability of proteins and is necessary to induce and control folding of the peptide chain, as well as the local and overall conformation of the enzyme (examples; alcohol dehydrogenase,

aspartate transcarbamylase and protein kinase C). A catalytic role indicates that Zn participates directly in enzyme catalysis. If Zn is removed by chelating or other agents, the enzyme becomes inactive and cannot function (examples; alkaline phosphatase, aminopeptidase, angiotensin converting enzyme, collagenase, carboxypeptidases, phospholipase C and dipeptidase, carbonic anhydrase) (Vallee and Falchuk, 1993; Auld, 2001). A coactive Zn site exists in Zn enzymes that contain two or more metal atoms in close proximity, which function as a catalytic unit (example; two Zn atoms in phospholipase C or Zn and Mg in alkaline phosphatase (Hough et al., 1989; Kim and Wyckoff, 1989)). The presence of the coactive Zn atom, although not absolutely essential for either enzyme activity or stability, can enhance or diminish the catalytic function in conjunction with the other active site metal atom (Vallee and Auld, 1992).

1.2.3 Zinc proteins

In addition to its function in enzymes, Zn also plays a critical role in a range of nucleoproteins that are directly involved in gene regulation. For example, when Transcription factor IIIA (TFIIIA) (the first Zn metalloprotein discovered) is coordinated to a Zn atom, the amino acids in the protein form loop structures called "Zn fingers" which contain the DNA-binding domain (Miller et al., 1985). If Zn is removed by chelating agents, TFIIIA cannot bind to DNA and regulate transcription (Hanas et al., 1983). Since the discovery of Zn finger proteins, around 45 proteins (e.g. transcription factors) have been reported to contain similar, yet conformationally

different Zn-binding motifs (Coleman, 1992; Laity et al., 2001). Differences in the mode of binding of Zn to these proteins, interatomic distances, and surrounding amino acid conformation, have led these motifs to be termed “Zn clusters” (e.g. in GAL4 protein from yeast) and “Zn twists” (e.g. in steroid hormone receptors for glucocorticoids and oestrogen) (Vallee et al., 1991). Similar to observations in TFIIIA, the removal of Zn from these proteins inhibits their function in gene regulation (Johnston, 1987; Freedman et al., 1988; Pan and Coleman, 1989).

Important for pregnancy is a relatively new group of Zn proteins containing Zn-finger motifs. The human Zic gene family comprises of five members encoding Zn-finger transcription factors (Zic1-Zic5), which have been found to play a crucial role in a variety of early developmental processes. These include the development of neural tissues and the neural crest, left-right axis patterning, somite development, formation of the cerebellum, cell fate specification and the promotion of cell proliferation. Mutations in Zic genes have recently been implicated in a wide range of congenital malformations, such as neural tube defects, heterotaxy, forebrain anomalies and holoprosencephaly (Aruga, 2004; Grinberg and Millen, 2005; Merzdorf, 2007). The requirement of Zn for the function of Zic proteins further indicates the importance of Zn for many processes involved in neural and early development.

1.2.4 Zinc absorption and transport

The adult human contains 2-3 grams of Zn, about 0.1 % of which is replenished daily. The Australian recommended daily intake of Zn is 12-16 mg/day for male adults, 4.5-6 mg/day for infants and 18-22 mg/day for lactating or pregnant women (King and Turnland, 1989). Only about one third of dietary Zn is absorbed in younger subjects (males 22-30) (Turnlund et al., 1986). Zn absorption is based on the quantity and bioavailability of Zn in food, with red meat being the richest common source of Zn. Zn bioavailability is enhanced by the presence of citrate, vitamin C, high dietary protein, prostaglandin E2, histidine, lactose, picolinic acid and pancreatic secretions, and is decreased from food with a high phytate content (Coyle et al., 1998). The absorbed Zn is transported within the body via the vascular compartment, which represents only 0.5% of total body Zn, and is distributed throughout all tissues (muscle 57%, bones 29%, skin 6% and liver 5%) (Jackson, 1989; Vallee and Falchuk, 1993; Coyle et al., 1998). Zn dependant proteins are found in the cytoplasm and within many organelles of the cell (i.e. nucleus, endoplasmic reticulum, golgi, secretory vesicles, mitochondria). Therefore transport of Zn into and out of cells and organelles require membrane transporter proteins to facilitate movement. Known eukaryotic Zn transporters come mainly from two families, the ZIP (SLC39) and CDF/ZnT (SLC30) proteins (see review: (Eide, 2006).

The absorption/transport of dietary Zn from the intestinal lumen is mainly thought to occur by transcellular pathways (passive and carrier-mediated processes) (Steel and

Cousins, 1985; Hoadley et al., 1987; Hoadley et al., 1988). In humans, the localization of Zn transporters ZnT-like transporter 1 (hZTL1)/ZnT5 (SLC30A5) and hZIP4 (SLC39A4) at the enterocyte apical membrane, indicate they have a potential role in the absorption of dietary Zn across the membrane (Ford, 2004). hZIP4 is mutated in most cases of the inherited Zn deficiency disorder acrodermatitis enteropathica, which is characterized by impaired Zn absorption from the intestine. Of the other transporters known to be expressed in the intestine, the localization of only ZnT1 (SLC30A1) at the basolateral membrane, along with evidence for Zn efflux from cells mediated by plasma-membrane associated ZnT1 (Palmiter and Findley, 1995), indicates that ZnT1 functions in the intestine to efflux absorbed dietary Zn from the enterocyte into the portal circulation.

The precise mechanisms of Zn transport across the placenta remains unclear. However, it is believed that Zn is acquired by placental syncytiotrophoblast from the serum Zn pool by a carrier-mediated process and from the Zn bound to serum protein, by an endocytotic mechanism (Bax and Bloxam, 1995). Localisation of hZTL1/ZnT5 at the apical membrane of the placental syncytiotrophoblast indicates a fundamental role in the transfer of Zn to the fetus (Ford, 2004).

1.2.5 Zinc Homeostasis

Zn homeostasis is primarily maintained via the gastrointestinal system, where dietary Zn absorption in the small intestine (mainly in the duodenum) is largely balanced by

the excretion of endogenous Zn from the pancreas and from serosa to lumen secretion across the intestinal wall (Cousins, 1985; Lonnerdal, 1989; Coyle et al., 1998). When dietary Zn is excessive, Zn absorption generally decreases and Zn excretion increases, to remove excess Zn from the body. At low dietary Zn intakes (Zn depletion), Zn absorption increases, and there is a reduction in Zn excretion and/or rate of growth (Smith and Cousins, 1980; Jackson et al., 1981; Menard and Cousins, 1983; Cousins, 1985; Coppen and Davies, 1987; King, 1990; Sian et al., 1993). During pregnancy and lactation, there are also changes in Zn metabolism that occur in order to meet the needs for Zn by the fetus. These adjustments include an increase in maternal intestinal absorption (i.e. fractional Zn absorption) (Davies and Williams, 1977b; Swanson and King, 1987; Fung et al., 1997), which is more significant in pregnant women with low dietary Zn intakes, (Donangelo et al., 2005; Hambidge et al., 2006), as well as increased placental transfer of Zn to the fetus at the end of gestation (Fosmire, 1990; Lindsay et al., 1994).

Whole body, tissue and plasma Zn concentrations remain relatively constant despite large variations in dietary Zn intake. In experimental rats, whole body Zn contents changed only when extremely low (<10 mg/kg) or extremely high (>100 mg/kg) intakes were consumed (Kirchgessner, 1993). Changes in plasma Zn concentrations (which are often used to assess Zn status and provide the conduit for Zn transport within the body) also do not occur until the homeostatic mechanisms become insufficient to maintain body Zn contents (King, 1990; Coyle et al., 1998; King et al.,

2000). Plasma Zn concentrations decrease only in severe Zn depletion, which has been suggested to occur as a "second line of defense" of homeostasis to maintain critical levels of Zn in tissues that are most susceptible to Zn deficiency (King et al., 2000). However, plasma Zn concentrations also decrease as an acute phase response to other metabolic changes such as stress, infection, inflammation and xenobiotics (including ethanol). The reduction in plasma Zn in these circumstances does not represent Zn deficiency, but a whole body redistribution with the removal of Zn from the plasma primarily into the liver in response to induction of hepatic Metallothionein (a Zn-binding protein) by inflammatory mediators and xenobiotics (i.e. redistribution of Zn to other tissues in response to a metabolic need) (King, 1990; Coyle et al., 1998). These changes in Zn distribution, although transient, can have a negative impact if it occurs during pregnancy. This response will be discussed in detail later in the introduction in relation to ethanol teratogenicity.

1.2.6 Zinc deficiency in adults

The importance of Zn is best demonstrated by the effects of Zn deficiency states (in particular acrodermatitis enteropathica) on normal body functioning. Zn deficient cells fail to divide and differentiate, resulting in poor growth and delayed development (Prasad et al., 1963). In children and adults, the organs affected include the epidermal, gastrointestinal, central nervous, immune, skeletal and reproductive systems (Hambidge and Walravens, 1982), with dermatitis, alopecia, diarrhea, emotional disturbances (Maverakis et al., 2007) and suppression of aspects

of cell mediated immunity all clinical features of acrodermatitis interopathica (Ripa and Ripa, 1995). Mild Zn deficiency, which results from insufficient dietary Zn intake, is likely to affect certain vulnerable groups; including those with poor food intake or those with high physiological requirements such as infants and young children, pregnant or lactating women.

1.2.7 Zinc deficiency during pregnancy

It is well established that Zn plays a critical role in fetal growth and development, reflected by an increase in Zn requirements throughout gestation with the growth of the fetus. It is therefore not surprising that maternal Zn deficiency during pregnancy can have a detrimental effect on fetal development. It has been shown that only one or two days on a low Zn diet is sufficient to reduce the plasma Zn levels of a pregnant dam to approximately 30% of control levels (Hurley and Swenerton, 1966; Dreosti et al., 1968). As the maternal plasma is the major source of Zn for the fetus, this can result in a decreased supply of Zn to the fetus. Animal studies have been valuable in demonstrating the link between fetal Zn deficiency and teratology (Hurley and Swenerton, 1966; Beach et al., 1982; Dreosti et al., 1986; Halas et al., 1986; da Cunha Ferreira et al., 1989). Similar to teratology found with ethanol exposure during pregnancy, the type and severity of malformations vary depending on the dietary Zn level, time and duration of exposure to Zn deficiency.

The dietary Zn level (i.e. deficiency state) necessary to affect fetal development is not known. In one study, total loss of the conceptus was demonstrated in mice fed diets containing less than 2.5 parts per million (ppm), with Zn intakes in the range of 5-10 ppm increasing post-implantation loss and incidence of teratogenesis (Dreosti et al., 1986). In comparison, other studies which fed rats Zn-deficient diets containing less than 1 ppm produced fetuses at term, albeit, with increased resorptions and significant congenital abnormalities (Hurley and Swenerton, 1966; Hurley et al., 1971; da Cunha Ferreira et al., 1989). Differences in fetal outcome could be explained by the rodent species used, or differences in the bioavailability of Zn from the diets used in these studies.

Long-term Zn deficiency in rats fed a diet containing 0.3-0.5 ppm of Zn throughout all stages of pregnancy, has been shown to impair reproduction and decrease fetal body weight, with 90 % of the fetuses demonstrating gross malformations affecting every organ system (Hurley et al., 1971). Short-term Zn deficiency was also shown to be teratogenic, but with altered incidences of abnormalities observed in accordance with the developmental events occurring at the time the deficiency was imposed (days 0-10, 22 % malformed; days 0-12, 56 % malformed; days 6-14, 50 % malformed). Zn deficiency in the first days of pregnancy is usually associated with defects of the head region, including the eyes, facial structures and brain. Later exposure to Zn deficiency results in a more frequent incidence of skeletal malformations (Hurley and Swenerton, 1966). Developmental abnormalities of the

urogenital system and tail are also common. A number of studies have also demonstrated that Zn deprivation during pregnancy and lactation can result in poor fetal activity, newborn motor development, learning, long-term, short-term and working memory in adult offspring (Lokken et al., 1973; Halas and Sandstead, 1975; Halas et al., 1977; Halas et al., 1979; Halas et al., 1983; Halas et al., 1986; Golub et al., 1995).

In humans, it has been estimated that 82 % of pregnant women world wide are likely to have inadequate intakes of Zn (Caulfield et al., 1998). Nevertheless, there are very few well-controlled studies evaluating the causal relationship between mild Zn deficiency and teratogenesis. It has been found that there is an increased risk of neural tube defects (NTD) with decreased total preconceptional Zn intake (Velie et al., 1999). Babies with NTD demonstrated lower hair Zn levels (Srinivas et al., 2001) and 43 % of NTD cases had lower serum Zn concentrations (Golalipour et al., 2006). In addition, children with cleft lip and their mothers had significantly lower red-blood cell Zn concentrations than controls (Krapels et al., 2004). Evidence that the human fetus is susceptible to the teratogenic effects of severe Zn deficiency is best demonstrated by pregnancy outcomes in women with AE. Hambridge and colleagues reported that out of every seven pregnancies, there was one abortion and two malformations (Hambidge et al., 1975).

Table 1.1. Similarities in fetal outcome following maternal Zn deficiency or ethanol exposure during pregnancy in rodents

	References	
Fetal abnormality	**Zn deficiency**	**Ethanol**
Resorptions	(Dreosti et al., 1986) (Hurley et al., 1971)	(Randall and Taylor, 1979) (Sauerbier, 1987)
Low birth weight	(Hickory et al., 1979) (Beach et al., 1982)	(Sauerbier, 1987) (Chernoff, 1977)
Ocular defects	(Dreosti et al., 1986) (Hurley and Swenerton, 1966)	(Sauerbier, 1987) (Sulik et al., 1981)
Cleft lip/palate	(Hurley et al., 1971) (Hurley and Swenerton, 1966)	(Sulik et al., 1981) (Kotch and Sulik, 1992)
Skeletal abnormalities	(da Cunha Ferreira et al., 1989) (Hickory et al., 1979)	(Randall and Taylor, 1979) (Lee and Leichter, 1983)
Brain abnormalities	(Hurley et al., 1971) (Dreosti et al., 1981b)	(Lopez-Tejero et al., 1986) (Berman and Hannigan, 2000)
Cognitive impairment	(Halas and Sandstead, 1975) (Halas et al., 1986)	(Riley, 1990) (Berman and Hannigan, 2000)
Heart defects	(Hurley and Swenerton, 1966)	(Webster et al., 1984) (Chernoff, 1977)
Postnatal mortality	(Beach et al., 1982) (Hurley and Mutch, 1973)	(Middaugh et al., 1988) (Lopez-Tejero et al., 1986)

1.2.8 Teratogenicity: zinc deficiency vs ethanol exposure

It is apparent that the teratogenic outcomes resulting from prenatal Zn deficiency are very similar to those resulting from prenatal ethanol exposure (Table 1.1). Both insults interfere with fetal growth and development, resulting in congenital malformations (e.g. craniofacial defects and brain impairments). They have also been shown to affect the same cellular processes, such as increasing embryonic cell death (apoptosis) in specific cell populations (Rogers et al., 1995; Jankowski-Hennig et al., 2000; Dunty et al., 2001). In addition, the exposure of rodents to Zn deficiency and ethanol simultaneously during pregnancy (short and long term exposure) is synergisitic, such that there is an increased incidence of fetal abnormalities compared to either insult alone (Ruth and Goldsmith, 1981; Miller et al., 1983; Keppen et al., 1985). These early findings are suggestive of a common underlying Zn-related aetiology, (i.e. ethanol-induced Zn deficiency). It has been shown that the Zn-binding protein metallothionein (MT), plays a critical role in this mechanism, as the link between maternal ethanol exposure and fetal Zn deficiency.

1.3 METALLOTHIONEIN (MT)

MT‟s are intracellular, low molecular weight (<7000Da) metalloproteins which are present in almost all living organisms. In mammals, the single-chain polypeptide consists of 61-68 amino acids, with a highly conserved distribution of 18-23 cysteine residues, a property which enables MT to bind to metals. While the metal-free form of MT (known as apo-MT) has a disordered structure, the binding of metal ions to

apo-MT results in the development of a well defined protein fold (Figure 1.2). MT‟s are structurally composed of two globular metal-binding domains (α and β) with differential capabilities in associating with metal ions. The stable C-terminal α domain (residues 31-62) contains 11 cysteine residues and can bind 4 divalent metal atoms. In comparison, the more reactive N-terminal β domain (residues 1-30) contains 9 cysteines and binds 3 (Romero-Isart and Vasak, 2002; Vasak, 2005). Therefore, under physiological conditions MT can bind a maximum of 7 Zn ions or 12 Cu ions, in tetrahedral and trigonal arrangements, respectively (Nielson et al., 1985). While MT binds Zn predominantly under normal physiological conditions, Zn can be readily displaced by Cu or other exogenous metals, such as Cd in excess (Shaw et al., 1991). Cu has the highest binding affinity for MT, followed by Cd and then Zn (Waalkes et al., 1984a).

1.3.1 Isoforms

There are four major isoforms of MT which have been identified in mammals, termed MT-1 to MT-4. In humans, there are at least 16 MT genes clustered on chromosome 16, including at least 11 MT-1 genes (MT1A, -B, -E, -F, -G, -H, -I, -J, -K, -L, and –X, some of which may encode RNAs that are not functional in directing production of detectable MT proteins), MT-2 (also known as MT-2A), MT-3 and MT-4. In the mouse, there are only four MT genes (one for each isoform) that reside on chromosome 8 (West et al., 1990; Quaife et al., 1994).

As the majority of MT research has been done on rodents, the following discussion refers to MT in mice. The four MT isoforms differ in amino acid residues (other than the shared 20 cysteine residues), their charge properties, the organ they are expressed and their inducibility. MT-1 and MT-2, the most abundant isoforms of MT, are ubiquitously expressed in most tissues, with the highest areas of expression being the liver, pancreas, kidneys and intestine. They are also expressed at all stages of development.

MT-3, which has been shown to have a specific neuronal growth inhibitory function, is expressed predominantly in gluatminergic neurons in the brain, although very low expression has been observed in the pancreas and intestine (Ebadi et al., 1995). MT-4 is expressed in differentiating stratified squamous epithelial cells (Palmiter, 1987) and has also been detected in the maternal deciduum (Liang et al., 1996).

1.3.2 MT Induction

MT-1 and MT-2 are rapidly induced by a variety of stimuli, whereas MT-3 and MT-4 are relatively unresponsive in similar conditions. Expression of MT1 is most dynamic in the liver, where metals, xenobiotics and acute phase or stress proteins (e.g. cytokines, oxidants) have demonstrated MT inductive properties. The promoter region of MT genes contains several response elements which are involved in the induction and transcriptional regulation of MT, as well as elements involved in basal

[1] Note: From this point of the thesis the term „MT" refers collectively to the MT-1 and MT-2 isoforms.

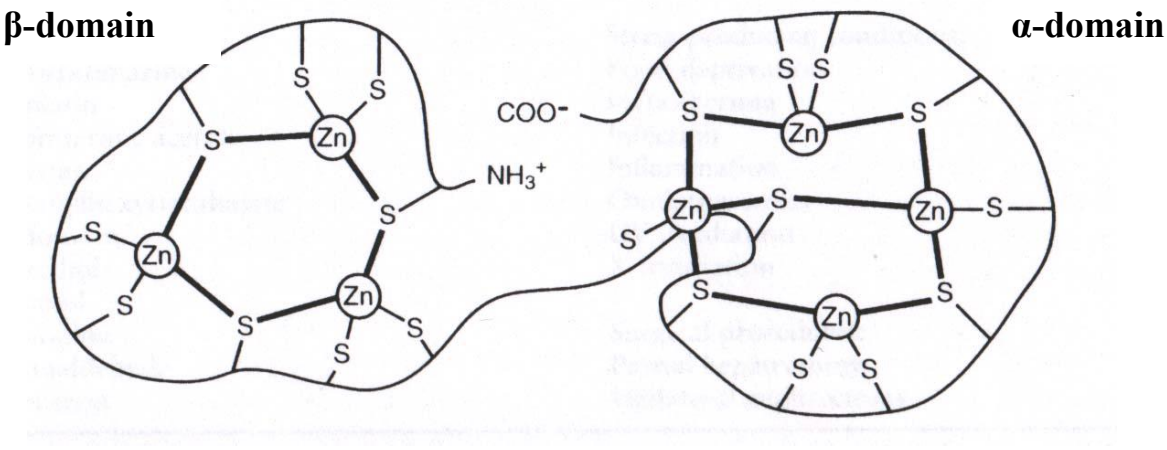

Figure 1.2. A schematic representation of the Metallothionein structure bound to 7 Zn2+ atoms

level transcription. Distinct pathways and response elements are responsible for the activation of the MT promoter by different inducing factors. Of the metals, Zn, Cu, Cd, Ag, Au, Hg and Bi have all been shown to induce MT (Palmiter, 1994). However, Zn is the primary physiological inducer. The binding of metal ions to metal transcription factor (MTF-1) allows the protein to bind to metal response elements (MRE) on the MT gene which initiates gene transcription (Westin and Schaffner, 1988).

Inflammation or infection results in the release of large concentrations of cytokines (e.g. interleukin (IL-6), IL-1 and tumor necrosis factor alpha (TNF-α), glucocorticoids and catecholamines), which are all effective inducers of MT in the liver (Karin and Herschman, 1980; Brady and Helvig, 1984; Bremner, 1987; Sato et al., 1992; Coyle et al., 1993). These acute phase proteins induce MT by interacting with response elements distinct from MRE‟s. Physical stressors such as cold, heat and restraint also increase hepatic MT levels, most likely via inflammatory intermediates (Oh et al., 1978; Hidalgo et al., 1988; Hernandez et al., 2000). However, restraint stress has also been shown to activate the glucocorticoid receptor, which consequently binds to glucocorticoid response elements (GRE) and activates MT transcription (Ghoshal et al., 1998; Jacob et al., 1999).

While a number of xenobiotics including ethanol, paracetamol, urethane, α-hederin, lipopolysaccharide (LPS) and carbon tetrachloride have been shown to induce MT,

the exact pathways involved are unknown (Waalkes et al., 1984a; Waalkes et al., 1984b; Brzeznicka et al., 1987; Wormser and Calp, 1988; Min et al., 1991; Liu et al., 1993; Carey et al., 2000b; Carey et al., 2003a). Response elements have not been identified to interact directly with these drugs, therefore it is possible that the release of inflammatory factors are responsible for the induction of MT (as discussed above).

The consequence of MT induction in all cases (metals, drugs or acute phase response) is the movement of Zn from the plasma into the liver, where it is subsequently bound by MT. This results in an increase in the concentration of Zn in the liver and consequently, a significant decrease in the plasma Zn concentrations from normal (approximately 13μmol/L in mice) (Daston et al., 1991; Daston et al., 1994; Carey et al., 2000b; Nishimura et al., 2001; Carey et al., 2003a). The extent of the response depends on the concentration and type of inducer and the duration of MT induction. Nevertheless, as the plasma compartment contains only 0.5% of total body Zn, even small changes in the distribution of Zn can have a significant effect on plasma Zn levels, without impacting on concentrations in other tissues with higher Zn content.

1.3.3 MT function

Despite a diverse range of functions of MT being discovered, a primary and essential role of MT has not been identified. The generation of mice lacking functional MT genes (MT-/- mice), as well as mice that overexpress MT, has contributed

significantly to our current understanding of the multifunctional roles of MT. It appears that MT is not essential for normal life, as MT-/- mice demonstrate normal reproductive capacity and long-term survival (Masters et al., 1994; Coyle et al., 2002). However, there is evidence that MT is involved in Zn homeostasis, as in situations of extreme Zn challenge (Zn excess or deficiency), MT+/+ mice are better able to regulate Zn absorption than MT-/- mice (Coyle et al., 2000). Furthermore, there is evidence of a survival advantage of MT in a range of stress situations. MT has been indicated to protect against heavy metal toxicity (Michalska and Choo, 1993; Masters et al., 1994), xenobiotics (Kondo et al., 1995; Deng et al., 1999; Liu et al., 1999; Hanada, 2000) and inflammation (Coyle et al., 2002). For more detail on MT function, see reviews (Palmiter, 1998; Coyle et al., 2002; Vasak, 2005).

1.3.4 MT induction during pregnancy and teratology

While it is clear that MT induction has advantages in certain situations, there is evidence that the induction of MT during pregnancy can have a negative effect on fetal development. Daston and colleagues first demonstrated that exposure of pregnant rats to urethane on GD 11 significantly induced liver MT, decreasing maternal plasma Zn concentrations by 30 %, and inhibiting the transfer of ^{65}Zn to fetal tissues by 50 % compared to controls (Daston et al., 1991). The induction of MT and subsequent decrease in fetal Zn supply (which is essential for fetal growth and development) resulted in fetuses that exhibited decreased weight and delayed skeletal ossification when examined on GD 18. A range of other teratogenic compounds with

different pharmacological actions, such as α-hederin, TNF-α, 2-ethylhexanoic acid, arsenic, and most important to this thesis ethanol, have since been demonstrated to have similar effects on MT, Zn distribution and fetal outcome (Daston et al., 1994; Taubeneck et al., 1994; Taubeneck et al., 1995; Bui et al., 1998).

1.3.5 Ethanol-induced Zn deficiency: A possible cause of teratology

Fetal Zn deficiency has for several decades been suggested as a key contributing factor in the aetiology of ethanol-related birth defects and is the mechanism currently being investigated in our laboratory. Flynn and associates were first to recognise that alcoholic mothers had significantly lower plasma Zn levels than non-alcoholic women and that an inverse relationship occurs between maternal plasma Zn levels and expression of FAS (Flynn et al., 1981). The similarities in congenital abnormalities produced by prenatal Zn deficiency and those found in FASD, as well as the fact that these insults are co-teratogenic further supported that there may be a common mechanism between ethanol and Zn-deficiency related teratology.

Studies in rats (Taubeneck et al., 1994) and by our laboratory using mice (Carey et al., 2000b) demonstrate that ethanol exposure at the beginning of organogenesis (day 8 of gestation, GD 8) results in fetal Zn deficiency, by the induction of MT in the mother‟s liver. This causes Zn to redistribute from the maternal plasma into the liver as it is sequestered by MT, resulting in a decrease in plasma Zn concentrations by up to 65 % over 16 h. The transient decline in plasma Zn limits the supply of Zn to the

fetus, as studies have demonstrated that maternal ethanol exposure impairs transfer of ^{65}Zn to the fetus (Carey et al., 2000a) and reduces fetal Zn uptake and total fetal Zn content (Ghishan et al., 1982). The role of MT and Zn deficiency in ethanol teratogenicity is best supported by our findings that ethanol exposure in MT+/+ mice increased the incidence of physical birth abnormalities (27 %) compared with saline-treated mice (6.4 %), in association with decreased plasma Zn concentrations. In the absence of MT, MT-/- mice did not demonstrate a fall in plasma Zn following ethanol exposure (Carey et al., 2000b), ethanol had no effect on the delivery of Zn to the fetus (Carey et al., 2000a) and MT-/- fetuses exhibited fewer abnormalities than saline-treated controls (2.2 %). In addition, we have recently shown that a subcutaneous Zn injection at the time of ethanol exposure on GD 8 can prevent birth defects and spatial memory impairments in mice offspring, by increasing plasma Zn concentrations following ethanol exposure (Carey et al., 2003b; Summers et al., 2006). These studies indicate that maternal plasma Zn levels are important in the aetiology of ethanol-related impairments and in addition, can be manipulated by treatment with the intention of limiting these impairments. However, in the human setting, the administration of Zn to mothers via subcutaneous injection is not a likely or desirable form of intervention. This thesis therefore questions the possibility of whether Zn treatment can be administered via the diet, and whether Zn supplementation can provide sufficient levels of Zn to the mother to prevent the effects of ethanol (i.e. binge drinking and chronic ethanol models) on fetal development.

1.4 SUMMARY

The consumption of ethanol during pregnancy can have severe adverse affects on fetal development, resulting in spontaneous abortion or a spectrum of birth defects (FASD). These include growth retardation, craniofacial abnormalities and brain deficits, manifested as behavioural impairments. The type and severity of these abnormalities is dependant on a number of factors, including the dosage, duration and timing of ethanol exposure. Most worrying is that FASD not only results from chronic alcoholism, but even by a single occasion of binge drinking during early pregnancy. Despite public warnings in Australia on the adverse affects of prenatal ethanol exposure, there is still a high incidence of pregnant women consuming ethanol during pregnancy. FASD is of major public health concern and a significant economic cost to the community. Understanding the mechanisms involved in ethanol teratogenicity is therefore crucial to develop intervention strategies to protect the fetus in situations where it is exposed to alcohol and minimize these negative outcomes. While a diverse range of mechanisms have been investigated, there are several lines of evidence, discussed herein, that support a hypothesis that ethanol-induced fetal Zn deficiency is one of the underlying mediators of teratology. Similarities between the teratogenic effects of prenatal ethanol and Zn deficiency imply a common aetiology. Ethanol (along with a number of other teratogens), has been shown to be a potent inducer of the Zn-binding protein MT, in the maternal liver, which precedes movement of Zn from the plasma into the liver (sequestered by

MT), thus depleting the plasma of Zn for up to 24 h. This compromises the fetal Zn supply and decreases Zn transfer to the fetus, thereby increasing the risk of teratology. Zn is essential for fetal development, being involved in a myriad of functions including its presence in a large number of enzymes and transcription factors that regulate gene expression. Thus, maintaining an adequate fetal Zn supply is critical during pregnancy. Key findings from our laboratory demonstrate that subcutaneous Zn injections at the time of ethanol exposure on GD8 can prevent physical impairments and spatial memory impairments in mouse offspring. These findings not only support our hypothesis that FASD is caused at least in part by a metallothionein-mediated mechanism, but indicate that the changes in maternal plasma Zn following ethanol exposure can be manipulated to limit ethanol-related impairments. Based on these results, this thesis focuses on whether a more natural approach of giving dietary Zn supplementation throughout pregnancy can provide similar therapeutic benefits against a range of abnormalities (including physical defects, growth, mortality and behaviour) caused by binge-drinking in early pregnancy or chronic ethanol exposure throughout pregnancy.

1.5 AIMS

Investigations in this thesis were directed towards extending the findings of earlier experiments (Carey et al., 2003, Summers et al., 2006) to examine the potential benefit of dietary Zn supplementation in protecting against ethanol teratogenicity. More specifically the major aims were to:

Determine whether dietary Zn supplementation throughout pregnancy can prevent physical birth abnormalities at GD 18 caused by ethanol exposure on GD 8.

Investigate the MT-Zn response in Zn-supplemented dams following ethanol exposure on GD 8.

Assess whether maternal ethanol exposure on GD 8 affects postnatal growth, survival, behavioural parameters (including anxiety, motor, exploratory function, spatial learning and memory and object recognition) or brain structure in adult offspring.

Examine the effects of dietary Zn supplementation throughout pregnancy on growth, survival and behavioural parameters in adult offspring, and whether it has a protective role against any negative effects of ethanol on these measures.

Develop a chronic ethanol mouse model to examine whether chronic ethanol exposure throughout pregnancy can affect postnatal growth, survival and spatial memory impairments in adult offspring and to assess whether dietary Zn supplementation can protect against any negative outcomes caused by ethanol.

CHAPTER 2

The effect of ethanol exposure in early pregnancy on birth abnormalities, pre- and post-natal growth, and on survival in offspring: The influence of dietary Zn supplementation and its effect on maternal MT-Zn response.

2.1 INTRODUCTION

The teratogenic nature of ethanol has been firmly established in clinical and experimental animal studies. The most apparent adverse fetal outcomes caused by prenatal ethanol exposure are the physical birth abnormalities, such as craniofacial dysmorphology, growth deficiency and cardiovascular defects, and in extreme cases prenatal or postnatal mortality (Jones, 1975; Clarren and Smith, 1978; Randall and Taylor, 1979; Streissguth et al., 1980; Becker et al., 1996). While these physical defects are perceived as the severe end of the spectrum of prenatal ethanol effects, a significant number of studies (including recent studies in our laboratory) have demonstrated that they can result from even a single "binge" of ethanol in early pregnancy (GD 7-10 in mice), a particularly sensitive period of fetal development marked by rapid cell division and differentiation (Webster et al., 1983; Padmanabhan et al., 1984; Cook et al., 1987; Sauerbier, 1987; Becker et al., 1996; Carey et al., 2000b; Carey et al., 2003b; Sulik, 2005). In humans, studies indicate that ethanol exposure and in particular reports of binge drinking, is often highest in early pregnancy (Kesmodel et al., 2003; Alvik et al., 2006; Ethen et al., 2008).

As discussed in the first chapter of this thesis, there is growing evidence supporting that physical abnormalities result, at least in part, from the adverse effect of ethanol on maternal/fetal Zn homeostasis. Briefly, ethanol exposure in early pregnancy induces maternal liver MT, which causes plasma Zn concentrations to decrease as Zn is redistributed to the liver and therefore limits the fetal Zn supply required for normal development (Taubeneck et al., 1994; Tanaka, 1998; Carey et al., 2000a; Carey et al., 2000b). Of particular interest are our recent findings that subcutaneous Zn treatment, when given concomitant with ethanol exposure on GD 8 in pregnant mice, can prevent physical birth abnormalities and spatial memory impairments, by increasing plasma Zn levels at the time of ethanol exposure and limiting the "typical" ethanol-associated decrease in fetal Zn supply (Carey et al., 2003b; Summers et al., 2006). An earlier study by Seyoum and Persaud (1995) using intraperitoneal Zn treatment also supports our findings that Zn treatment has a protective influence against ethanol teratogenicity, as ethanol + Zn-treated fetuses had a higher number of somites, cardiac development were more advanced and embryonic protein content was higher than ethanol alone (Seyoum and Persaud, 1995). While these studies demonstrate that maternal plasma Zn levels can be altered to limit ethanol teratogenicity, the administration of Zn via injections is not a desirable method of delivery for a long-term therapy. Furthermore, subcutaneous Zn treatment transiently increases the maternal plasma Zn to levels that may be viewed as being non-physiological (5 times higher than normal) and perhaps even deleterious (Carey et al.,

2003b). This raises the question of whether dietary Zn supplementation, a more generally accepted and less invasive form of Zn treatment, can also protect against ethanol teratogenicity.

A number of studies have previously examined the influence of dietary Zn supplementation on ethanol-related birth defects. Tanaka and colleagues demonstrated that excess Zn in the diet in ethanol-treated pregnant rats resulted in an increased fetal body weight, increased protein content of the cerebrum and prevented the resorptions obtained with ethanol alone (Tanaka et al., 1982). Zn supplementation with ethanol also increased the metabolic activity in the hippocampus (evidence of prevention of the brain dysfunction by ethanol treatment) and increased cerebral weight and RNA compared to ethanol alone, however, not to the level observed in controls (Tanaka et al., 1983). Contrary to early findings, they later found no benefit of Zn supplementation (Tanaka et al., 1988; Tanaka, 1998). Keppen and colleagues (1990) also found that supplemental Zn (four times the recommended daily allowance) was not protective against the effects of ethanol on fetal development, and appeared to have an adverse effect on fetal weight and prenatal mortality (Keppen et al., 1990). These inconsistent findings, however, were all from studies which used a chronic alcohol model (i.e. where ethanol is continuously consumed in the diet throughout all stages of pregnancy), rather than a "binge" model where ethanol is given as an acute dose on a single day of gestation. It is yet to be examined whether

dietary Zn supplementation is protective against the deleterious effects of acute ethanol exposure in early pregnancy.

The first aim of this chapter is to examine the effects of dietary Zn supplementation throughout pregnancy on the development, growth and survival of offspring during gestation and postnatal life, of mothers acutely exposed to ethanol on GD 8. GD 8 in mice occurs during the critical period of organogenesis, and the formation of specific areas of the CNS with neurogenesis and migration of cells in the fore-, mid- and hindbrain. Ethanol exposure at this time in rodents has been shown by numerous studies to cause mortality, growth restriction, craniofacial, cardiac, ocular, brain and behavioural impairments, as well as cell death in developing regions of the CNS (Becker et al., 1996; Carey et al., 2000b; Dunty et al., 2001; Kronick et al., 1976; Minetti et al., 1996; Molina et al., 1984; Sauerbier., 1987; Sulik et al., 1981; Summers et al., 2006; Webster et al., 1984; Weston et al., 1994). GD 8 was specifically chosen as the day of ethanol administration, as it results in a wide range of the features observed in FASD. This chapter will focus on physical defects (including craniofacial dysmorphology and heart defects), growth deficiency and mortality and the influence of dietary Zn supplementation determined in a normal pregnancy setting. It is hypothesized that dietary Zn supplementation will have a beneficial effect in protecting against the negative outcomes of GD 8 ethanol exposure.

The second aim of this chapter is to determine whether dietary Zn supplementation can alter the effects of ethanol exposure on maternal Zn homeosasis (a possible mechanism for the prevention of ethanol-related defects). It has previously been discussed (Carey et al., 2003b) that bypassing the gastrointestinal processing step may be necessary to obtain an increase in the resultant plasma Zn concentration. Indeed, in the gastrointestinal tract, MT is thought to play a role in restricting Zn absorption in times of excess (Davis et al., 1998). While it is unlikely that oral Zn supplementation can increase the maternal plasma Zn to levels comparable to those achieved by subcutaneous Zn injection, several studies have indicated that high dietary Zn intakes during pregnancy can increase plasma Zn levels to some degree (Goldenberg et al., 1995; Neggers et al., 1997). In addition, Mendeleson and Huber (1979) found that the reduction in fetal Zn caused by long-term ethanol exposure (6 % ethanol in drinking water) throughout pregnancy was prevented by diets fortified with Zn (Mendelson and Huber, 1979). Whether dietary Zn supplementation during pregnancy is sufficient to prevent the "typical" ethanol-induced decrease in plasma Zn (as shown using subcutaneous Zn treatment) (Carey et al., 2003b) or alter the associated increase in liver MT and liver Zn concentrations will be examined in this study. These findings will provide a better understanding of the actions of ethanol on the embryo and possible protective mechanisms of Zn supplementation on fetal development.

2.2 METHODS

2.2.1 AIN-93G diet (for growth, pregnancy and lactation in rodents)

The diet used for all experimental phases of our studies was casein-based and composed as seen in Table 2.1. The components of the AIN-93M mineral mix and AIN-93-VX vitamin mix used in this diet are shown in Tables 2.3 and 2.3 respectively.

The constituents of the diet were combined in a large commercial dough mixer (OEM VE201, Italy) in the following order:

Cornstarch, casein, dextrinized cornstarch and sucrose added and mixed for 20 min.

Choline bitartrate, L-cystine, vitamin mix, mineral mix and cellulose added and mixed for 30 min.

Soybean oil gradually added over 5-10 min, then mixed for 20 min.

Water containing $ZnS0_4$ added (see below) and mixed until a smooth consistency was obtained.

The basic mixture (with no added Zn) was analysed by flame atomic absorption spectroscopy (Perkin Elmer Analyst 300, Überlingen, Germany) and found to have a Zn content of approximately 5 mg/kg. The required amounts of aqueous $ZnSO_4$ were added to the mixture to produce a control and Zn-supplemented diet with Zn levels of 35 and 200 mgZn/kg respectively. For each kg of dry mix, approximately 600 ml of water (containing $ZnSO_4$) was added. The mixture was placed in plastic trays up to a thickness of 2 cm, and dried for 1-2 days at room temperature. The concentrations of

Zn in each diet were confirmed via flame atomic absorption spectroscopy. The diets were cut into squares and stored in the freezer (-20 °C) until required.

2.2.2 Animals and mating procedure

C57BL/6J mice were purchased from the Institute of Medical and Veterinary Science (IMVS, Adelaide). The C57BL/6J mouse strain is established as the standard animal model of FAS, as it is the strain most able to metabolically process ethanol, it exhibits similar patterns of physiological abnormalities following prenatal ethanol exposure as humans (ranging from behavioural abnormalities to congenital heart defects and dysmorphology) and its effects are seen at BAC‟s comparable to those seen in binge drinking women (e.g. Becker et al., 1996; Randall and Taylor., 1979; Sulik et al., 1981; Webster 1983). It is well suited for experimentation in determining Zn-related effects of maternal ethanol exposure, as a MT-knockout mouse has been generated from this strain (e.g. Carey et al.,2000a; Carey et al., 2000b). Mice were maintained in an animal house at 22 °C, subject to a 12 h light/ 12 h dark cycle, with lights on at 6:00 am. All animals were given unrestricted access to water and were fed a commercial diet (Milling Industries, Adelaide, Australia) until the commencement of mating, where they were placed on an AIN-93G casein-based control diet.

Mating was carried out by pairing nulliparous females (aged 9-14 weeks) with a proven male. Females were examined every morning for the presence of a vaginal

Table 2.1. Composition of AIN-93G Diet (for growth, pregnancy and lactational phases of rodents).

Ingredient	% of diet	G /kg mixture
Cornstarch	40%	400 g
Casein	20%	200 g
Dextrinized cornstarch	13.2%	132 g
Sucrose	10%	100 g
Soybean Oil	7%	70 g
Cellulose	5%	50 g
L-Cystine	0.3%	3 g
Choline Bitartrate	0.25%	2.5 g
Mineral Mix (AIN-93M-MX)	3.5%	35 g
Vitamin Mix (AIN-93-VX)	1%	10 g

Table 2.1. Components of the mineral mix used for the preparation of the AIN-93G diet

AIN-93M Mineral Mix	
Ingredient	**g or mg/kg mixture**
Calcium carbonate	357 g
Monopotassium Phosphate	196 g
Potassium Citrate Monohydrate	70.8 g
Sodium Chloride	74 g
Potassium Sulfate	46.6 g
Magnesium Oxide	24 g
Ferric Citrate.$6H_20$ (16-17 % Fe)	6.06 g
Manganese Carbonate (43-48% Mn)	0.63 g
Copper Carbonate (53-55% Cu)	0.3 g
Powdered Sugar	220.97 g
Sodium Metasilicate. $9H_20$	1.45 g
Potassium Iodate	10 mg
Sodium Selenate, Anhydrous	10.3 mg
Ammonium Molybdate.$4H_20$	7.95 mg
Chromium Potassium Sulfate.$12H_20$	275 mg
Lithium Chloride	15.1 mg
Boric Acid	81.45 mg
Sodium Fluoride	63.5 mg
Nickel Chloride. $6H_20$	63.6mg
Ammonium Vanadate	6.6 mg
Zinc Sulphate	Added to diet to required amount

Table 2.3. Composition of the vitamin mix used for the preparation of the AIN-93G diet

AIN-93-VX Vitamin Mix (g/kg)	
Ingredient	**g or mg/kg mixture**
Nicotinic Acid	3 g
D-Calcium Pantothenate	1.6 g
Pyridoxine HCL	700 mg
Thiamine HCL	600 mg
Riboflavin	600 mg
Folic Acid	200 mg
D-Biotin	20 mg
Vitamin B^{12}	2.5 g
Alpha Tocopherol Powder	30.0 g
Vitamin A Polmitate	1.6 g
Vitamin D^3	250 mg
Phylloquinone	75 mg
Powdered Sucrose	959.66 g

plug. Confirmation of a plug was designated as GD 0, at which time females were separated from males and housed individually until the time of experimentation. All experimental procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee.

2.2.3 GD 8 Ethanol and dietary Zn supplementation

On GD 0, pregnant dams were assigned to one of four treatment groups: 1) Saline + control diet, 2) Ethanol + control diet, 3) Saline + Zn supplemented diet, or 4) Ethanol + Zn supplemented diet (n=11 per treatment). Mice were fed the control diet (35mg Zn/kg) or the Zn-supplemented diet (200mg Zn/kg) from GD 0–GD 18. A well-described ethanol exposure regimen followed (Webster et al., 1980), based on an acute high exposure to ethanol on GD 8, a critical period during organogenesis. Pregnant mice received two intraperitoneal injections, separated by 4 h, of either saline (0.85% w/v NaCl) or 25% ethanol (v/v) in saline solution (0.015 ml/g body weight). This dosage of ethanol results in blood alcohol concentrations that peak at approximately 350 mg/dL within 30 minutes after the first injection (Carey et al., 2000b) and at 500 mg/dL following the second injection. Blood alcohol concentrations remain above 100mg/dL for approximately 10 h (Sulik, 2005). Because animals receiving ethanol were inebriated for approximately 2 h after each injection (similar to the lethargy and ataxia seen in humans with comparable BAC"s), food was removed from all groups immediately after the first injection and was returned 2 h following the second injection to standardise food intake. All mice were inspected regularly following treatment to ensure that the subjects in our study did

not develop any negative side-effects from the injections (e.g. external infection, rapid weight loss).

2.2.4 Fetal dysmorphology

On GD 18, mice were anaesthetized with halothane and approximately 1ml of blood was collected via cardiac puncture. Blood was stored on ice in ependorf tubes containing 5 µl heparin, until later processing. Following cervical luxation, uteri were immediately excised and examined for resorption sites. Individual fetuses and their placentas were then separated and weighed and the fetal crown-rump length (CRL) was measured. Fetuses were examined for external abnormalities (e.g. anopthalmia, microphthalmia, micrognathia, limb defects, haemorrhaging). As previous studies have demonstrated that the head size can be reduced by prenatal ethanol exposure, head dimensions (height (h), width (w) and depth (d)) were measured to determine fetal head volume. Head volume was calculated using the equation $\frac{\pi(h \times w \times d)}{6}$ mm^3 for ellipsoid structures and presented as head volume/CRL ratios. All fetuses were then stored in 10% buffered formalin for later examination.

2.2.5 Congenital heart defects (preliminary study)

10 fetuses from the ethanol and saline alone groups (n=10 litters) were randomly selected to examine whether ethanol causes congenital heart defects in our mouse

model. As there was not a sufficient number of physically abnormal fetuses in the saline control group to enable comparison between treatment groups (i.e. less than 10 abnormal fetuses), all fetuses examined for heart defects were therefore free of external physical abnormalities. Nevertheless, it has previously been shown that the incidence of heart defects caused by ethanol on GD 8 is similar in externally abnormal and normal fetuses (Webster et al., 1984). For each fetus, the head, arms and legs were excised, prior to processing in an automated tissue processor. Fetuses were then embedded in paraffin blocks and serially sectioned in the frontal plane at 10 μm (approximately 15 sections per fetus). The sections were placed on glass slides and stained with haematoxylin and eosin as follows: xylene (MERCK Pty Ltd.) (2 min), xylene (2 min), alcohol (MERCK Pty Ltd.) (2 min), alcohol (2 min), rinse with water, haematoxylin (SIGMA) (5 min), rinse with water, acid alcohol (5 dips), rinse with water, lithium carbonate (SIGMA) (10 s), rinse with water, eosin (SIGMA) (1 min), alcohol (1 min), alcohol (1 min), histolene (1 min), histolene (1 min). Slides were cover-slipped and later examined using a light microscope by 2 researchers blinded to treatment group, for heart defects (e.g. ventricular septal defects, atrial septal defects, overriding aorta).

2.2.6 Maternal Plasma Zn analysis

Blood samples were centrifuged at 14 000 x g for 4 mins and the plasma was collected and stored at -20°C until Zn analysis. For analysis, 300μl of each sample was added to 2 ml of Zn diluent (diluent = 50 ml butan-1-ol, 1.5 ml 30 % Bridge 35

and 8.9 ml concentrated HCl made to 1 L with distilled H_20) and Zn concentrations in the plasma were determined by flame atomic absorption spectroscopy.

2.2.7 Postnatal growth and survival

In a separate group of pregnant mice, dams were allocated to one of the four treatment groups on GD 0 and were subjected to the same GD 8 ethanol or saline treatment as described above (n=13-16). The control or Zn-supplemented diet, however, was fed to mice from GD 0-20. From GD 19, cages were inspected twice daily for the appearance of pups. At birth (postnatal day (PD) 0), the number of live and dead pups were recorded. To determine the effect of ethanol and Zn supplementation on postnatal growth and survival, pups from each litter were weighed on PD 3 and 21 and counted on PD 3 and 21 (weaning) and 60, so that the number of dead pups for each litter could be calculated. Care was taken to minimise human contact with pups (particularly in the first week following birth) to prevent the dams from cannibalising their pups.

2.2.8 Zn supplementation and ethanol response: GD 8 time-course

To examine the effect of dietary Zn-supplementation on liver MT, Zn, and plasma Zn response following ethanol exposure, a separate cohort of pregnant mice were allocated to either a control diet or a Zn-supplemented diet from GD 0. On GD 8, mice were treated with ethanol as described above. Mice were anaesthetised with

halothane and blood was taken via cardiac puncture prior to cervical luxation, at several time-points following ethanol exposure (0, 8, 14 and 24 hours, n = 3-7 for each time point). Maternal liver and muscle samples were also removed, stored on ice and then analysed for MT or Zn content.

2.2.9 Zinc analysis

Maternal plasma was obtained, stored and prepared as described above (2.2.6 maternal plasma Zn analysis) and Zn concentrations were determined by flame atomic absorption spectroscopy.

Maternal liver and muscle samples (100mg) were dried at approximately 65 ^{o}C for 48 h and then digested with 1 ml concentrated nitric acid (whilst heating at 100-120 ^{o}C to evaporate the liquid). 1.5 ml 1M hydrochloric acid was then added to dissolve the remaining solid material (at 50 ^{o}C for 15 mins) and the resultant solution was analysed for Zn content as described above for plasma samples.

2.2.10 Metallothionein analysis

Maternal livers were diluted 1:5 (w/v) with TRIS-HCl buffer (10mM, pH 8.2), and homogenised using a motor driven Potter-Elvehjem homogeniser (Wheaton, NJ, USA). After homogenisation, aliquots were boiled for 2 min, cooled (in ice-cold water) and then centrifuged for 4 min at 14 000 x g. The resultant supernatant was stored at -80 ^{o}C until further MT analysis. The supernatant was then diluted 1:25 with

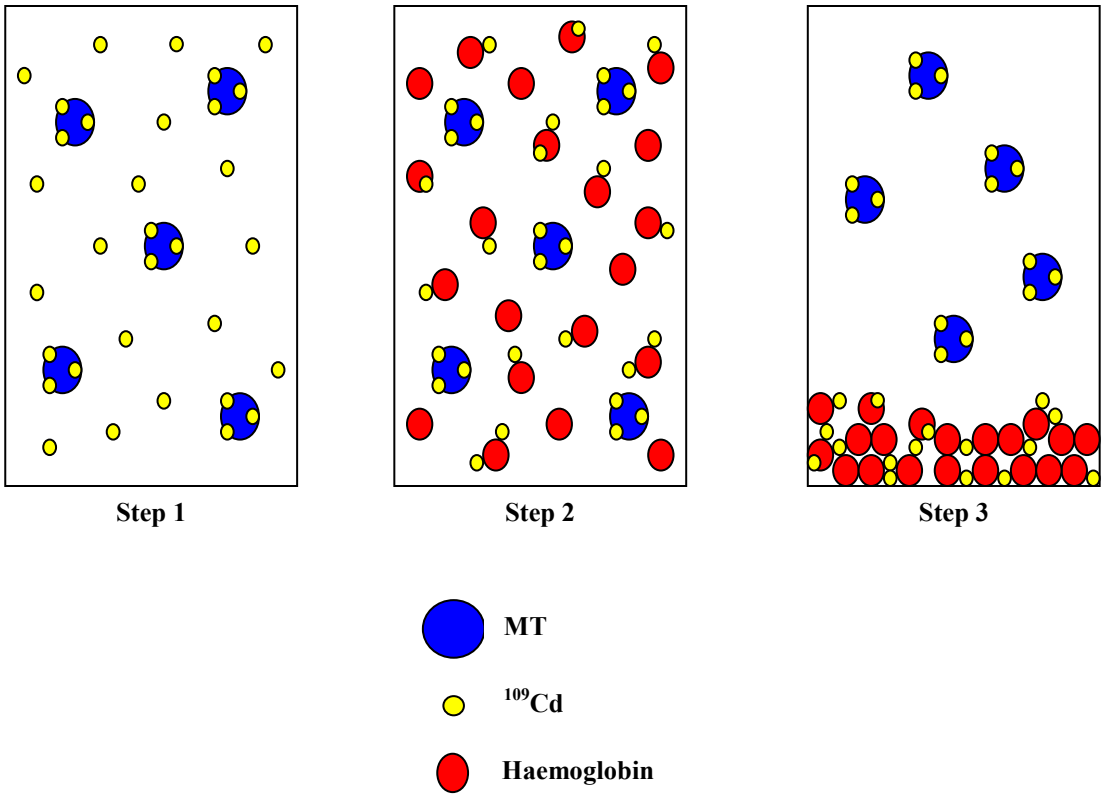

Figure 2.1. Key steps in the quantification of metallothionein.

Step 1: sample is incubated with ^{109}Cd which binds to MT, displacing zinc atoms in a 1:1 ratio.

Step 2: haemoglobin is added to the sample, binding any non-MT bound ^{109}Cd.

Step 3: sample is centrifuged, and the ^{109}Cd-MT complex in the supernatant is passed through a gamma counter.

TRIS-HCl buffer. 200µl of the diluted sample was added to 200 µl of radioactively labelled cadmium (2µl ^{109}Cd + 10ml Cd buffer) and incubated at room temperature for 15 min. 100µl of human red cell haemolysate (containing 4g/ dL haemaglobin) was added to each sample, boiled (2 min), cooled and centrifuged (1 min at 13 000 x g) and then another 100µl of red cell haemolysate was added to samples, boiled (2 min), cooled and centrifuged (4 min at 13 000 x g). 450µl of the sample was analysed for MT concentration using the gamma-counter (Packard Auto-Gamma R S650). The key steps in this process are illustrated in Figure 2.1.

2.2.11 Statistical analyses

Fetal data on GD 18, including placental and fetal weight, CRL and head size were compared by two-way ANOVA, with fixed effects treatment (ethanol or saline) and diet (normal Zn or supplemented Zn). Plasma Zn levels on GD 18 and plasma, liver and muscle Zn levels and liver MT concentrations for the GD 8 ethanol time-course were analysed by general linear model (GLM) with fixed effects treatment, diet and time (0, 8, 14, 24 h) and with all combination of interactions these effects. GLM was also used to compare postnatal pup weights at PD 3 and 21, to investigate the effect of treatment on postnatal growth. As there were no differences in weights between male and female offspring, data were combined for each group for analysis. We determined post hoc significance using Tukey‟s post hoc test. Data concerning litter size, fetal resorptions and abnormalities at GD 18 were non-normally distributed and therefore analysed with the Mann-Whitney U test. To compensate for possible

confounding within-litter treatment effects, each litter was viewed as a single unit of analysis. Statistical analysis was performed with Minitab software (Minitab Inc., State College, PA). Results are presented as means ± SEM. Postnatal survival (the proportion of dead pups within each litter at birth, PD 3, 21 and 60) was analysed as a binomial distribution by hierarchical mixed generalised linear regression analysis. The cumulative postnatal mortality are presented as the median cumulative percentage of pups dead at each time point. All differences were considered to be significant at $p < 0.05$.

2.3 RESULTS

2.3.1 GD 18 Fetal dysmorphology

The maternal and fetal data as assessed on GD 18 are presented in Table 2.4. Maternal ethanol treatment on GD 8, either by ethanol alone or with dietary Zn supplementation, had no effect on litter size or the percentage of fetal resorptions compared with saline treatment alone. In addition, there was no effect of GD 8 treatment or diet throughout pregnancy on fetal weight, placental weight, fetal CRL and fetal head volume/CRL ratio (Table 2.4).

Fetuses exposed to ethanol alone on GD 8 exhibited a significantly higher incidence of external abnormalities compared with saline, saline + Zn and ethanol + Zn fetuses ($p<0.05$) (Table 2.4). The ethanol group had the greatest number of litters affected,

with 11 out of 11 litters containing abnormal fetuses, compared to saline (8/11), saline + Zn (7/11) or ethanol + Zn (7/11) (Table 2.4). In addition, 6 out of 11 of the litters from the ethanol group contained 2 or more abnormal fetuses, compared to ethanol + Zn (1/11), saline and saline + Zn groups (each 0/11). Sixty percent of the total abnormalities observed in all groups involved malformations of the eye, with microphthalmia being the most frequent of all abnormalities, followed by anophthalmia, haemorrhaging of the cranium or dorsal region and limb abnormalities (Table 2.5, examples in Figure 2.2).

2.3.2 GD 18 Congenital heart defects

10 fetuses from each group exposed to ethanol or saline alone on GD 8 were examined for congenital heart defects. While none of the control fetuses showed any heart malformations, the ethanol group had 2 out of 10 fetuses that displayed evidence of heart anomalies. One fetus had a ventricular septal defect (hole in the septal wall separating the left and right ventricle), while the other showed an abnormal heart structure, with the left ventricle opening into the right atrium (Figure 2.3). There was no clear evidence using histology of any abnormalities of the great vessels (i.e. overriding aorta or pulmonary artery or transposition of vessels) which have been previously linked to prenatal ethanol exposure (Webster et al., 1984). Atrial septal defects (hole in the septal wall separating the left and right atrium) were not able to be detected on GD 18, due to the presence of the foramen ovale, a natural opening between the atrium that only closes after birth). Zn-supplemented fetuses

Table 2.4. GD18 data from dams treated with saline or ethanol on GD8 ± dietary Zn supplementation during pregnancy

	Maternal Treatment			
	saline	saline + Zn	ethanol	ethanol + Zn
Maternal data				
No. litters	11	11	11	11
No.fetuses	75	81	86	67
Average litter size[b]	6.8 ± 0.7	7.3 ± 0.3	7.8 ± 0.6	6.1 ± 0.5
No. resorptions	12	17	17	15
% resorptions/ per litter[b]	15.5 ± 5.1	16.8 ± 2.4	16.3 ± 4.0	15.9 ± 3.9
Plasma Zn (umol/L)[a]	10.5 ± 0.8	21.5 ± 0.6* #	10.8 ± 0.3	29.12 ± 0.6 *#
Fetal Data				
No. abnormal fetuses	8	7	24	8
No. litters with abnormal fetuses	8	7	11	7
% abnormal fetuses/ per litter[b]	10. 0 ± 2.3	9.1 ± 2.2	26.4 ± 3.5*†¶	12.2 ± 3.3
Body weight (mg)[a]	777.3 ± 25.2	833.4 ± 18.8	813.5 ± 32.5	790.3 ± 40.4
Crown-rump length (CRL) (mm) [a]	18.6 ± 0.3	18.7 ± 0.02	19 2 ± 0.4	18.9 ± 0.4
Head volume (mm3)/CRL [a]	12.0 ± 0.4	12.4 ± 0.5	11.4 ± 0.02	11.3 ± 0.3
Placental weight (mg) [a]	76.1 ± 4.3	79.2 ± 2.9	76.0 ± 1.7	81.1 ± 1.9

Values represent the means ± SEM where applicable.

[a]Data analysed by two-way ANOVA, with Tukeys post-hoc test.

[b]Non-parametric data analysed by Mann-Whitney U test.

* Significantly different from saline group, $p < 0.05$; † Significantly different from saline + Zn group, $p < 0.05$; # Significantly different from ethanol group, $p < 0.05$; ¶ Significantly different from ethanol + Zn group, $p < 0.05$.

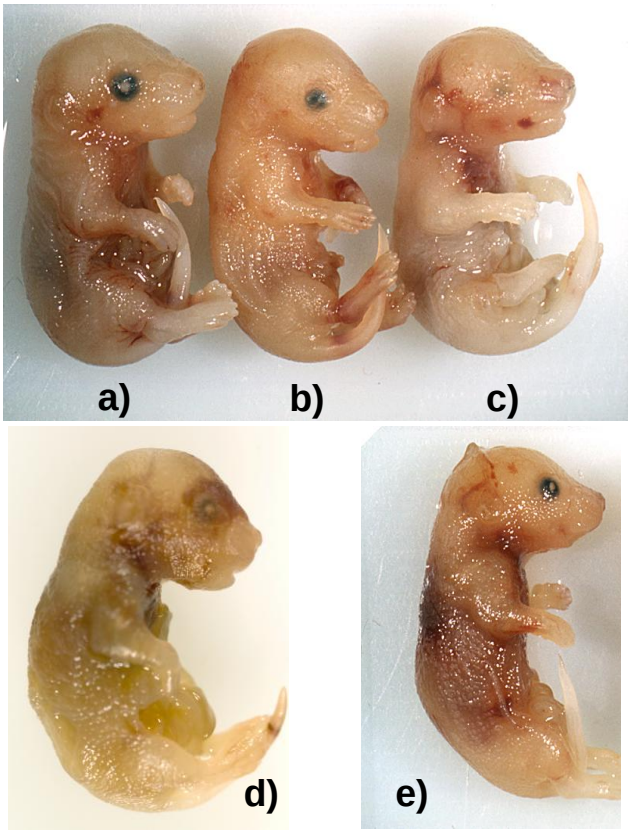

Figure 2.2. Examples of physical abnormalities observed in GD 18 fetuses exposed to saline or ethanol on GD 8 ± dietary Zn supplementation throughout pregnancy; a) a normal fetus, b) microphthalmia (small eye), c) anophthalmia (no eye), d) haemorrhage of cranium (in this incidence, near the frontal region), e) micrognathia (small jaw).

Table 2.5. Type and Frequency of Abnormalities in GD 18 Fetuses

Treatment[a]	No.	Microphthalmia	Anophthalmia	Micrognathia	Haemorrhage[b]	Limbs[c]	Tail[d]
saline	8	3	2		3		
saline + Zn	7	4	1			2	
ethanol	24	7	6	1	4	5	1
ethanol + Zn	8	4	1		2	1	

[a]Treatment of mother on gestational day (GD) 8. Dietary Zn supplementation (200 mgZn/kg) was given from GD 0-18.

[b]Refers to haemorrhaging of the cranium or dorsal region of the fetus

[c]Limb abnormalities include local areas of haemorrhage on the distal limbs

[d]Refers to a bent tail

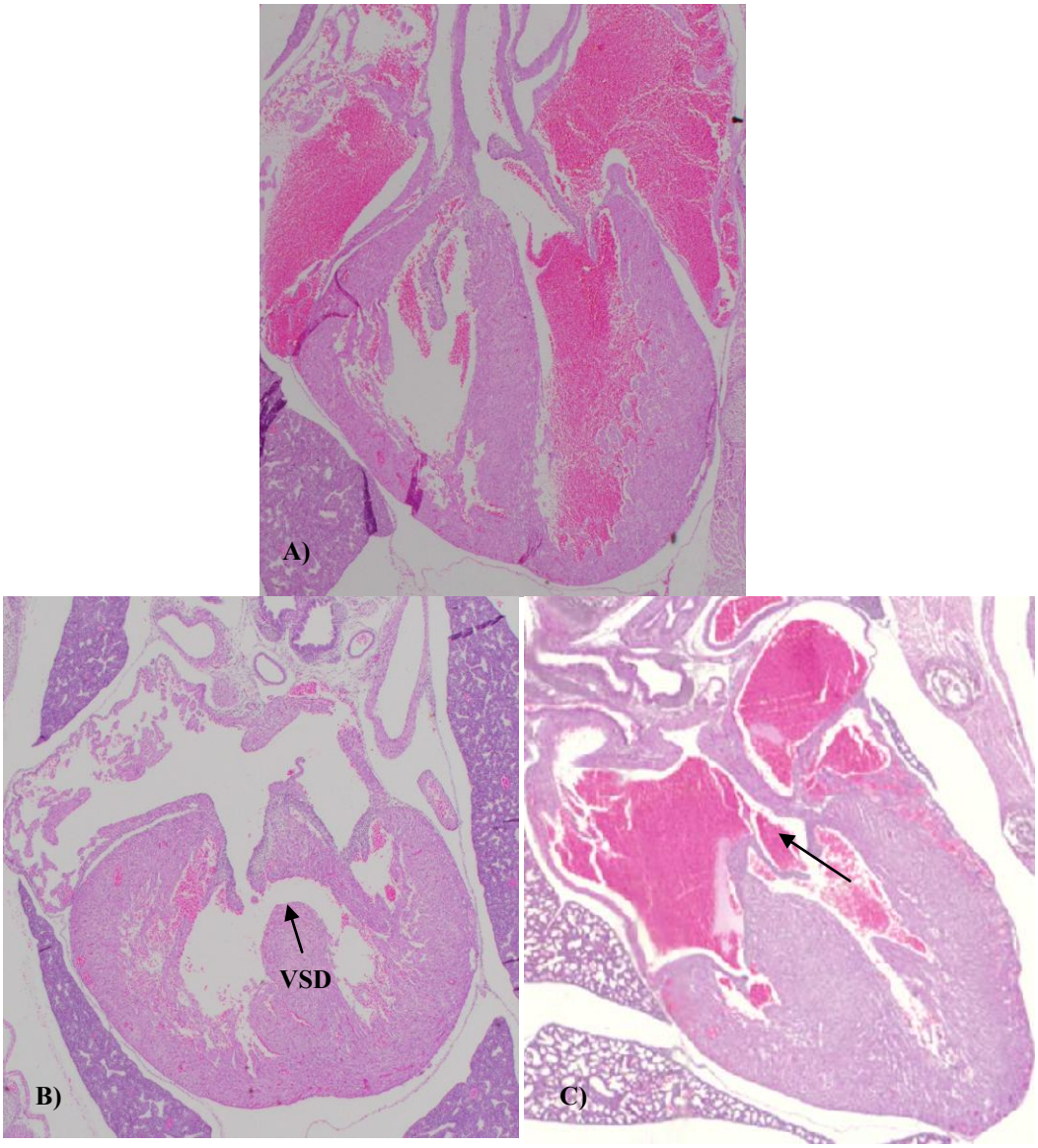

Figure 2.3. Congenital heart defects observed in GD 18 fetuses that were exposed to ethanol on GD 8 of pregnancy. Histological sections were serially sectioned in the coronal plane (5µm) and stained with haematoxylin and eosin. A) A normal heart from a saline-treated fetus; B) a Ventricular Septal Defect (VSD) in an ethanol-treated fetus; and C) an abnormal heart from an ethanol-treated fetus, in which blood is shown to be transferred between the left ventricle and the right atrium.

were not examined for the prevention of heart defects due to the small number of cardiac malformations caused by ethanol in our study.

2.3.3 GD 18 Maternal plasma Zn concentrations

On GD 18, there was no difference in maternal plasma Zn concentrations between dams treated with saline or ethanol alone on GD 8. However, both saline + Zn and ethanol + Zn dams that were supplemented with dietary Zn from GD1-18 had higher plasma Zn concentrations (>100 % higher) than the un-supplemented dams (Table 2.4).

2.3.4 Postnatal growth and survival

In a separate study, pregnant dams were allowed to deliver their offspring to investigate the effect of ethanol exposure and dietary Zn supplementation on postnatal growth and survival. There was no difference between treatment groups in the length of the gestational period (days) (saline: 20.0 ± 0.1, saline + Zn: 19.9 ± 0.2, ethanol: 20.1 ± 0.1, ethanol + Zn: 20.0 ± 0, means ± SEM).

The average litter size at birth and postnatal body weights of offspring for each treatment group are presented in Table 2.6. There was no effect of maternal ethanol treatment or dietary Zn supplementation on litter size at birth or the postnatal growth of offspring, as offspring from all groups demonstrated similar weights on PND 3 and 21 (Table 2.6).

Table 2.6. Postnatal growth and body weights of offspring between PD 3 and PD 21

Treatment[a]	No. of litters	Litter size at birth	PD3 weight (g)	PD21 weight (g)
Saline	13	6.0 ± 0.6	1.4 ± 0.07	7.1 ± 0.07
Saline + Zn	14	5.7 ± 0.5	1.5 ± 0.07	7.9 ± 0.07
Ethanol	16	6.3 ± 0.6	1.4 ± 0.06	6.9 ± 0.06
Ethanol + Zn	15	5.5 ± 0.5	1.5 ± 0.06	6.9 ± 0.06

Values represent the means ± SEM.

[a]Treatment of mother on GD 8. Dietary Zn supplementation (200mg Zn/kg) was given from GD 0-18.

Data was analysed by GLM.

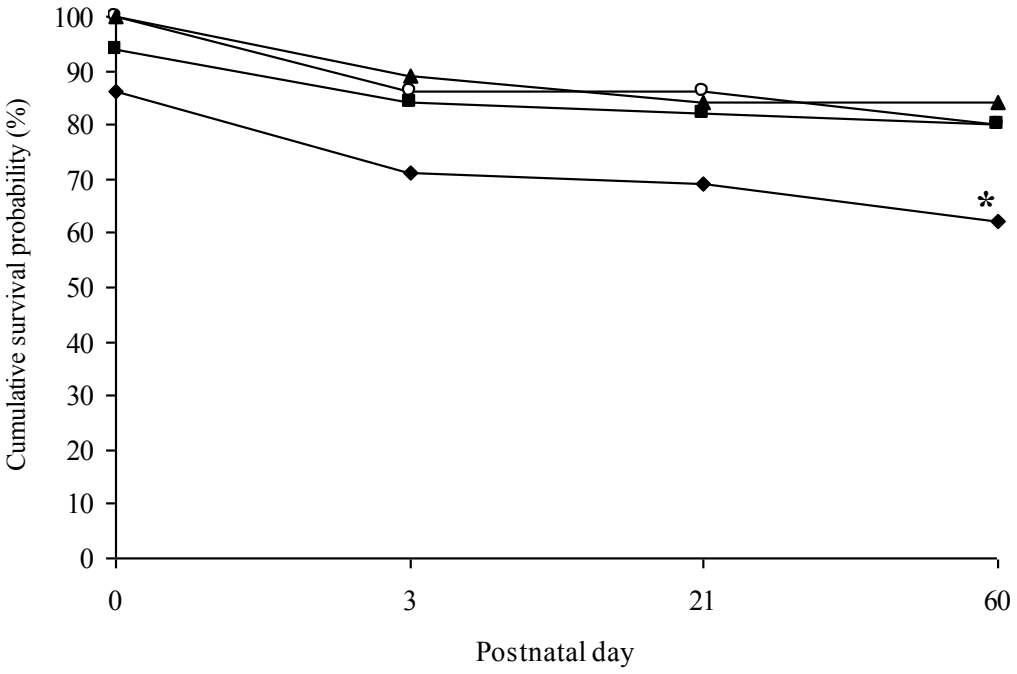

Figure 2.4. The effect of dietary Zn supplementation throughout pregnancy on the postnatal survival probability of offspring exposed to saline or ethanol treatment on GD 8. (saline (o), saline + Zn (▲), ethanol (♦), ethanol + Zn (■)). *Significantly different from saline, saline + Zn and ethanol + Zn groups, $p<0.05$. n = 78-100 offspring (from 13-16 litters) per treatment at birth.

The cumulative survival probability (from birth until PD 60) of offspring from each treatment group is presented in Figure 2.4. There were 7 stillbirths in 4 out of 16 litters in the ethanol group compared to one stillbirth in 15 of the litters in the ethanol + Zn group. There were no stillbirths in the saline or saline + Zn. Here, the proportion of deaths at birth were significantly greater in the ethanol-treated group compared to the saline-treated groups. While there were less deaths in the ethanol + Zn group than the ethanol group at birth, this difference did not reach significance. Postnatal mortality from birth until PD 60 was significantly greater in the ethanol group. By PD 60, 38 % of offspring had died in the ethanol group, compared to only 16-20 % in the other groups ($p<0.05$). 69 % of litters in the ethanol group were affected by mortality, compared to between 27-54% in the other groups. The greatest risk of mortality occurred in the first three days of life, with between 10-15 % of offspring from all groups dying between PD 0 and 3, (Figure 2.4).

2.3.5 GD 8 maternal plasma, liver and muscle Zn and liver MT

In a separate experiment, pregnant dams fed either a control or Zn-supplemented diet from GD 0 were injected with ethanol on GD 8, to examine the effect of dietary Zn levels on maternal plasma, liver and muscle Zn and liver MT concentrations following ethanol exposure. After ethanol injection, plasma Zn concentrations in dams fed a control diet decreased significantly by 25 % from 10.7 μmol/L to a minimum of 8 μmol/L at 8 h and gradually returned to normal over the following 16

h ($p<0.05$). In contrast, plasma Zn concentrations in Zn-supplemented dams were not different from baseline values at 8 h after ethanol exposure (13.3 μmol/L), but significantly increased by 20 % with peak values at 14 h (16.0 μmol/L) ($p<0.05$), after which they returned to normal values by 24 h. Zn-supplemented dams had significantly higher plasma Zn concentrations at baseline and at all time-points examined following ethanol exposure (8, 14 and 24 h) compared to dams fed a control diet ($p<0.05$) (Figure 2.5).

Maternal liver MT increased from baseline levels (31.6 nmol Cd bound /g wet wt) 8 h after ethanol exposure in dams fed the control diet (to 113.9 nmol Cd bound /g wet wt) or the Zn-supplemented diet (to 130.6 nmol Cd bound /g wet wt) ($p<0.05$). Liver MT concentrations in both these treatment groups remained significantly elevated at 14 and 24 h after ethanol treatment ($p<0.05$). There was no significant difference in liver MT concentrations between control and Zn-supplemented dams at any time-point examined (Figure 2.6).

Liver Zn concentrations increased from baseline values by 30 % in control dams following ethanol exposure, with maximum levels attained at 8 h (359.5 - 475.2 nmol /g wet wt), returning to pre-treatment levels by 14 h ($p<0.05$) (Figure 2.7). Although the liver Zn concentrations were not different from baseline at 24 h, the mean values were nevertheless 55 nmol/g higher and this amount can account for the Zn bound to liver MT, which was 73 nmol/g higher at 24 h than before ethanol was administered (Figures 2.6 and 2.7) In comparison, Zn-supplementation had no effect on liver Zn

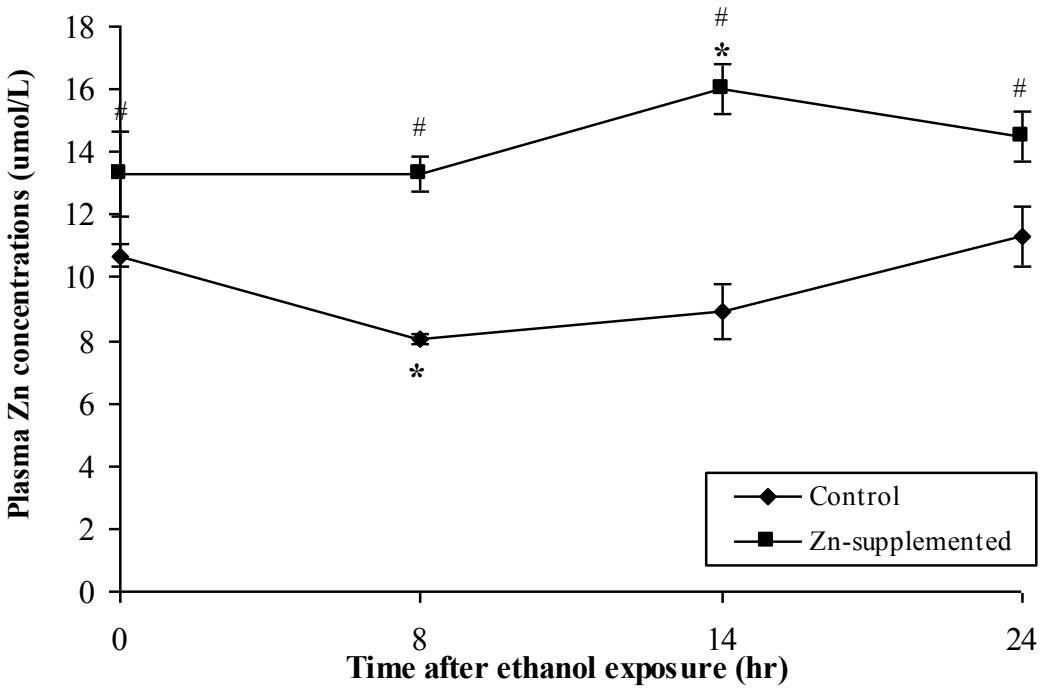

Figure 2.5. The effect of GD 8 ethanol treatment on maternal plasma Zn concentrations in pregnant mice fed either a control (35 mgZn/kg) or Zn-supplemented (200 mgZn/kg) diet from GD 0. Points represent the means ± SEMS, n = 3-7 for each time point (i.e. control: 0 h =4, 8h =7, 14 h =4, 24 h = 4 and Zn-supplemented: 0 h =3, 8 h =5, 14 h =5, 24 h =3). *Plasma Zn concentrations in control mice were significantly lower than basal levels at 8 h, whereas in Zn-supplemented mice, they were significantly higher at 14 h, ($p < 0.05$). # Significantly different between control and Zn-supplemented mice ($p < 0.05$).

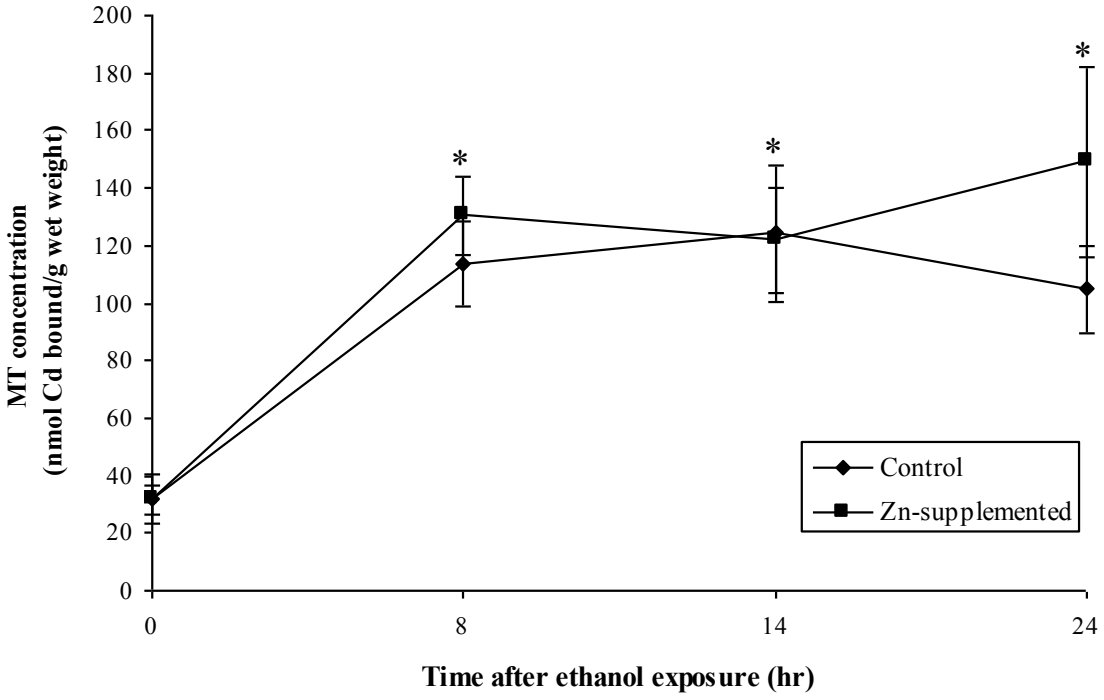

Figure 2.6. Effect of GD 8 ethanol exposure on maternal liver MT concentrations in pregnant mice fed either a control (35 mgZn/kg) or Zn-supplemented (200 mgZn/kg) diet from GD 0. Points represent the means ± SEM, n = 3-7 for each time point (i.e. control: 0 h =4, 8 h =7, 14 h =4, 24 h = 4 and Zn-supplemented: 0 h =3, 8 h =5, 14 h =5, 24 h =3). *Significantly higher than basal levels (t = 0 h) for each treatment ($p<0.05$).

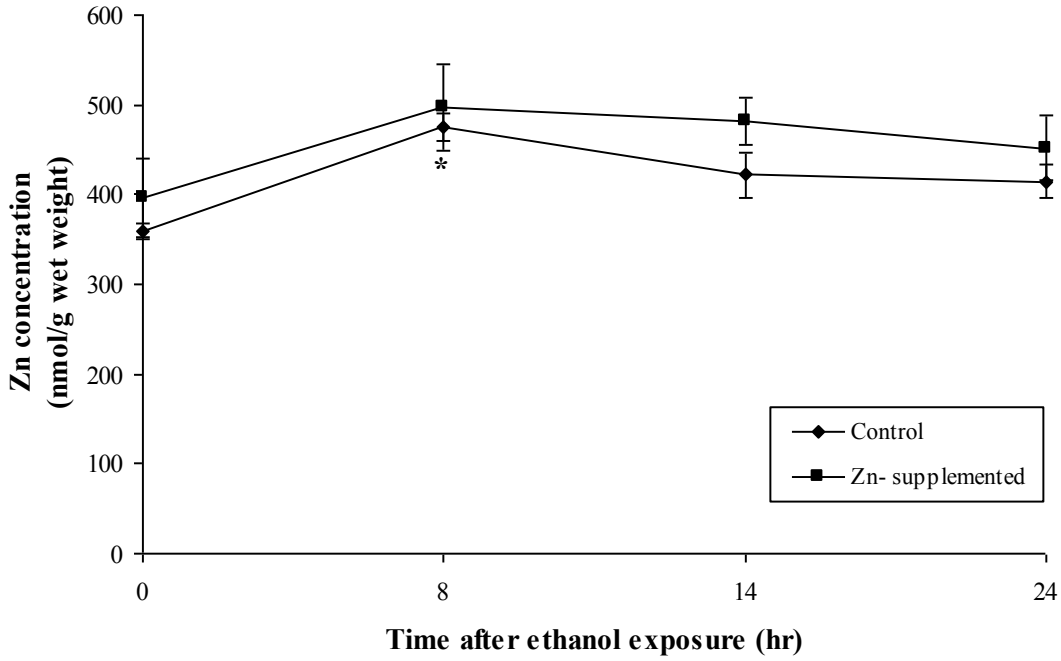

Figure 2.7. The effect of GD 8 ethanol treatment on liver Zn concentrations in pregnant mice fed either a control (35 mgZn/kg) or Zn-supplemented (200 mgZn/kg) diet from GD 0. Points represent the means ± SEM, n = 3-7 for each time point (i.e. control: 0 h =4, 8 h =7, 14 h =4, 24 h = 4 and Zn-supplemented: 0 h =3, 8 h =5, 14 h =5, 24 h =3). *Liver Zn concentrations in control/ethanol mice were significantly higher than basal levels (i.e. t = 0 h) ($p<0.05$).

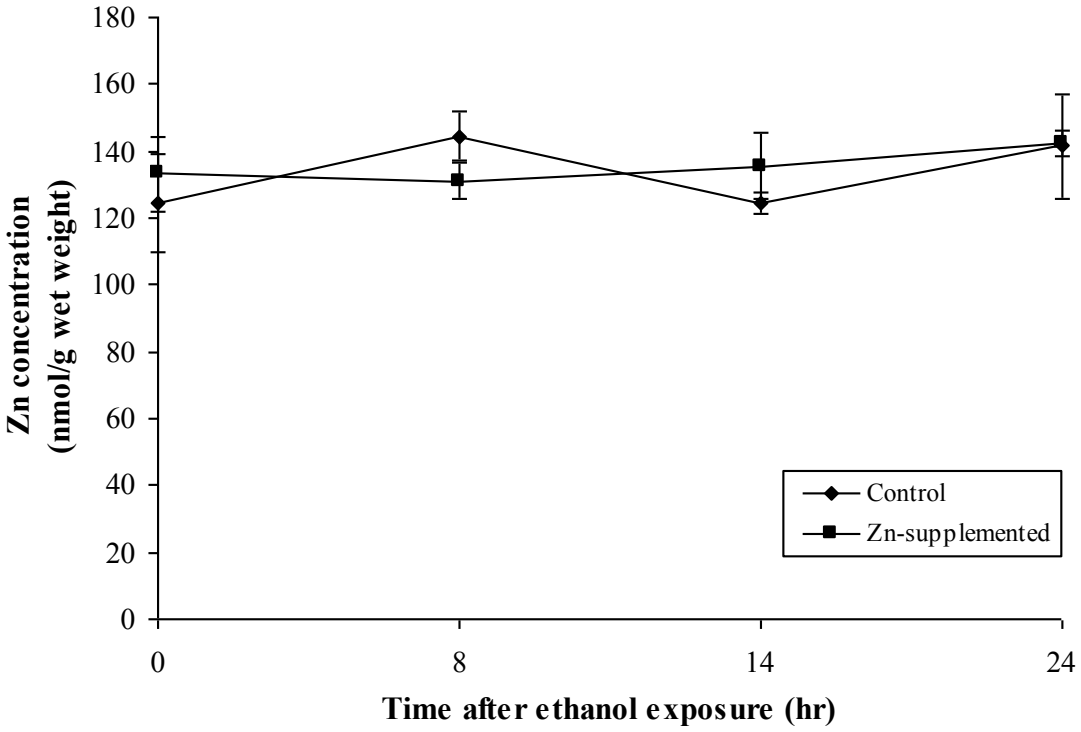

Figure 2.8. The effect of GD 8 ethanol treatment on maternal muscle Zn concentrations in pregnant mice fed either a control (35 mgZn/kg) or Zn-supplemented (200 mgZn/kg) diet from GD 0. Points represent the means ± SEM, n=3-7 for each time point (i.e. control: 0 h =4, 8h =7, 14 h =4, 24 h = 4 and Zn-supplemented: 0 h =3, 8 h =5, 14 h =5, 24 h =3).

concentrations after ethanol exposure.While there was an overall effect of diet on maternal liver Zn concentrations after ethanol exposure (i.e. Zn-supplemented > control Zn), there was no significant difference in liver Zn concentrations between control and Zn-supplemented dams at any specific time-points examined.

Muscle Zn concentrations did not change from base-line values after ethanol exposure, in control or Zn-supplemented dams (Figure 2.8).

2.4 DISCUSSION

Supplementing pregnant dams with Zn via a subcutaneous injection at the time of ethanol exposure has been shown to prevent the deleterious physical effects on the fetus (Carey et al., 2003b; Summers et al., 2006). In the present study, we found that dietary Zn supplementation throughout pregnancy can also prevent physical birth abnormalities caused by ethanol exposure on GD 8. This was demonstrated by the finding that while fetuses exposed to ethanol alone had an increased incidence of external abnormalities, the incidence of those also supplemented with Zn (i.e. ethanol + Zn group) was not different from saline-treated fetuses.

Of the abnormalities that were observed on GD 18, eye malformations (similar to those reported in children with FAS (Clarren and Smith, 1978; Webster et al., 1983) were the most common. This is consistent with the findings of identical studies

involving ethanol exposure on GD 8 (Carey et al., 2000b) and GD 7 (Sulik et al., 1981), where 30 out of 72 C57BL/6J fetuses had eye defects. This high incidence is due to the fact that the period of ethanol exposure (GD 7-9) occurs during a particularly critical stage of ocular development. Furthermore, the C57BL/6J mouse has a genetic predisposition for eye malformations, such as microphthalmia and anophthalmia, with an incidence as high as 13 % (Cook and Sulik, 1986). In the present study, however, the incidence of eye defects in the saline-treated fetuses was only 7 %. In comparison to our previous findings where the basal incidence of spontaneous abnormalities in controls was reduced with subcutaneous Zn treatment (Carey et al., 2003b), no similar beneficial effect was observed with dietary Zn supplementation in normal pregnancy.

While it is recognised that one of the major outcomes of prenatal ethanol exposure is impaired intrauterine growth, we did not observe any detrimental effect of ethanol on fetal body weight or other growth measurements (i.e. CRL and head volume) on GD18, which is in agreement with previous investigations that used an identical ethanol dosage on GD 8 (Carey et al., 2000b; Carey et al., 2003b). In addition, prenatal ethanol exposure did not affect postnatal growth of offspring in this study. It could be argued that as we did not weigh the offspring until PD 3 (to minimize adverse effects of handling), that any ethanol-mediated growth restriction may have been underestimated particularly if the smallest and most severely affected offspring had died in the first few days of life. However, as there was no evidence of body

weight differences between fetuses on GD 18, it would seem unlikely that growth retardation would only become evident at parturition. That several studies using a higher dose of ethanol during organogenesis did show a decrease in fetal body weight (Kronick, 1976; Sauerbier, 1987), suggests that the dose of ethanol (0.015 ml/g) we used is at or below the threshold required to affect fetal or postnatal growth. In addition, ethanol exposure during the later portion of pregnancy (GD 12-17) appears to be more critical for this pregnancy outcome (Becker et al., 1996).

While measurements on the percentage of fetal resorptions on GD 18 indicate that ethanol did not increase prenatal mortality (as demonstrated by previous acute ethanol studies (Kronick, 1976; Carey et al., 2000b), a major finding in this study was that ethanol exposure on GD 8 significantly increased postnatal mortality between birth and PD 60, with the most significant loss occurring in the first 3 days of life. Most importantly, we demonstrated that in addition to its prevention of physical abnormalities, dietary Zn supplementation throughout pregnancy also helped to protect against the high incidence of postnatal mortality caused by ethanol. The majority of studies that have previously shown an effect of ethanol on postnatal survival have involved chronic ethanol intake rather than acute ethanol exposure during pregnancy (Lopez-Tejero et al., 1986; Middaugh et al., 1988; Middaugh and Boggan, 1995). In chronic studies, other maternal factors resulting from long-term ethanol exposure can also contribute to postnatal death (e.g. direct toxicity to ethanol, poor maternal care after birth and deficits in caloric intake). However, the lack of a

difference in postnatal pup weights suggests that maternal care was consistent across treatment groups after birth in our study. Furthermore, there are several studies where prolonged ethanol exposure throughout pregnancy did not affect maternal behaviour or care after birth (Marino et al., 2002; Allan et al., 2003), indicating that it is unlikely that a single dose of ethanol to the mother on GD 8 would affect any of these parameters.

We were unable to identify the cause of death in offspring at post mortem, as the mothers often cannibalised parts of their body soon after their death and those examined displayed no obvious dysmorphology. However, the finding that the ethanol group had more stillbirths and a significant number of offspring died in the first few days of life, but not during gestation, suggests that ethanol may impair the development of tissues whose function are supported by the mother during gestation and only become critical after parturition. Furthermore, the prevention of mortality by Zn supplementation indicates that Zn is protective against other abnormalities not specifically examined in this study. One possible example is cardiac malformations, as cardiac defects are common in children with Fetal Alcohol Syndrome (Loser and Majewski, 1977; Clarren and Smith, 1978) and a study identical to ours demonstrated that ethanol exposure on GD 8 resulted in 60% of fetuses (6/10 externally abnormal fetuses, 6/10 normal fetuses) developing ventricular septal defects (many of which were associated with other abnormalities of the great vessels) (Webster et al., 1984). In this regard, it has been shown that embryos from ethanol + Zn treated rats had

more advanced cardiac development compared to ethanol treated embryos (Seyoum and Persaud, 1995). We undertook a separate preliminary study with the aim to determine whether dietary Zn supplementation could prevent ethanol-related cardiac malformations. However, in comparison to the findings by Webster and colleagues (1984), only 2 out of 10 (20%) ethanol-exposed fetuses from independent litters were found to have abnormalities using histological techniques in our study. While this is a significant incidence, a much larger study would be desirable to achieve a sufficient number of heart defects to examine the effect of Zn supplementation. Future research could also examine whether other internal systems or specific structures affected by ethanol are protected by Zn supplementation (e.g. defects of the facial region, brain, ocular, skeletal, renal or auditory systems). This will determine whether Zn protects against all ethanol-related impairments, or only those affecting specific organs.

The present study examined the premise that dietary Zn supplementation is protective against birth defects by altering the effects of ethanol on maternal Zn homeostasis. We demonstrated that ethanol exposure on GD 8 in mice fed a control diet caused the induction of liver MT which persisted for more than 24 hours after exposure. This increased MT synthesis was associated with an increase in liver Zn and a 25 % reduction in plasma Zn concentration at 8 h after ethanol exposure. This plasma Zn response was not as severe or prolonged as our previous findings, which remained significantly lower than normal for more than 16 h after ethanol exposure (Carey et al., 2000b). Whether this reduction in plasma Zn status is of sufficient magnitude and

duration to disrupt developmental processes solely on its own is unclear. Interestingly, in our previous studies where ethanol was administered on GD 12, we found evidence that fetal Zn accrual may be impaired by ethanol independently of MT induction (Carey et al, 2000). This finding agrees with an earlier study where it was reported that chronic ethanol exposure during pregnancy or acute administration on GD 19 can impair the placental transfer of ^{65}Zn to late gestation rat fetuses (Ghishan et al, 1982). Thus it is reasonable to suggest that a combination of MT-dependant (low plasma Zn) and independent (impaired fetal Zn uptake) mechanisms may contribute to the developmental toxicology and further that the low plasma Zn in early pregnancy caused by ethanol-mediated MT induction may potentiate Zn deficiency in the fetus, which may already be compromised by impairment in placental Zn transfer. Differences in feeding times, gut transit rate and fractional absorption of Zn may all contribute to the availability of Zn and the dynamics of repletion of the plasma Zn after MT induction by ethanol and other toxins and hence may further explain the wide variation in plasma Zn levels that are associated with fetal dysmorphology (Daston et al., 1991; Daston et al., 1994; Carey et al., 2000b).

In the present study, dietary Zn supplementation during pregnancy increased plasma Zn concentrations above controls on the normal Zn diet, but did not affect liver MT concentration, or alter the ethanol-mediated liver MT response on GD 8. However, Zn Supplementation prevented the fall in plasma Zn associated with ethanol-induction of hepatic MT, with the ethanol + Zn group having plasma Zn 20 % higher

than in the controls. It has long been recognized that maternal Zn homeostasis changes during pregnancy (Cousins, 1985), presumably to meet the increased demands for fetal growth (Donangelo et al., 2005). Reduced excretion, changes in tissue Zn distribution and an increase fractional absorption of Zn have all been found in late pregnancy (Donangelo et al., 2005; Harvey et al., 2007). Our findings that the plasma Zn concentration is higher in Zn-supplemented dams on GD 8 and late pregnancy (GD 18) suggests these mice are in a more positive Zn balance throughout pregnancy than mice fed normal levels of Zn in their diet. That plasma Zn increases rather than falls in response to MT-induction in Zn-supplemented mice, may be explained by dynamics of plasma Zn homeostasis. Plasma Zn is an exchangeable zinc pool that represents only a 0.1% of total body Zn and hence at any given time reflects the equilibrium between tissue requirements, secretion and intestinal Zn absorption (Cousins 1985). Thus mice fed a Zn-supplemented diet presumably have a larger Zn reserve possibly bound-to or internalised within the mucosa of the gut wall to be mobilised and replete the plasma compartment after liver MT-sequestration than those fed normal Zn diets.

While the plasma Zn levels are not as high after dietary Zn supplementation as those in studies where subcutaneous Zn was co-administered with ethanol (Seyoum and Persaud, 1995; Carey et al., 2003b; Summers et al., 2006), our findings nevertheless demonstrated that the higher-than-normal plasma Zn levels with dietary Zn supplementation are sufficient to protect the fetus against ethanol-mediated

dysmorphology. There are several possible mechanisms by which a positive Zn balance may be protective. As previously mentioned, Zn supplementation may prevent the fetal Zn deficiency arising from the fall in plasma Zn levels which we have shown to be transiently decreased by up to 65 %, due to the ethanol-mediated induction of MT in the mothers liver (Carey et al., 2000b). That Zn treatment overwhelms the MT response so that Zn can be accessed and utilised by the fetus, is supported by our findings from this study, where plasma Zn concentrations did not decline, but increased by 20% above baseline after ethanol exposure in Zn-supplemented dams. Our earlier findings that a Zn injection given with ethanol on GD 8 which markedly increases Zn concentrations also prevented physical and cognitive impairments, further supports the premise that Zn deficiency is a key mediator of ethanol-related teratology (Carey et al., 2003b; Summers et al., 2006). However, the possibility that Zn has MT-independent effects cannot be overlooked. Ethanol is known to generate free radicals which are key factors involved in the induction of apoptosis (Henderson et al., 1995; Goodlett and Horn, 2001). Apoptosis has been well characterised in various fetal tissues following ethanol exposure during pregnancy and is suggested by other studies to be the cellular basis for ethanol-related birth defects (Dunty et al., 2001). Zn is involved in a number of anti-apoptotic pathways (Allington et al., 2001; Truong-Tran et al., 2001; Fernandez et al., 2003) and Zn treatment has been shown to promote cell survival after exposure to other teratogenic agents (Fernandez et al., 2003). Thus, Zn treatment may influence repair

mechanisms in the fetus by preventing apoptosis and protecting against ethanol-generated oxidative stress.

Although dietary Zn supplementation was shown to be protective against the effects of ethanol exposure, there did not appear to be any benefits of excess Zn in the diet on the growth or survival of offspring in the normal pregnancy setting. This agrees with the current body of knowledge on Zn supplementation, which has not yet shown any consistent benefits of supplementation in normal pregnancy (i.e. non Zn-deficient mothers), despite the importance of Zn for fetal development (Shah and Sachdev, 2006; Mahomed et al., 2007). Alternatively, there was no evidence of toxicity to excess Zn in this study. Mice fed the high Zn diet had no obvious side effects and produced pups with similar growth, development and survival measurements as dams on the control Zn diet. However, prolonged exposure to excess Zn in the diet can cause anaemia through trace element imbalances (Porter et al., 1977; Prasad et al., 1978; Fosmire, 1990) and other studies have shown adverse effects of long-term dietary Zn supplementation during pregnancy on fetal weight, prenatal mortality (Keppen et al., 1990) and short-term memory (Moazedi Ahmad et al., 2007). Consequently, for any potential clinical behefit there is a need to minise the amount of Zn in the diet while provide adequate protection against ethanol. Whether a lower level of dietary Zn supplementation would suffice to protect against ethanol-related birth abnormalities warrants further investigation. We have recently shown that dietary Zn supplementation of 100 μg Zn/g was sufficient to prevent birth

abnormalities caused by lipopolysaccharide administration (which also induces MT and decreases plasma Zn concentrations) given on GD8 in dams. Interestingly, in that study the incidence of abnormalities per litter due to LPS exposure were inversely proportional to zinc concentrations in the range 5-100 μg Zn/g (Chua et al., 2006).

2.5 CONCLUSION

This is the first study to demonstrate that dietary Zn supplementation throughout pregnancy can prevent physical abnormalities caused by ethanol exposure on GD8. It is also the first to demonstrate that ethanol exposure on GD8 decreases postnatal survival and this can also be prevented by dietary Zn supplementation. It appears that maternal hyperzincaemia associated with Zn supplementation ameliorates teratogenicity and mortality either by preventing the ethanol-associated decrease in fetal Zn supply, or by providing excess Zn during critical periods of development to compensate for any effects of ethanol. The findings of this study further support maternal/fetal Zn deficiency as a possible mediator of ethanol-related teratology.

CHAPTER 3

The effect of ethanol exposure in early pregnancy on the phenotype, cognition and behaviour, and on structural anatomy of the hippocampus of adult offspring; The influence of dietary Zn supplementation on neurobehavioural impairments.

3.1 INTRODUCTION

Cognitive and behavioural impairments are one of the major features of children diagnosed with FAS. Children with FAS demonstrate a broad spectrum of neurobehavioural deficits, including memory impairments, learning disabilities, hyperactivity, attention deficits and motor dysfunction (Mattson and Riley, 1998; Mattson et al., 2001). In addition, it is well known that FAS (or the consumption of ethanol during pregnancy in general) ranks with Down‟s Syndrome as a leading cause of mental retardation (Abel and Sokol, 1986). While these cognitive impairments and behavioural difficulties reflecting brain dysfunction, are not as obvious as the physical abnormalities discussed in Chapter 2, they are the most consistently observed problems in children exposed to ethanol during pregnancy and can be seen in children without the dysmorphic craniofacial features in FAS (diagnosed as Alcohol-Related Neurodevelopmental Disorder (ARND)). These outcomes can result from diverse drinking patterns, ranging from chronic ethanol consumption in extreme cases, to the episodic “binge” of large quantities of ethanol

over a short time period. Furthermore, there is increasing evidence that despite the fact that the most intense period of central nervous system development occurs later in pregnancy, consumption of ethanol in early pregnancy (organogenesis) can still impair the initial stages of brain development (Kotch and Sulik, 1992; Becker et al., 1996; Dunty et al., 2001; Jing and Li, 2004), leading to cognitive and behavioural impairments (Viglieccа et al., 1986; Diaz Perez et al., 1991; Dumas and Rabe, 1994; Becker et al., 1996; Minetti et al., 1996; Summers et al., 2006). Considering the significant incidence and costs to both the individual and community to address these problems, understanding the aetiological mechanism behind neurobehavioural impairments is therefore essential to develop appropriate strategies for intervention or prevention.

While many molecular mechanisms have been proposed to account for ethanol-induced behavioural deficits (including those discussed in *Chapter 1, 1.1.7. Aetiology of ethanol teratogenicity*), several lines of evidence support that Zn deficiency may also be a contributing factor to neurobehavioural impairments. In addition to the abundance of evidence already discussed in Chapter 1 (*1.2.9. Teratogenicity: Zn deficiency vs ethanol exposure; 1.3.5. Ethanol-induced zinc deficiency: A possible cause of teratology*) and Chapter 2, which supports a role for MT-induced Zn deficiency as a mediator of ethanol teratogenicity, several studies have demonstrated associations between prenatal ethanol exposure, maternal Zn status and fetal neurodevelopment. Zn is essential for over 1000 enzymes in the body, many of which

are critical for processes involved in central nervous system development, such as neurogenesis, neuronal migration and synaptogenesis. A deficiency of Zn during pregnancy has been demonstrated to impair brain and behavioural development (Pfeiffer and Braverman, 1982; Frederickson et al., 2000; Bhatnagar and Taneja, 2001). In humans for example, mild maternal Zn deficiency, assessed by low levels of Zn in serum and hair, is associated with an increased frequency of neural tube defects, which can subsequently impair brain development (Srinivas et al., 2001). Similarities have been identified between the cognitive impairments produced by prenatal Zn deficiency or Zn deprivation in immature animals and those found in FAS, including impaired learning, attention and memory (Halas and Sandstead, 1975; Halas et al., 1986; Golub et al., 1995; Becker et al., 1996; Mattson and Riley, 1998; Mattson et al., 2001). The hypothesis that ethanol impairs Zn availability during fetal brain development is supported by findings that prenatal ethanol exposure reduces Zn content in the hippocampal mossy fibers in a rodent model system (Savage et al., 1989). Studies that have previously investigated low Zn status as a potential mediator of ethanol-related brain dysfunction have shown that Zn supplementation with ethanol throughout pregnancy in rats results in increased cerebral weight and cerebral RNA content compared with ethanol alone. An increase in metabolic activity in the fetal hippocampus following Zn supplementation further indicated possible protection from impaired brain development (Tanaka et al., 1983). We have recently demonstrated that a subcutaneous Zn injection at the time of ethanol exposure on GD 8 prevents birth defects and spatial memory impairments in mice offspring by

increasing plasma Zn concentrations during the transient period of Zn deficiency (Carey et al., 2003b; Summers et al., 2006). In addition, we reported in Chapter 2 that dietary Zn supplementation throughout pregnancy can prevent severe physical defects and postnatal mortality caused by prenatal ethanol exposure. However, whether increased Zn in the diet is also protective against ethanol-related cognitive and behavioural impairments has not been explored.

The aim of this study was to investigate the effect of ethanol exposure on GD 8 on a range of cognitive processes in mice offspring, and to determine whether dietary Zn supplementation throughout pregnancy can protect against cognitive and behavioural impairments. To examine specific aspects of cognition and behaviour in mice and identify neurobehavioural impairments, a wide range of tests are available which model human cognitive function. In the first part of this study, all adult offspring were examined and screened in a series of these tests for the presence of physical or behavioural defects, including growth, visual, sensory, anxiety, exploratory and motor impairments. The major cognitive processes assessed in this study, however, were learning and memory, as deficits in these processes are one of the most commonly reported cognitive effects of prenatal ethanol exposure (Mattson and Riley, 1998; Mattson et al., 2001) and can occur in the absence of physical abnormalities (Dumas and Rabe, 1994; Mattson et al., 1998; Summers et al., 2006). More specifically, spatial learning and memory impairments have been widely reported in children with FAS and in animal models using various versions of spatial

maze tasks (Reyes et al., 1989; Gianoulakis, 1990; Dumas and Rabe, 1994; Uecker and Nadel, 1996; Kim et al., 1997; Matthews and Simson, 1998; Uecker and Nadel, 1998; Berman and Hannigan, 2000; Summers et al., 2006). While findings related to object recognition memory are not as consistent, Uecker and Nadel (Uecker and Nadel, 1996) have shown that children with FAS were able to perform immediate but not delayed object recall and recent findings by Popovic and colleagues (Popovic et al., 2006) demonstrate that ethanol exposure during the prenatal period impairs object recognition in rats. Therefore, in the second part of this study, phenotypically-normal offspring (i.e. offspring that did not display any physical or behavioural deficits in the screening process) were randomly selected and tested using a cross-maze escape task for spatial learning and memory impairments and an object recognition memory task. In addition, we commenced preliminary examination of the hippocampus of offspring (a brain structure widely demonstrated to be involved with spatial learning and memory (O'Keefe et al., 1975; O'Keefe and Nadel, 1978; Berman and Hannigan, 2000) and affected by prenatal ethanol exposure (Barnes and Walker, 1981; West et al., 1981; Diaz Perez et al., 1991; Berman and Hannigan, 2000)), to determine if ethanol caused any obvious structural abnormalities.

3.2 METHODS

3.2.1 Animals and mating procedure

C57BL/6J mice were purchased from the Institute of Medical and Veterinary Science (IMVS, Adelaide). Housing and mating procedures for mice from these experiments were conducted as described in Chapter 2, Methods, 2.2.2. Animals and mating procedure.

3.2.2 Treatment

On GD 0, pregnant dams were assigned to one of four treatment groups: 1) Saline + control diet, 2) Ethanol + control diet, 3) Saline + Zn supplemented diet, or 4) Ethanol + Zn supplemented diet (n=18-20/treatment). Dietary Zn supplementation (AIN-93G- 200 mg Zn/kg) was given to assigned dams from GD 1-18, whereas the remaining dams were maintained on the control diet (AIN-93G- 30 mg Zn/kg) for this period. Diets were made as described in Chapter 2, Methods, 2.2.1. AIN-93G diet (for growth, pregnancy and lactation in rodents).

Pregnant dams were treated with either ethanol or saline on GD 8 (a critical period of organogenesis and neuronal growth) as described in Chapter 2, Methods, 2.2.3. GD 8 Ethanol and Dietary Zn supplementation. Maternal weights were recorded at GD8 and GD 14. This weight data was used to determine if mice put on weight after treatment (which is usually a good indicator of successful pregnancy progression). A

pregnancy success rate of only approximately 60% is achieved in dams with confirmed vaginal plugs. Therefore this information is helpful in determining which treatments to allocate to pregnant mice during the breeding schedule and to ensure that sufficient numbers of litters are born from each treatment group relatively close in age.

3.2.3 Litter size and neonatal growth

From GD 19, cages were inspected twice daily for pups. The date of birth was noted as postnatal day (PD) 1 and litter-size was recorded. Each litter was culled to a maximum of 7 pups. Viable pups were not handled until PD 3 to minimise cannibalism by the dams. Pup weights were recorded on PD 3 and 21, when they were also weaned and housed according to sex and treatment until behavioural testing.

3.2.4 Physical and behavioural screening

All offspring were physically examined at PD 40 and body weight, body length, coat colour, hair morphology, skin colour, tail morphology, gait, pelvic elevation (normal elevation (3mm)), limb defects (polydactyly, ectrodactyly) and eye abnormalities (microphthalmia, anophthalmia, cataracts) were noted. Each mouse was also subjected to a range of behavioural tests for vision (corneal reflex, visual placement), olfactory, sensory (righting reflex) and motor (wire hang test, swimming ability, open-field test) impairments. This was to identify any possible effects of prenatal

ethanol on these parameters and to exclude mice with obvious physical abnormalities from subsequent behavioural tests (i.e. spatial memory and object recognition tasks) which are dependent on many of these systems tested. Timed tasks were limited (as indicated below) and non-responders were given the maximum score. Observations were made by 2 observers, who were blinded to the treatment groups.

Brief details of the behavioural tests are as followed:

- *Corneal reflex*, an automated blinking of the eyelids elicited by stimulation of the eyeballs cornea with a cotton bud.

- *Visual Placement*, a test in which a mouse is held 30cm above a surface and its ability to stretch out its arms to the surface before its nose touches indicates visual sensory.

- *Righting reflex***,** an indication of motor and vestibular function in which a mouse is placed on its back and its ability to immediately return to the prone position is noted.

- *Olfactory test*, where a mouse is placed in a clean cage containing buried Whiskas® Cat Food (dry pellets) and the latency to find the novel object (indicated by digging up the food) is measured (maximum of 2 min).

- *Swimming*, a measure of motor strength and coordination in which a mouse is placed in a box of water and given 30 s to swim.

- *Wire-hang test***,** an indication of neuromuscular strength in which the mouse is placed on a wire cage lid and the lid is gently turned upside down and latency to fall on the bedding (6 inches below) is recorded (maximum of 1 min).

- *Open-field test*, a measure of motor function, anxiety and exploratory locomotion in a novel environment. The square open-field arena (length, 50 cm; width, 50 cm; height, 25 cm) contained a white laminated floor divided into 25 squares as shown in Figure 3.1. Letters were marked on each square to indicate their position in the field: X, centre square; I, inner squares; O, outer squares and C, corner squares. A video camera was mounted overhead, to record each trial and to allow viewing of mouse activity on a monitor located outside of the testing area. Individual mice were released in the centre of the arena and their behaviour and position of squares crossed were recorded for 5 min. Behavioural parameters measured included: Total number of squares crossed (motor ability and exploration), percentage of squares covered which were adjacent to the wall (wall zone), number of fecal boli and urination (anxiety), frequency of jumps, rearing onto back feet, sitting and grooming. The frequency of sitting or grooming was scored as a multiple of 5 s intervals.

C	**O**	**O**	**O**	**C**
O	**I**	**I**	**I**	**O**
O	**I**	**X**	**I**	**O**
O	**I**	**I**	**I**	**O**
C	**O**	**O**	**O**	**C**

Figure 3.1. A diagram of the floor of the open-field apparatus used to test exploratory behaviour and anxiety in mouse offspring. Letters were used to represent the position of squares- C, Corner; O, Outside; I, Inside; X, Centre square.

3.2.5 Selection for spatial learning/ memory and object recognition tests

12 male and 12 female adult offspring (PD 50) were randomly selected per treatment (n=24/treatment) from offspring that were demonstrated to be free of obvious physical impairments during prior testing. No more than 1 male and 1 female were selected from the same litter (number of litters represented = saline (13), ethanol (13), saline + Zn (14), ethanol + Zn (14)). Mice were handled every day until the beginning of testing, when offspring were assessed for impairments in spatial learning and memory and object recognition memory.

3.2.6 Cross-maze escape task: Spatial learning and memory

Spatial learning and memory were assessed using a cross-maze escape task as previously described (Summers et al., 2006). The apparatus (shown in Figure 3.2) consisted of a clear plastic cross-maze placed in a circular pool of water (1 m diameter) maintained at 23 ^{o}C. The pool was surrounded by a 90 cm high wall covered in black plastic. The water was made opaque by powdered milk to conceal an escape platform (EP) which was submerged 0.5 cm below the surface of the water in the distal half of the North arm. Constant visual (spatial) cues were placed around the maze and were provided by various objects around the room and by the experimenter who always stood at the South arm.

Habituation (PD60); During a habituation trial with no EP placed in the pool, the animals were allowed to swim for 60 s.

Learning (PD61-66); Mice were given a 6 d training period in which they were required to learn the position of the submerged EP from the other three (East, South, West) arms that did not contain the EP. Each mouse was given 6 daily trials (2 blocks of 3 trials separated by a 30 min rest), in which each of the 3 arms were chosen as a starting point in a semi-randomised pattern (twice daily). For each trial, the mouse was placed in the distal end of an arm facing the wall and allowed 60 s to reach the EP, where it remained for 10 s. Mice that did not climb onto the EP in the given time were placed on the EP for 10 s. The mouse was then placed in a cage for 10 s and subsequent trials were continued.

Memory (PD 78 and 105); Animals were tested on two occasions (12 and 28 d after training) to assess their memory of the EP location, which was located in its initial position during training. Mice were given 6 daily trials, as described for training.

Data were recorded for each mouse for each trial on their escape latency (i.e. time taken to swim to the platform) and number of correct trials (i.e. if a mouse found the platform on the first arm entry).

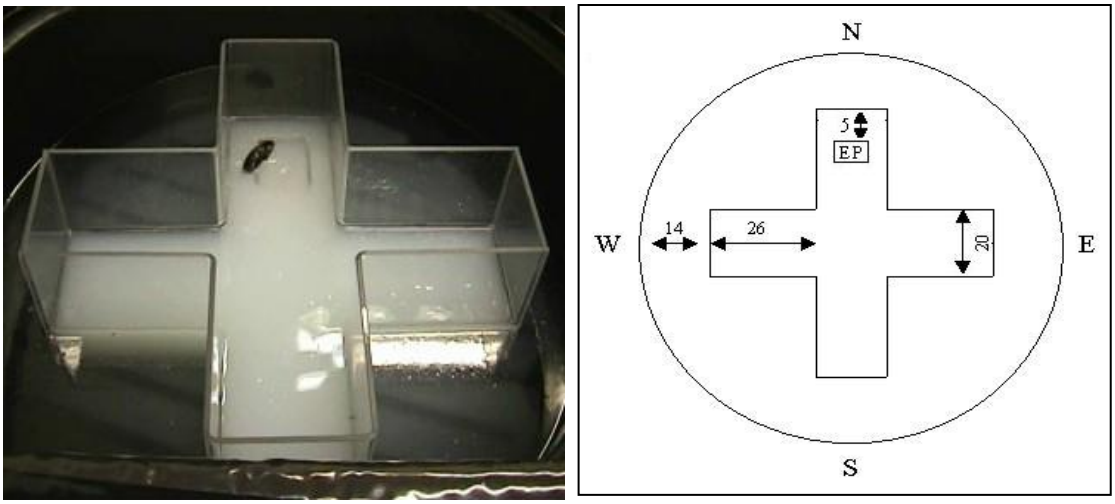

Figure 3.2. The cross-maze apparatus used to test spatial learning and memory in mice. Dimensions of the maze are indicated in the diagram. EP represents the Escape platform; N, North; E, East; S, South; W, West.

3.2.7 Object recognition task

Object recognition memory was assessed in offspring 15 days after testing in the cross-maze escape task (approx. PD 120). The object recognition task (ORT) (modified from (Sik et al., 2003) measures a specific form of episodic memory and takes advantage of the natural affinity of mice for novelty; mice that recognise a previously seen (familiar) object will spend more time exploring novel objects. Briefly, the apparatus (shown in Figure 3.3) consisted of a plastic arena (length, 50 cm; width, 35 cm; depth, 20 cm) filled with bedding. Two different sets of objects were used; a yellow-capped plastic jar (height, 6 cm; base diameter, 4.3 cm) and a red plastic bulb (length, 8 cm; width, 4 cm). A mouse could not displace the objects and preliminary experiments in a separate cohort of animals demonstrated that mice had no preference for either object or location in the arena. Mice spent equal amounts of time when presented with both of these objects, regardless of the position they were placed in the arena (data not shown).

Habituation (PD120); The day before the actual test, animals were habituated to the test box (i.e. allowed to explore the apparatus without any objects present for 5 min).

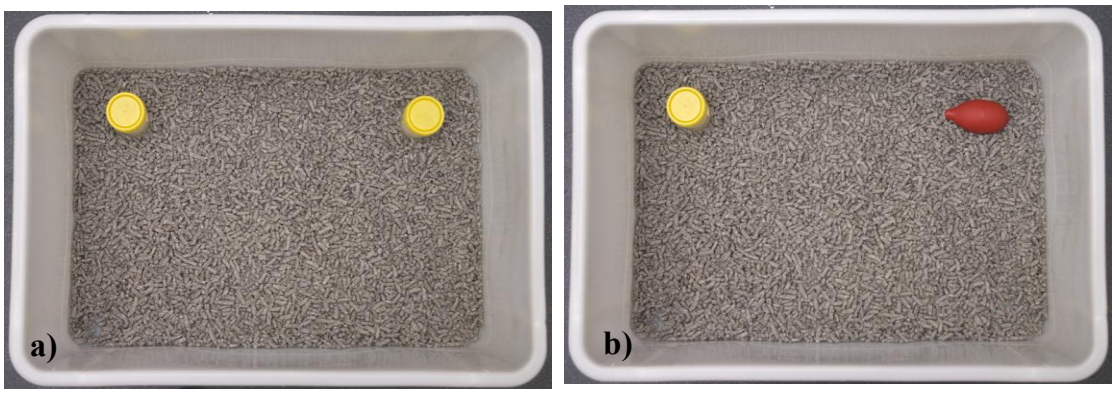

Figure 3.3. The apparatus used to test object recognition memory in mice.

a) The sample phase (containing two identical „sample" objects), and b) the choice phase of the object recognition task (containing a sample and a novel object).

Object recognition memory (PD 121); Mice underwent the testing session comprised of two trials. The duration of each trial was 3 min. During the first trial (the sample phase), the box contained two identical objects (samples) which were placed in the north-west (left) and north-east (right) corners of the box (5 cm away from the walls). A mouse was always placed in the apparatus facing the south wall. After the first exploration period, the mouse was placed back in its home cage. After a 15-min retention interval, the mouse was placed in the apparatus for the second trial (choice phase), but now with two different objects, a familiar one (sample) and a novel one. The objects were cleaned thoroughly with alcohol between sessions to remove any lingering scents. The times spent exploring each object during trial 1 and trial 2 were recorded. Exploration was defined as either touching the object with the nose or within 2 cm of the object.

The basic measures in the object recognition task were the times spent by the mice in exploring an object during trial 1 and trial 2. An overview of the different variables are given in Table 3.1. e1 and e2 are measures of the total exploration time of both objects during trial 1 and trial 2 respectively. $h1$ is an index of habituation measured by the difference in total exploration time from trial 1 to trial 2. $d1$ and $d2$ were considered as index measures of discrimination between the novel and the familiar

Table 3.1. Measures involved in the object recognition test

Exploration	Habituation	Discrimination
$e1 = a1 + a2$	$h1 = e1 - e2$	$d1 = b - a$
$e2 = a + b$		$d2 = d1/e2$

objects. In fact, *d*2 is a relative measure of discrimination which corrects d1 for exploratory activity (*e*2). A discrimination index above zero describes animals exploring the novel object more than the familiar object. An animal with no preference for either object will have an index near zero. All animals achieved the minimum exploration time required (i.e. 7 s for trial 1 or trial 2) to be included in the analysis.

3.2.8 Preliminary brain examination

Approximately two weeks after behavioural testing, male offspring were sacrificed so their brains could be obtained for preliminary analysis. Mice were first anaesthetised using isofluorane and then whole-body perfused with 10 % formalin fixative. This was performed by making a small incision in the right atrium of the heart and inserting the injection of formalin into their left ventricle, to enable the heart to pump the fixative around the body and perfuse the brain. Approximately 2 h following perfusion, whole brains were carefully removed and stored in formalin. After 2-3 days, the olfactory lobes were removed from each brain and then brains were sectioned into 6 (3 mm) coronal samples. Samples were placed in cassettes in pairs (2 anterior (containing frontal lobe), 2 mid-brain (containing hippocampus) and 2 posterior (containing cerebellum)) and processed routinely for paraffin embedding overnight. Serial sections (5 μm thickness) of the mid-brain samples were cut on a sliding microtome and placed on glass slides (approximately 15 slides). Slides were stained with haematoxylin and eosin as described in Chapter 2, Methods, 2.2.5.

Congenital heart defects (preliminary study). Slides were qualitatively examined by Professor Peter Blumbergs, Head of Neuropathology (Hanson Institute Centre for Neurological Diseases) to determine if prenatal ethanol exposure caused any overt changes to brain structures (i.e. obvious abnormal size, position, arrangement of structures) associated with memory function (e.g. hippocampus, dentate gyrus). No quantitative measurements were made on these structures. The brains of female offspring were used for separate gene expression and microarray experiments (not a part of this thesis).

3.2.9 Statistical analysis

Maternal weights and litter-size were analysed by one-way ANOVA. A general linear model (GLM) was used to analyse PD 3 and 21 body weights and all physical and behavioural parameters measured at PD 40, to determine an effect of treatment. PD 3, 21 and 40 body weights were compared to examine for differences in rates of growth. For spatial learning and memory (12 d after training), escape latency data were analysed using GLM. However, due to an imbalance in the data for memory at 28 d after training (i.e. loss of one mouse prior to testing), escape latency data were analysed using restricted maximal likelihood (REML) linear mixed modelling. Wald tests were used to determine a significant effect of treatment. Correct trials which return a binary value (yes/no, whether they went directly to the escape platform), were analysed using a logistic regression model. Correct trials were presented as predicted proportions (the number of correct trials/the total number of trials).

Deviance tests were used to determine the significance of the treatment effect, followed by least significant difference (LSD) tests. Object recognition data were analysed using GLM to determine location preference (comparing a1 and a2 for each object) and interactions of the dependant variables (e1, e2, d1 and d2). One-way ANOVA was used to analyse treatment differences, followed by Tukey‟s post hoc test. Differences between d2 and zero were determined for each group using one-sample t-statistics (Sik et al., 2003) (Minitab Statistics Software, Minitab Inc., State College PA). Also, the number of animals with a discrimination index (d2) > 0.3 was determined within each experimental group. As no treatment-gender interactions were found in this study, male and female data were combined for each treatment and results were presented as mean ± SEM where applicable. Differences were considered to be significant at $p<0.05$.

3.3 RESULTS

3.3.1 Postnatal growth and physical/behavioural development

There was no significant difference in maternal weights at GD 14 between treatment groups (control: 27.4 ± 0.6, ethanol: 26.9 ± 0.4, saline + Zn: 27.2 ± 0.6, ethanol + Zn: 27.0 ± 0.5). Table 3.2 summarises the measured offspring data including litter size, body-weights and physical and behavioural screening data. There was no effect of treatment on litter-size, postnatal body weights at PD 3, 21 or 40 or body length at PD 40. This confirms that all treatment groups had similar rates of growth. There

were no differences between treatment groups for any of the physical parameters (coat colour, hair morphology, skin colour, tail morphology, gait, pelvic elevation, limb defects and eye abnormalities) or behavioural parameters examined on PD 40 (vision (visual placement and corneal reflex), olfactory, sensory (righting reflex) and motor function (swimming ability, wire-hang test)). Furthermore, in the open-field test, there was no effect of treatment on the number of squares crossed in 5 minutes (motor and exploratory function), the percentage of squares that were adjacent to the wall zone and number of fecal boli and urination (anxiety) and frequency of jumps, rearing onto back feet, sitting and grooming (Table 2).

Only a small number of mice (2-8 offspring/treatment) were eliminated from further behavioural testing due to eye abnormalities (microphthalmia, anophthalmia, cataracts) and two with tail defects (gnawed by litter-mate). These abnormalities had the potential to impair their ability to perform in the spatial memory or object recognition tasks, which require intact vision and swimming ability.

3.3.2 Spatial learning and memory

The escape latency and correct trials scores for each of the 6 d of spatial learning are shown in Figure 3.4. Mice from all treatment groups learnt the cross-maze escape task, indicated by a reduction in the time taken to find the platform position (Fig 3.4.B) and an increase in the number of correct trials across the period of training (Fig 3.4.A).

Table 3.2. Physical and behavioural measures of offspring exposed to ethanol ± zinc in early pregnancy

	Treatment			
	saline	**saline + Zn**	**ethanol**	**ethanol + Zn**
Litter-size at birth	5.50 ± 0.41	5.67 ± 0.46	5.72 ± 0.48	6.33 ± 0.45
Number of litters at birth (n=)	20	18	18	18
Physical measures				
Body weight (g) - PD3	1.29 ± 0.04	1.30 ± 0.04	1.29 ± 0.04	1.21 ± 0.03
- PD21	6.61 ± 0.02	6.45 ± 0.02	6.87± 0.03	6.20 ± 0.02
- PD40	16.5 ± 0.04	16.5 ± 0.05	17.2 ± 0.05	16.1 ± 0.05
Body length (mm)- PD40	76.9 ± 0.09	77.7 ± 0.10	78.6 ± 0.17	76.1 ± 0.10
Behavioural measures				
Olfactory test (sec)	43.8 ± 4.08	47.8 ± 4.81	43.4 ± 4.72	35.0 ± 3.73
Wire hang test (sec)	52.1 ± 1.86	51.2 ± 2.06	53.3 ± 1.95	53.0 ± 1.79
Open field test (measures per 5min trial)				
-Number of squares crossed	177.6 ± 6.07	179.3 ± 6.47	169.4 ± 6.90	164.9 ± 4.75
-% of squares crossed which were in wall zone	82.4 ± 0.63	83.6 ± 0.78	84.4 ± 0.69	83.4 ± 0.68
-Number of jumps	0.10 ± 0.07	0.02 ± 0.01	0.32 ± 0.17	0.16 ± 0.07
-Number of rears	42.1 ± 1.20	41.6 ± 1.60	39.5 ± 1.80	44.9 ± 1.67
-Number of sits	3.30 ± 0.58	4.32 ± 0.85	4.35 ± 0.90	4.59 ± 0.74
-Number of grooming bouts	2.68 ± 0.23	3.00 ± 0.30	2.80 ± 0.25	2.65 ± 0.22
-Frequency of urination	0.08 ± 0.03	0.10 ± 0.04	0.12 ± 0.04	0.10 ± 0.04
-Frequency of defecation	1.24 ± 0.12	1.03 ± 0.14	1.15 ± 0.15	1.21 ± 0.16

Measures are from offspring exposed to ethanol or saline on GD 8 with or without dietary Zn supplementation from GD0-18 (n=18-20 litters/treatment).

Values represent means ± SEM.

All behavioural parameters listed were measured at PD 40.

[a]Wall zone, refers to the squares of the open field box that are adjacent to the surrounding wall.

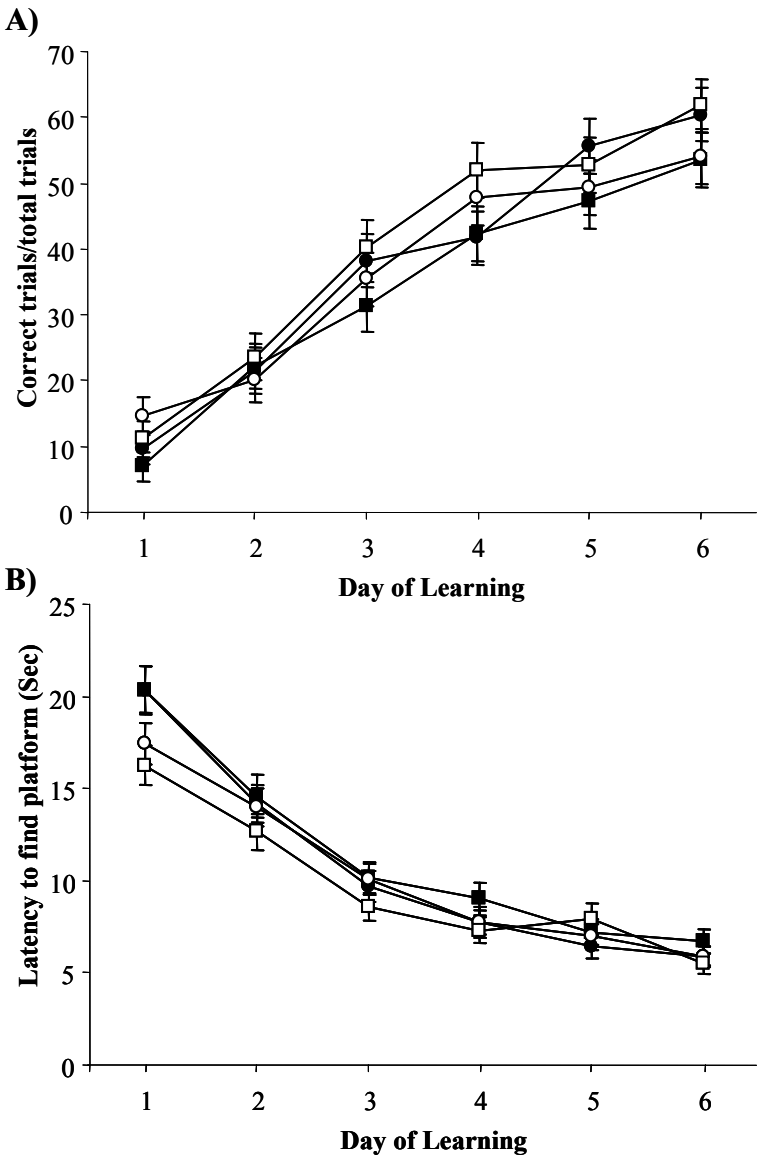

Figure 3.4. Spatial learning: The effect of treatment on correct trials and escape latency. Correct trials (A) and escape latency (B) data over 6 d of spatial learning testing for offspring exposed to saline (●), ethanol (■), saline + Zn (○) or ethanol + Zn (□) on GD 8. Dietary Zn supplementation was given from GD0-18. Correct trials are presented as predicted proportions (i.e. the number of correct trials/total observations for each treatment) ± SEM. Escape latency is presented as means ± SEM. Each point is the treatment groups mean of the sum of six daily trials for each mouse (n=24/treatment).

There were no statistically significant differences between treatment groups in the escape latency scores [F (15, 440) = 0.47, p =0.955] or the number of correct trials [χ^2_{15} =9.43, p = 0.85] over the 6 d of training (i.e. no significant treatment × day interaction), indicating that mice from all treatment groups learnt the task to the same level.

The correct trials and escape latency scores for spatial memory (tested at 12 and at 28 d after mice learnt the initial platform position) are shown in Figure 3.5. Deviance tests on correct trial data demonstrated a significant effect of treatment on spatial memory performance at 12 d [χ^2_3 = 32.45, p<0.001], with offspring exposed to ethanol during pregnancy having significantly fewer correct trials compared with saline treatment. In comparison, the performance of offspring exposed to dietary Zn supplementation with ethanol during pregnancy was not different from saline-treated mice (Fig 3.5.A). The escape latency of ethanol-treated control mice was not different from saline-control mice at this time-point, but was longer than both Zn-supplemented groups [F(3, 87) = 3.17, p = 0.028] (Fig 3.5.B).

When spatial memory was tested 28 d after training, there was a significant effect of treatment on correct trials [χ^2_3 = 39.20, p<0.001], and escape latency scores [χ^2_3 = 27.91, p<0.001]. Ethanol-treated mice demonstrated spatial memory impairments, having significantly fewer correct trials, as well as taking longer to find the escape platform compared to saline-treated mice.

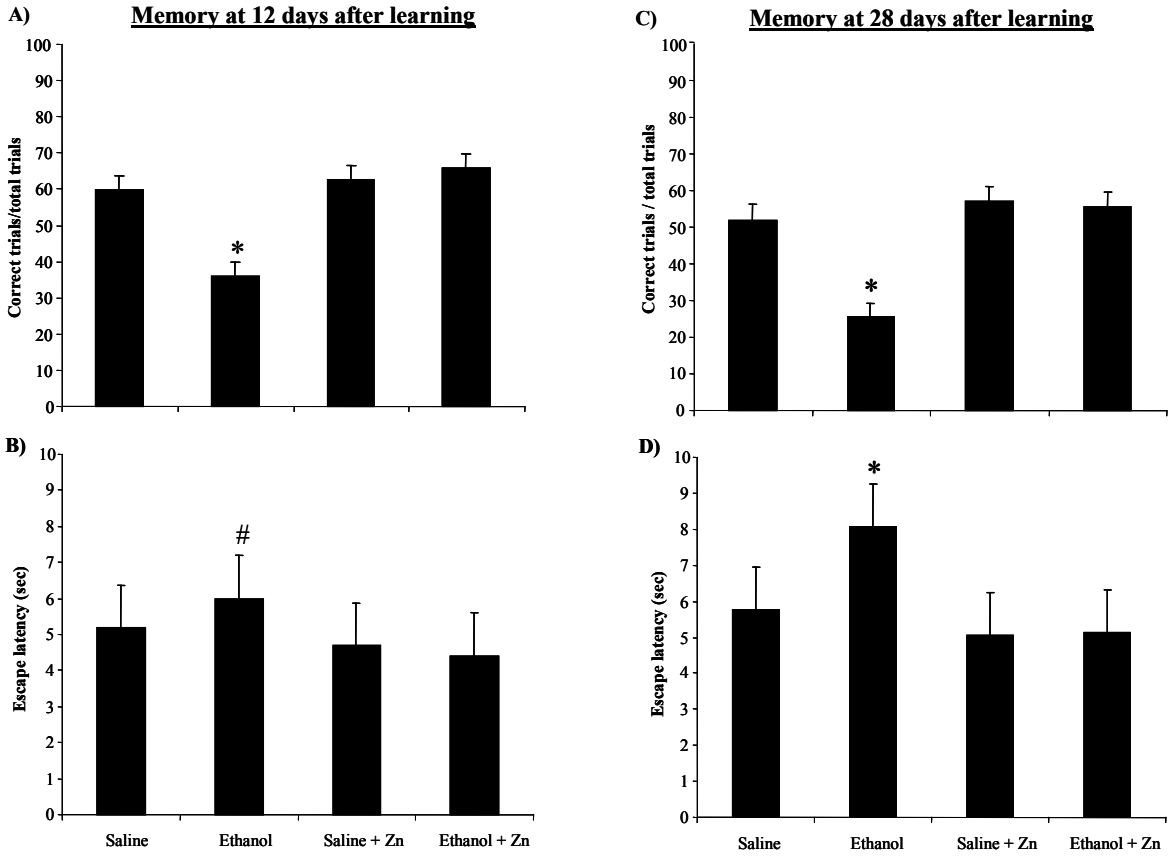

Figure 3.5. The effect of treatment on spatial memory tested at 12 d and at 28 d after mice learnt an escape task. Correct trial (A and C) and escape latency (B and D) data for spatial memory testing of offspring exposed to saline or ethanol on GD8, with or without dietary Zn supplementation throughout pregnancy. Correct trials are presented as predicted proportions (i.e. the number of correct trials/total observations for each treatment) ± SEM. Escape latency is presented as means ± SEM. *Significantly different from all other treatment groups at $p<0.05$. # Significantly different from Zn-supplemented treatment groups only at $p<0.05$. N=23-24/treatment.

Ethanol + Zn mice performed to the level of saline-treated mice with shorter escape latencies and increased correct trials compared with ethanol treatment alone (Fig 3.5.C, D).

3.3.3 Object recognition memory

During trial 1 of the object recognition task, none of the treatment groups demonstrated a preference for either of the identical objects, with mice spending an equal percentage of time exploring the object on the left as on the right side of the arena (overall mean (% of total exploration time): left; 49.9 ± 1.2, right; 50.1 ± 1.2) (Figure 3.6). This confirms that there was no bias towards either corner of the testing arena in any group.

The total exploration time towards both objects in trial 1 was not different between groups (overall mean: 12.4 ± 0.2 s). In trial 2, there was an effect of treatment [F (3, 91) = 5.94, p = 0.001] with a lower object exploration time observed in ethanol (10.5 ± 0.6 s) and ethanol + Zn (11.6 ± 0.8 s) mice compared to saline-treated mice (15.6 ± 1.3 s). Saline + Zn mice (12.5 ± 0.8 s) were not different from the other treatment groups. The index measure of habituation of exploratory behaviour *h*1 showed that there were no differences between groups from trial 1 to trial 2 (mean, -0.3 ± 0.4 s). In general, the h1 indices were negative, reflecting an increase in the total exploratory activity from trial 1 to trial 2 for all groups.The relative discrimination index (*d*2)

between the novel and familiar object for each group in trial 2 is shown in Figure 3.7. There was a clear effect of treatment on object recognition memory performance [$F(3, 91) = 72.78$, $p = 0.001$]; *d*2 was significantly lower in the ethanol-treated mice compared with the other treatment groups and in saline + Zn offspring compared to ethanol + Zn mice. However, neither of these Zn-supplemented groups was different from saline mice. The One-sample t-test demonstrated that *d*2 in ethanol-treated mice was not different from zero [$t(23) = 0.73$, $p=0.23$], confirming that these mice had poor object recognition and had no preference for the novel object in the choice phase. In comparison, the *d*2 index was significantly different from zero for the saline [$t(22) = 9.32$, $p<0.0001$], saline + Zn [$t(23) = 7.65$, $p<0.0001$], as well as ethanol + Zn mice [$t(23) = 9.59$, $p<0.0001$], indicating that these mice had good object recognition memory and a clear preference for the novel object. The number of mice with an index higher than 0.3 was; 23/out of 23 with saline treatment; 19/24 for saline + Zn; 21/24 with ethanol + Zn and only 2/24 for ethanol alone (Figure 3.7).

3.3.4 Preliminary brain examination

Preliminary qualitative examination of brain sections from saline and ethanol-treated offspring tested in the cross-maze escape task and object recognition task, demonstrated that ethanol did not result in any significant gross malformations in the structures of the hippocampus (CA1, CA2 and CA3 areas) or dendate gyrus (Figure 3.8).

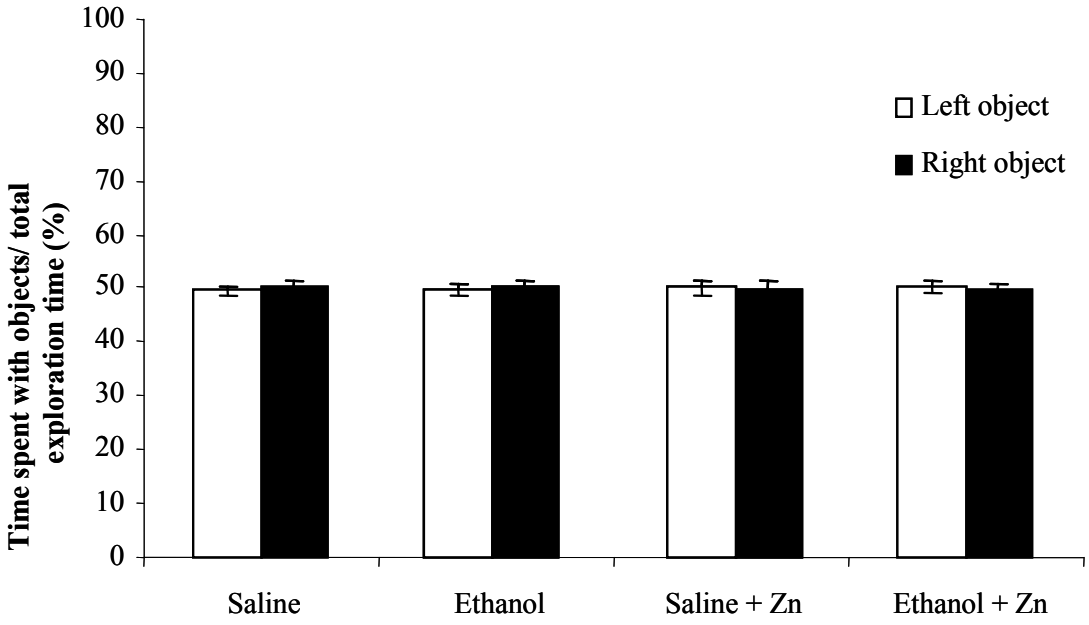

Figure 3.6. Percentage of total exploration time spent with each of the objects during the sample phase (trial 1) of the object recognition task. None of the groups showed a preference for either identical object on the left or right side of the testing arena. Values represent the mean ± SEM of each group, (n =23-24/treatment).

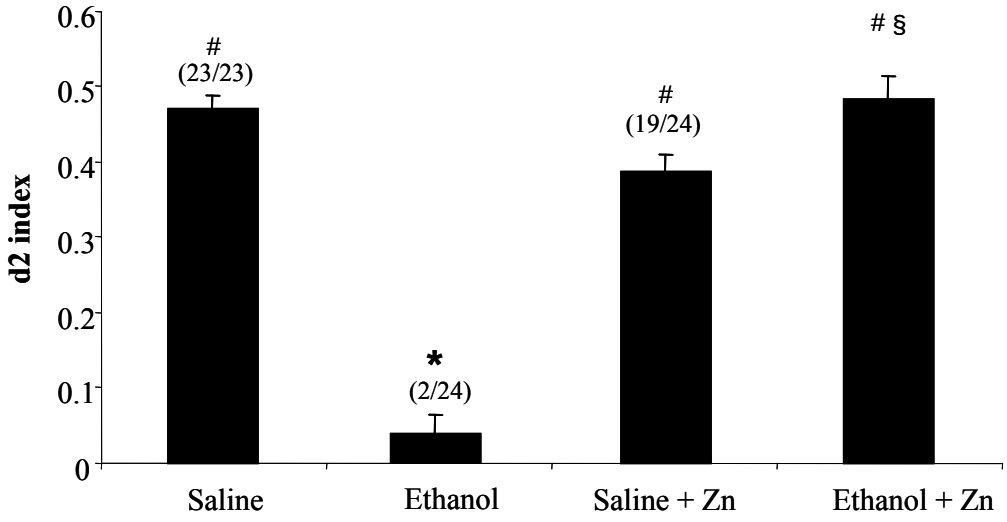

Figure 3.7. The discrimination index (d2) between the novel and familiar object for offspring exposed to saline or ethanol on GD8, with or without dietary Zn supplementation throughout pregnancy. Values represent the mean index ± SEM. Indicated between the brackets; number of mice with a d2 index higher than 0.3 (from the total group of mice in each experimental group). d2=d1/e2, where d1 is the measure of discrimination between the novel (a) and familiar (b) object (d1= b-a); e2 is the time spent exploring both the familiar and novel object in the choice phase (e2 = a + b). *Significantly different from all other treatment groups at $p<0.05$; §Significantly different only from saline + Zn at $p<0.05$; # is significantly different from zero at the $p<0.001$.

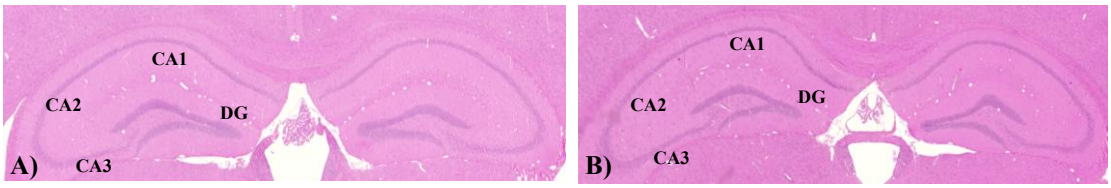

Figure 3.8. Haematoxylin and Eosin-stained sections of the hippocampus from the brains of A) saline-treated offspring, and B) ethanol-treated offspring. There were no obvious structural differences in the arrangement of pyramidal neurons in the CA1-3 regions or granule cells of the Dendate Gyrus (DG) between treatment groups.

3.4 DISCUSSION

The results of this study demonstrate that ethanol exposure to pregnant mice on GD 8 causes impairments in spatial and object recognition memory in adult offspring. Furthermore, dietary Zn supplementation to mothers throughout pregnancy prevented both these memory impairments as assessed by the cross-maze escape task and object recognition task. This is evident from the fact that offspring from mothers exposed to ethanol and dietary Zn supplementation during pregnancy, performed at the level of control offspring for the parameters measured for each memory task.

The present study supports previous findings in both humans and experimental animals that prenatal ethanol exposure impairs performance on spatial tasks (Dumas and Rabe, 1994; Minetti et al., 1996; Matthews and Simson, 1998; Uecker and Nadel, 1998; Berman and Hannigan, 2000; Summers et al., 2006). In particular, these results are consistent with our previous study (Summers et al., 2006) and findings by Minetti and collegues (Minetti et al., 1996) that a binge of ethanol on GD 8 in mice and rats respectively, is sufficient to cause spatial memory deficits in adult offspring. In the present study, spatial memory was tested at 12 and at 28 d after offspring learnt an escape task. Ethanol-treated offspring had significantly fewer correct trials than the other treatment groups at both of these time-points, clearly indicating they had problems remembering the platform location. While it would be expected that mice with fewer correct trials also take longer to find the platform (i.e. longer escape

latency scores), this was only the case for memory testing at 28 d after training. Despite ethanol-treated mice having fewer correct trials at 12 d compared to the other treatment groups, their escape latency scores were not different. This could have been due to differences in swim speed (which affects escape latency without affecting choice accuracy), however, this measure was not examined in this study. It is also possible that the differences in both correct trials and escape latency at 28 d and not at 12 d, are attributed to the longer delay between training and memory testing, which may have posed more of a challenge to the animals.

The spatial memory impairments observed in this study were demonstrated in adult offspring that were of normal physical appearance. This complements recent studies in rodents (Dumas and Rabe, 1994; Summers et al., 2006) and with monkeys (Clarren et al., 1992) that embryonic binge exposure to ethanol can impair cognition in offspring that are free of obvious abnormalities. In an attempt to remove factors from this study that could influence performance in the spatial navigation task and object recognition test, all mice underwent a physical and behavioural screening process, to exclude mice with obvious physical impairments from memory testing. It has been noted that growth, motor, visual and attention deficits can occur as part of the spectrum of defects resulting from intrauterine ethanol exposure (Becker et al., 1996; Mattson and Riley, 1998). However, there were no significant differences between treatments for any of these parameters (or the other cognitive processes measured in this study), and only a small number of mice were removed from testing due to eye malformations (a defect towards which C57BL6J mice have an innate

preference) (Cook and Sulik, 1986). In addition, ethanol-treated offspring demonstrated consistent learning of the cross-maze escape task similar to control mice, supporting the premise that the spatial memory impairments observed were not due to other underlying deficits caused by ethanol (including those affecting motivation and reactions to immediate stress).

In addition to spatial memory impairments, our study supports previous findings that object memory is also impaired in adult offspring exposed to ethanol during pregnancy (Uecker and Nadel, 1996; Garcia-Moreno et al., 2002; Popovic et al., 2006). Offspring that were exposed to ethanol alone on GD8 were not able to identify the previously investigated object and thus explored the novel and familiar objects for the same duration during the choice phase of testing (in comparison to the other treatment groups which clearly demonstrated object recognition by spending more time interacting with the novel object). As the place of the objects were not counter-balanced between consecutive mice, there is a possibility that the ethanol-treated mice didn‟t show preference for the novel object on the same side of the box, if they considered both sides of the box to be novel as a result of their deficit in spatial memory. However, in the cross-maze escape task, spatial deficits were only observed after a 12 day interval between learning and memory testing. As treatment groups had the same improvement in learning on each day of the 6 day training phase would indicate that memory at least in the 24 hour interval between the learning trials was not affected. The lack of spatial cues within the arena of the object recognition test,

the short time period between trials and exploratory time that each mouse spent in the box, would make it unlikely that spatial memory deficits greatly influenced the result in the object recognition task. The object recognition task used in this study is similar to those used to test object memory in humans (MacKay-Soroka et al., 1982) and allows testing of rodents without external stress and is not based on positive or negative reinforcers (e.g. electric shock, food deprivation) which make the interpretation of drug effects on memory difficult. In addition the *d*2 discrimination index used in this study corrects for total exploratory activity, such that despite any differences in total object exploration time between subjects, it does not affect the validity of object recognition performance. This is indicated by the fact that while both ethanol and ethanol + Zn offspring had decreased exploratory activity, only ethanol treatment alone caused impairments in object recognition, with a discrimination index not different from zero and only 8 % of offspring with an index higher than 0.3 (compared to 79-100 % in other groups).

This is the first study to demonstrate that dietary Zn supplementation throughout pregnancy can prevent cognitive impairments caused by ethanol exposure on GD 8. This supports our previous findings that subcutaneous Zn treatment with ethanol on GD 8 prevents spatial memory impairments and physical defects (Carey et al., 2003a; Summers et al., 2006) and that dietary Zn supplementation throughout pregnancy prevents alcohol-related physical abnormalities and mortality (Chapter 2).

Furthermore, these results support the proposed mechanism that the availability of Zn plays a significant role in mediating alcohol-induced cognitive impairments.

Zn is essential for many critical processes in brain development and a transient limitation in fetal Zn (which we have shown to occur following binge ethanol exposure (Carey et al., 2000b)), has the potential to impair critical stages of early neuronal development. In support of this, ethanol exposure during organogenesis has been demonstrated to inhibit neuronal differentiation and proliferation (Jing and Li, 2004) and cause apoptosis in cell populations, such as the developing neural plate and primitive streak (Dunty et al., 2001). Suppression of normal neural cell development has been similarly demonstrated by a deficiency of dietary Zn throughout pregnancy (Rogers et al., 1995; Wang et al., 2001). A delay in brain development can lead to impairments in specific brain structures and associated cognitive processes, such as the memory impairments observed in this study. Accurate spatial memory performance depends on the medial temporal lobe hippocampal formation (O'Keefe and Nadel, 1978), as hippocampal legions impair spatial processes (O'Keefe et al., 1975). Ethanol exposure during pregnancy has been demonstrated to alter hippocampal anatomy (e.g. abnormal arrangement of hippocampal mossy fibers, decrease in number of neurons) (Barnes and Walker, 1981; West et al., 1981; Diaz Perez et al., 1991; Berman and Hannigan, 2000), even when ethanol was administered as early as GD7, a time which anticipates the period of initial hippocampal cell generation (Diaz Perez et al., 1991). While the

hippocampus does play a role in object memory (most apparent when complex tasks are used), the simple object recognition task used in this study is also dependant on the function of additional brain regions, specifically the perirhinal cortex (Buffalo et al., 1998; Cowell et al., 2006). Previous studies suggest that the structures underlying spatial memory are more vulnerable to the effects of prenatal ethanol than those underlying object memory (Kim et al., 1997; Uecker and Nadel, 1998). However, the occurrence of both spatial and object memory impairments in this study indicate that ethanol exposure on GD8 is a sensitive period for the development of brain regions involved in both of these processes. Preliminary examination of brain sections indicated that prenatal ethanol exposure did not cause any obvious structural abnormalities in the hippocampus of offspring tested in the study. Studies are therefore warranted to correlate specific molecular alterations with these memory impairments, to increase our understanding of the aetiology of ethanol-related cognitive impairments. In addition, determining whether Zn supplementation can prevent these molecular changes is important to elucidate the mechanism by which Zn is protective. For example, current work in our laboratory has indicated that ethanol exposure on GD 8 causes a 20% increase in brain-derived neurotrophic factor (BDNF) gene expression in adult offspring, a marker that plays a critical role in mediating long term potentiation (a mechanism underlying learning and memory formation). Elevated BDNF expression may be involved in cognitive impairment, possibly by causing hyperexcitability in the circuits involved in memory, leading to a loss of synaptic refinement. BDNF expression is regulated by Zn, and Zn

supplementation was shown to prevent increase in BDNF caused by ethanol (unpublished data).

As discussed in Chapter 2, there are a number of general mechanisms by which excess dietary Zn could protect against ethanol-related cognitive impairments. Zn supplementation could overwhelm the maternal MT-response (i.e. prevent the fall in plasma Zn concentrations caused by ethanol exposure (Chapter 2)) so that Zn can be accessed and utilised by the fetus and prevent ethanol-induced changes in brain development (i.e. possibly BDNF expression). Alternatively, Zn may have an MT-independent affect on the developing brain. The higher score of pups in the object recognition task from the Zn-supplemented ethanol group compared with the Zn-supplemented saline treated mice, may reflect an influence of excess Zn on repair mechanisms associated with ethanol-related damage. Zn is not only essential for growth and development but is also involved in anti-apoptotic pathways (Allington et al., 2001; Truong-Tran et al., 2001; Fernandez et al., 2003). Therefore, Zn supplementation may promote cell survival (as shown when given with other teratogenic agents) (Fernandez et al., 2003) to compensate for the ethanol-related brain deficits, rather than directly limiting the actions of alcohol.

3.5 CONCLUSION

This study demonstrates that a single binge of ethanol in early pregnancy can cause neurobehavioural impairments (spatial and object recognition memory deficits) and

that these can occur in the absence of physical abnormalities. More significantly, dietary Zn supplementation throughout pregnancy can ameliorate these impairments. Application of a binge-drinking model in this study is highly relevant to the clinical setting where more than 5 drinks in one session in the first 3-9 weeks of pregnancy places the fetus at considerable risk. While Zn supplementation during pregnancy did not further improve cognition or behaviour in control offspring, our findings do emphasis the importance of Zn in brain development and suggest that exogenous Zn may either offset ethanol-induced Zn deficiency or act through an independent mechanism to limit the effects of ethanol on cognitive development. In either case, our findings further support the hypothesis that Zn availability is a contributing factor to the development of ethanol-related birth abnormalities. These findings may be beneficial in the future development of appropriate intervention strategies for FAS or ARND.

CHAPTER 4

The effect of chronic ethanol exposure (via liquid diet) and dietary Zn supplementation throughout pregnancy on postnatal growth, survival and spatial learning and memory in mice offspring.

Preliminary studies:

- **The effect of various dietary Zn concentrations fed to dams throughout pregnancy on maternal tissue Zn distribution and fetal outcome.**
- **The effect of maternal exposure to various ethanol dosages throughout pregnancy on fetal outcome (i.e. size of litters, postnatal growth and survival).**

4.1 INTRODUCTION

The proposal that alterations in maternal and fetal Zn metabolism secondary to maternal ethanol consumption contribute to FASD, has been investigated for almost three decades. However, the evidence supporting a role for MT as a mediator of ethanol teratogenicity (i.e. MT-induced Zn deficiency) has only recently been demonstrated in rodents exposed to acute (binge-type) ethanol in early pregnancy (Carey et al., 2000a; Carey et al., 2000b). Only one study to our knowledge has investigated the effect of chronic ethanol exposure throughout pregnancy on maternal liver MT and found that it did not induce MT when examined late in pregnancy

(Harris, 1990). However, possible explanations for this lack of an effect were that MT may have been induced soon after initiation of ethanol consumption, but other adaptions to ethanol and a return to normal MT levels may have occurred following long-term exposure to ethanol, or the level of blood ethanol required to induce MT may not have been reached in the study. Nevertheless, while the maternal liver MT response to chronic ethanol administration throughout pregnancy remains unknown, there are several lines of evidence supporting that long-term ethanol exposure alters Zn metabolism in pregnant humans and experimental animals.

Pregnant alcoholic women have been shown to have lower plasma Zn levels than pregnant non-alcoholic women (Flynn et al., 1981). Lower cord-blood Zn concentrations have also been reported in women who drank during pregnancy compared to non-drinking controls (Halmesmaki et al., 1985). Infants born to the alcoholic mothers had lower fetal cord-plasma Zn levels and a much higher incidence of abnormalities than those born to non-alcoholic mothers (Flynn et al., 1981). This is supported by findings that infants with FAS had lower plasma Zn concentrations than normal infants (Assadi and Ziai, 1986). In animal studies, there are similarities in the fetal outcomes resulting from prenatal exposure to ethanol or Zn deficiency (*see Chapter 1: 1.2.8. Teratogenicity: zinc deficiency vs ethanol exposure and Table 1.1*) which suggests a common Zn-related aetiology between these insults. In rats, long-term ethanol exposure during pregnancy has been shown to decrease Zn content of the maternal serum (Ghishan et al., 1982) and muscle (Suh and Firek, 1982). While

some studies have found that ethanol had no effect on whole body fetal Zn concentrations (Keppen et al., 1985; Zidenberg-Cherr et al., 1988) others have shown that the availability of Zn to the fetus is decreased by ethanol. Ghishan and colleagues demonstrated that chronic ethanol exposure during pregnancy interferes with placental transport, as it depressed ^{65}Zn uptake in the placenta and fetus and decreased total Zn concentrations in the fetus (Ghishan et al., 1982). Further studies have also demonstrated significant reductions in fetal Zn concentrations following prenatal ethanol exposure (Mendelson and Huber, 1979; Suh and Firek, 1982) and in specific organs, such as the hippocampal mossy fibers of offspring (Savage et al., 1989).

Considering this evidence supporting an effect of ethanol on fetal Zn homeostasis, a number of studies have investigated the possibility of Zn supplementation as a preventative measure against the negative outcomes caused by ethanol. While Zn treatment (intraperitoneal (Seyoum and Persaud, 1995), subcutaneous (Carey et al., 2003b; Summers et al., 2006) or dietary Zn supplementation (Chapter 2 and 3)) has only recently been shown to have a protective influence against physical defects, postnatal mortality and memory impairments in offspring exposed to acute ethanol in early pregnancy, findings from much earlier studies in chronic ethanol rodent models have not been as consistent, with both positive and negative effects of Zn supplementation. Zn supplementation in rats was first shown to prevent the decrease in fetal Zn and maternal femur Zn caused by ethanol consumption throughout

pregnancy (Mendelson and Huber, 1979). Experiments by Tanaka and colleagues demonstrated that administration of excess Zn with an ethanol diet during pregnancy in rats increased maternal serum Zn concentrations and resulted in a better effect of fetal body weight, protein content in the cerebrum and on preventing resorptions than administration of ethanol alone (Tanaka et al., 1982). They subsequently found that Zn supplementation with ethanol increased metabolic activity in the fetal hippocampus, (a repair mechanism against the inhibitory effect on RNA and protein synthesis of ethanol). Although cerebral weight and RNA content was increased compared to ethanol alone, it did not reach the level of controls (Tanaka et al., 1983). Furthermore, the positive effects of Zn on body and cerebral weights of ethanol fetuses were not replicated in a later experiment (Tanaka, 1998). Other studies indicate that excess dietary Zn supplementation does not overcome the defect in placental Zn caused by prenatal ethanol consumption in rats (at 10 to 40 mg Zn/L) (Ghishan and Greene, 1983) or have a protective effect against ethanol teratogenicity in mice (appearing to have an adverse effect on fetal weight and prenatal mortality) (Keppen et al., 1990).

While further studies are required to fully determine whether Zn supplementation has a role in protecting against chronic-ethanol mediated teratogenicity, it is yet to be examined whether Zn supplementation is protective against postnatal outcomes, such as low birth-weight, growth retardation, neonatal mortality and behavioural impairments (which are hallmark signs for offspring of alcoholic women or ethanol-

exposed rodents) (Abel, 1985; Middaugh et al., 1988; Riley, 1990; Becker et al., 1996; Mattson and Riley, 1998; Mattson et al., 2001). That ethanol decreased Zn in the hippocampus of offspring (Savage et al., 1989) and Zn supplementation was shown to be protective against alterations in developmental processes in the fetal hippocamus caused by chronic ethanol exposure (Tanaka et al., 1982; Tanaka et al., 1983), suggests that behavioural deficits associated with hippocampal dysfunction (e.g. spatial learning and memory impairments) could potentially be limited by Zn supplementation. It is well established that children with FAS display impairments in spatial memory (Uecker and Nadel, 1996; Uecker and Nadel, 1998) and chronic ethanol consumption during pregnancy in rats impairs spatial processes (learning and memory) (Reyes et al., 1989; Gianoulakis, 1990; Kim et al., 1997; Matthews and Simson, 1998; Berman and Hannigan, 2000; Popovic et al., 2006). Therefore, the main objective of this chapter was to develop a chronic ethanol mouse model and demonstrate whether dietary Zn supplementation could protect against adverse postnatal outcomes in offspring caused by long-term ethanol exposure during pregnancy (i.e. growth deficits, mortality and most importantly spatial learning and memory impairments). Spatial learning and memory were tested on PD 60 in a randomly selected cohort of offspring that were free of physical abnormalities using a cross-maze escape task (as described in Chapter 3).

In a chronic ethanol rodent model, food and water consumption is limited to an ethanol-containing liquid diet where ethanol is added so that ~18 % (low dose) or

~35 % (high dose) of total daily calories are provided by ethanol (as rodents do not normally voluntarily drink enough ethanol to maintain chronically high blood alcohol levels). As we have not previously applied this chronic ethanol model (i.e. liquid diet) to our C57BL/6J mice, preliminary studies were conducted prior to the main behavioural experiment to determine the effect of various levels of Zn in the liquid diet throughout pregnancy on pregnancy/fetal outcomes and maternal tissue Zn distribution (i.e. plasma, liver and muscle Zn). In addition, we also investigated the effect of various dietary ethanol dosages (20, 27 and 35 % ethanol derived calories (EDC)) throughout pregnancy on the number, growth and survival of offspring in order to select an ethanol dosage suitable for the behaviour study. As the behavioural study requires 1 male and 1 female per litter without physical impairments, the dosage chosen must ensure that sufficient numbers of offspring survive after birth to be available for testing.

4.2 MATERIALS AND METHODS

4.2.1 Animals and mating procedure

C57BL/6J mice were purchased from the Institute of Medical and Veterinary Science (IMVS, Adelaide). All animals were given unrestricted access to water and were fed a commercial diet (Milling Industries, Adelaide, Australia) until the commencement of mating (at which they were placed on a Weinberg/ Keiver liquid diet as described below). Housing and mating procedures for mice from these experiments were conducted as described in Chapter 2, Methods, 2.2.2. Animals and mating procedure.

4.2.2 Weinberg/Keiver liquid diet

The diets used for all experiments in this chapter were purchased from DYETS Inc. The control (DYET# 715253) and ethanol (DYET# 715254) versions of the Weinberg/Keiver high protein liquid diet (with EDTA-extracted casein and Rx minerals without zinc) were stored in powder form at -20 °C. Diets were made into liquid form as required each morning during experiments. To make one litre of the the ethanol diet, ethanol (95 % v/v) was added to 156.1 g of powdered diet and made up to one litre with water and mixed in a blender. The volume of ethanol added was as specified by the product suppliers to produce a diet with a specific percentage of ethanol derived calories (i.e. 35 % EDC). At lower ethanol levels (i.e. 25 % and 27 % EDC), the volume of ethanol added was decreased accordingly. For the control diet, 245.76 g of the powdered control diet was made up to one litre with cold deionized water and mixed. Mice on the control diet were given the same volume of diet containing isocaloric amounts of maltose dextrose substituted for ethanol. As the control and ethanol diets did not contain any Zn, the required amounts of ZnSO4 were therefore added to the mixtures before blending, to produce diets with specific Zn levels (e.g. 4, 8, 25 or 50 mgZn /L). The levels of Zn and/or ethanol used in the diets in this chapter are specified for each study in the methods below. Mice were given fresh supplies of their assigned diet each morning (approximately 30 ml) in 50 ml inverted plastic graduated bottles fitted with metal feeding tubes, which were cleaned daily. Preliminary trials found that in bottles containing a ball in the metal

feeding tube, the diet often became blocked and limited consumption of the diet. We therefore used lids that did not contain a ball at the tip of the feeding tubes to ensure efficient delivery of the diet.

4.2.3 Dietary Zn concentrations, tissue Zn distribution and fetal outcomes (preliminary study)

To examine the effect of various dietary Zn concentrations on tissue Zn distribution and fetal outcomes, dams that were identified as pregnant (via detection of a vaginal plug) were fed a control liquid diet containing 4, 8, 25 or 50 mg Zn/ L from GD 0 to GD 18 (n = 9-13). 8 mg Zn/L was selected as the control Zn level for the liquid diet, which is the amount recommended for reproduction and used previously in control diets (Keppen et al., 1985). Diets containing 4 mg Zn/ L were classified as Zn-limiting (compared to the control level) and those containing 25 mg Zn/ L and 50 mg Zn/ L were classified as Zn-supplemented. On GD 18, mice were anaesthetized using halothane and blood was collected via cardiac puncture. Mice were then killed via cervical luxation and the maternal liver, muscle and uteri were immediately removed and stored on ice[2]. The conceptus was examined for resorption sites. Fetuses and their placentas were then separated and weighed and fetuses were briefly examined for external abnormalities as previously described *(Chapter 2, Methods 2.2.4. Fetal dysmorphology).* The maternal blood, liver and muscle tissue were processed for Zn

[2] Note: As only 50-60 % of mice identified as having a vaginal plug contained fetuses on GD 18, plasma, liver and muscle samples were also collected from non-pregnant mice to compare tissue Zn concentrations with those from pregnant dams.

analysis as previously described (*Chapter 2, Methods 2.2.6. Maternal plasma Zn analysis and 2.2.9. Zinc analysis)*. Fetuses were processed for Zn analysis by the methods used for liver and muscle tissue.

4.2.4 Ethanol dosage and pregnancy outcomes (preliminary study)

Pregnant dams (n = 12) were originally fed a 35 % EDC liquid diet (8 mg Zn/ L) beginning from GD 0 to GD 18. On GD 18, mice were anaesthetized, killed via cervical luxation and uteri were immediately excised and examined for resorption sites. Individual fetuses and their placentas were separated and weighed and fetuses were briefly examined for external abnormalities as previously described *(Chapter 2, Methods 2.2.4. Fetal dysmorphology).* Out of 12 dams identified as pregnant by plug detection, only 2 contained fetuses when examined on GD 18. While each had 8 pups per litter (with 3 and 1 resorptions respectively), over 50 % of these had severe abnormalities including microphthalmia (4), anophthalmia (1), abnormal head (3), micrognathia (2), with others displaying lesser degrees of small mouth. This indicated that the level of ethanol in the 35% EDC diet was too severe an insult for this study. The possibility that ethanol given in the first few days of pregnancy may have prevented implantation from occurring, was also considered. Therefore, a second protocol was adopted where pregnant dams were first placed on a control liquid diet (8 mg Zn/ L) from GD 0 to GD 5 and then an ethanol liquid diet (25, 27 or 35% EDC; 8 mg Zn/ L) from GD 6 to GD 18. Dams were either killed on GD 18 and the conceptus, placenta and fetuses were weighed or they were returned to the control

liquid diet on GD 18 until pups were born. The litter-size at birth was recorded and offspring weights were examined on PD 3, 9, 15 and 40. Postnatal mortality was recorded during this period.

Based on the results from this preliminary study (*see Results 4.3.2***,** *Ethanol dosage and pregnancy outcomes (preliminary study)*), the 27 % EDC liquid diet was selected as the ethanol dosage for the remaining studies in this chapter.

4.2.5 Blood alcohol concentration (BAC) and plasma Zn timeline: 27 % EDC liquid diet

To determine the BAC‟s and corresponding plasma Zn levels achieved by consuming a 27 % EDC diet, pregnant mice were placed on the 27 % EDC diet (8 mg Zn/ L), for 2 weeks (from GD 0 to GD 14). On GD 14, mice were anaesthetised at 11:00, 4:00 or 10:00 pm (n = 3 per time point), blood samples were obtained via cardiac puncture and mice were then killed via cervical luxation. The 10:00 pm time point corresponded with 4 hrs after lights-out, which has previously been shown to occur during peak BAC‟s (Allan et al., 2003). Blood was processed for Zn analysis as previously described (*Chapter 2, Methods 2.2.6. Maternal plasma Zn analysis).* BAC‟s were determined on plasma using an Emit ethyl alcohol kit (Behring Diagnostics, Cupertino, CA) on a Hitachi 911 analyser (Boehringer-Mannheim, Castle Hill, Australia). Liver samples were collected post mortem and processed for

MT analysis (stored at -70°C). However, unfortunately due to a freezer breakdown, samples were spoiled and unable to be analysed.

4.2.6 Chronic ethanol behaviour study: Diets, postnatal growth and survival

Following plug detection, all pregnant dams were placed on the control liquid diet (8 mg Zn/ L) from GD 0 to GD 5. On GD 6, pregnant dams were assigned to one of four dietary treatment groups: 1) control liquid diet (8 mg Zn/ L), 2) ethanol liquid diet (8 mg Zn/ L), 3) control + Zn-supplemented liquid diet (50 mg Zn/L), or 4) ethanol + Zn-supplemented liquid diet (n = 28-44)[3]. Mice were fed these diets until GD 18, at which they were returned to the control liquid diet (8 mg Zn/L) for the remainder of their pregnancy. Dietary consumption was measured to the nearest ml every day during pregnancy for each mouse, by weighing bottles containing fresh diet each morning and then again the following morning (i.e. (bottle + fresh diet) – (bottle + remaining diet) = diet consumed for that day). Maternal weights were only recorded on GD 0 and 10 to minimise handling.

From GD 19, cages were inspected twice daily for pups. The date of birth was noted as postnatal day (PD) 1 and litter-size was recorded. Viable pups were not handled until PD 3 to minimise human scent and reduce cannibalism by the dams. Each litter was culled to a maximum of 7 pups on PD 3. Pup weights were recorded on PD 3, 14

[3] Note: More pregnant dams were allocated to the ethanol treatment groups than control groups to allow for the possibility of ethanol decreasing pregnancy success in this cohort.

and 21, when they were also weaned and housed according to sex and treatment until behavioural testing. Postnatal survival was examined by counting the surviving pups on PD 3, 14, 21 and 40.

4.2.7 Chronic ethanol behaviour study: Selection for spatial maze task

[4]All offspring were examined for obvious physical defects, such as limb defects (polydactyly, ectrodactyly), tail morphology, eye abnormalities (microphthalmia, anophthalmia, cataracts) and swimming impairments, which could possibly impair their ability to perform in the spatial maze task. 12 male and 12 female adult offspring (PD 50) were randomly selected per treatment (n = 24/treatment) from offspring that were demonstrated to be free of obvious physical impairments during prior testing. No more than 1 male and 1 female were selected from the same litter. Offspring were weighed on PD 50. Mice were handled every day until the beginning of testing, when offspring were assessed for impairments in spatial learning and memory.

[4]Note: This chronic ethanol study was undertaken before the development of the object recognition task and the comprehensive screening process used for subject selection in Chapter 3. The comprehensive screening of all animals in Chapter 3 was only possible with the involvement of 2 investigators. This study therefore used a simpler method for subject selection where mice were examined for only obvious physical impairments and tested in the spatial maze task (which can be completed with one investigator).

4.2.8 Chronic ethanol behaviour study: Spatial learning and memory

Spatial learning and memory was assessed using a cross-maze escape task as previously described in Chapter 3, Methods, 3.2.6. Cross-maze escape task: Spatial learning and memory. However, the duration of the learning period was 5 days, as in our previous study (Summers et al., 2006).

At the completion of the study, brains were collected from all mice that were tested in the cross-maze escape task and weighed.

4.2.9 Statistical Analyses

Fetal weights, placental weight and fetal body Zn concentrations were compared by one-way ANOVA, as were plasma Zn concentrations and BAC‟s in the time-line study. Plasma, liver and muscle Zn concentrations were compared between pregnant and non-pregnant mice using General Linear Model (GLM). Dietary consumption during the behaviour study was also analysed using GLM. Brain weights were expressed as a ratio of their body weights and were analysed using one-way ANOVA Significance was determined using Tukey‟s post hoc test. Data concerning litter-size, fetal resorptions and abnormalities at GD 18 were non-normally distributed and analysed with the Mann-Whitney U test. All these statistical analysis were performed with Minitab statistics software (Minitab Inc., State College, PA). Results are presented as mean ± SEM. Differences were considered to be significant at $p < 0.05$. Postnatal survival was analysed as previously described in (Chapter 2, 2.2.11.

Statistical analyses) and was presented as the cumulative probability of survival of the total pups available after culling from birth to PND 40. For spatial learning and memory escape latency data were analysed using GLM. Wald tests were used to determine a significant effect of treatment. Correct trials which return a binary value (yes/no, whether they went directly to the escape platform), were analysed using a logistic regression model. Correct trials were presented as predicted proportions (the number of correct trials/the total number of trials). Deviance tests were used to determine the significance of the treatment effect, followed by least significant difference (LSD) tests.

4.3 RESULTS

4.3.1 Dietary Zn concentrations, tissue Zn distribution and fetal outcome (preliminary study)

GD 18 maternal and fetal data from dams fed various dietary Zn levels throughout pregnancy are displayed in Table 4.1. There were no differences in litter-size and percentage of fetal resorptions between treatment groups. However, the placental weight of fetuses exposed to 4, 25 or 50 mg Zn/ L diet throughout pregnancy were significantly lower compared to fetuses from the control 8 mg Zn/L group ($p < 0.05$). In addition, the incidence of abnormalities in these groups (although similar to levels observed in C57BL/6J mice (Carey et al., 2000b) and reported in the control groups in Chapter 2), were significantly greater than the control 8 mg Zn/ L group, which did

not result in abnormalities ($p < 0.05$). All abnormalities involved the eye (microphthalmia, anophthalmia), except one case of total body haemorrhage in the 4 mg Zn/L group. Fetuses from the Zn supplemented groups (25 and 50 mg Zn/L) had lower body weights than those in the control group ($p < 0.05$). Fetal body Zn concentrations were only increased in the 50 mg Zn/ L group compared to controls ($p < 0.05$) (Table 4.1).

Plasma, liver and muscle Zn concentrations in pregnant and non-pregnant mice fed various dietary Zn levels for 18 days are shown in Figure 4.1. Plasma Zn concentrations significantly increased with each increment in dietary Zn level in pregnant mice (i.e. $4 < 8 < 25 < 50$ mg Zn/ L) ($p < 0.05$). Thus, in comparison to the control 8 mg Zn/ L diet, the Zn-limiting 4 mg Zn/ L diet decreased plasma Zn levels and the Zn-supplemented diets (25 and 50 mg Zn/ L) increased plasma Zn levels. However, dietary Zn level had no effect on plasma Zn concentrations in non-pregnant mice. Thus, while there were no differences between pregnant and non-pregnant mice on the 4 mg Zn/ L diet, pregnant mice fed the 8, 25 or 50 mg Zn/ L diets had higher plasma Zn concentrations on GD 18 compared to non-pregnant mice on the corresponding diet ($p < 0.05$).

Table 4.1. GD 18 maternal and fetal data from dams fed various dietary Zn levels throughout pregnancy (GD 0-18)

	Zn concentration of diet (mg Zn/L)			
	4 mg Zn/L	8 mg Zn/L	25 mg Zn/L	50 mg Zn/L
GD 18 fetal data				
No. litters	7	5	6	6
No. fetuses	41	35	48	39
Litter-size[b]	5.9 ± 0.7	7.0 ± 1.2	8.0 ± 1.0	6.5 ± 0.9
% resorptions/per litter[b]	21.0 ± 4.8	12.2 ± 5.6	16.7 ± 9.2	12.1 ± 5.1
% abnormalities/per litter[b]	6.2 ± 3.0†	0 ± 0.0	9.5 ± 4.5†	5.0 ± 3.4†
Fetal weight (g)[a]	1.07 ± 0.02	1.12 ± 0.02	1.04 ± 0.01†	1.03 ± 0.02†
Fetal body Zn concentration (nmol/g)[a]	180.0 ± 10.7	258.0 ± 19.5	224.2 ± 22.6	275.6 ± 22.2*
Placental weight (mg)[a]	63.7 ± 0.003†	74.0 ± 0.003	57.1 ± 0.002†	63.6 ± 0.003†

Values represent the means ± SEM where applicable.

[a]Data analysed by one-way ANOVA, with Tukeys post-hoc test.

[b]Non-parametric data analysed by Mann-Whitney U test.

*Significantly different from 4 mg Zn/L group, $p < 0.05$.

†Significantly different from 8 mg Zn/L group, $p < 0.05$.

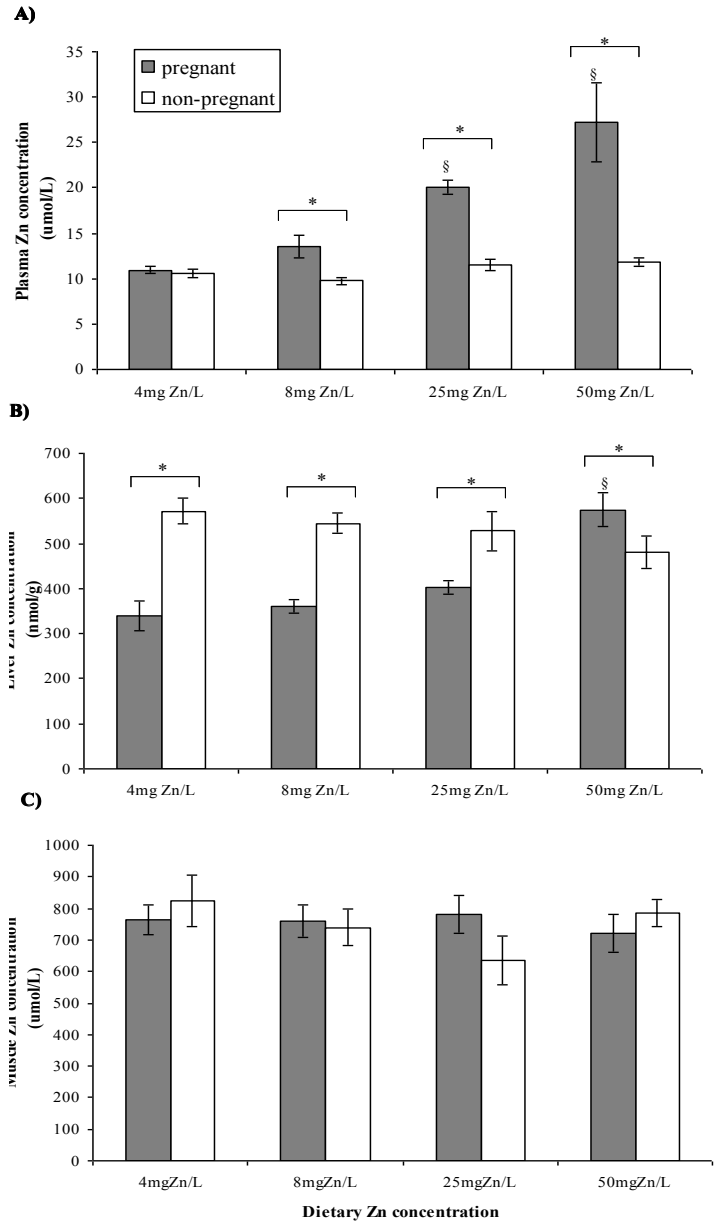

Figure 4.1. A) Plasma, B) liver and, C) muscle Zn concentrations in pregnant dams fed various dietary Zn levels throughout pregnancy (GD 0-18), and in non-pregnant dams fed the same diets for 18 days. Values represent means ± SEMs. §Significantly different from all other pregnant treatment groups, $p < 0.05$; *Significantly differences between the pregnant and non-pregnant groups on the same dietary Zn level , $p < 0.05$.

There was no effect of dietary Zn level on liver Zn concentration in non-pregnant mice. However, in pregnant dams, liver Zn concentrations was higher in those fed the 50 mg Zn/ L diet compared to the other groups ($p < 0.05$). While liver Zn concentration was significantly higher in non-pregnant mice fed the 4, 8 or 25 mg Zn/ L diets than pregnant dams on the corresponding diet, at 50 mg Zn/ L they were lower than those in pregnant dams ($p < 0.05$).

4.3.2 Ethanol dosage and pregnancy outcomes (preliminary study)

Pregnant dams fed liquid diets containing 0, 20, 27 or 35 % EDC throughout pregnancy were either killed on GD 18 (i.e. fetal parameters) or delivered their offspring (i.e. postnatal growth and survival). The fetal and postnatal data from these dams are shown in Table 4.2.

On GD 18, there were no differences in litter-size or percentage of fetal resorptions between treatment groups. However, fetuses from dams exposed to diets containing ethanol had significantly lower body weights compared to control fetuses. Furthermore, fetuses exposed to the 27 % and 35 % EDC diets were lighter than those exposed to the 20 % EDC diet ($p < 0.05$). Placental weights were lower in the 20 and 27 % EDC groups compared to the control group ($p < 0.05$) (Table 4.2).

When pregnant dams were allowed to deliver their offspring, there was a significant effect of ethanol on litter-size observed at birth, with the 35 % EDC group having a smaller average litter-size than the 20 and 27 % EDC groups ($p < 0.05$). While 25 and 15 % of offspring died between birth and PD 40 in the 20 and 27 % EDC groups respectively, 100 % of offspring exposed to the 35 % EDC died during this period (found either dead at birth or died within the first 2 days after birth). In addition, 2 pregnant dams had to be killed due to bad health. It was therefore concluded that the 35 % EDC liquid diet was too high to produce sufficient numbers of viable offspring for the behavioural study. There were no differences in the postnatal growth of offspring (i.e. weights at PD 3, 9, or 15) between the 20 and 27 % EDC groups. However, males in the 27 % EDC group had lower body weights than those in the 20 % EDC group ($p < 0.05$). The 27 % EDC liquid diet was selected from these results for the behavioural experiments, as this level of ethanol was shown to affect the fetus (i.e. weight) without significantly compromising the number and survival of offspring after birth (Table 4.2).

4.3.3 BAC and plasma Zn timeline: 27 % EDC liquid diet

BAC‟s and plasma Zn concentrations of pregnant mice fed a 27 % EDC liquid diet from GD 0-14 are shown in Figure 4.2. BAC‟s were significantly higher at 10 pm (peak levels 0.24 g/100ml) compared to concentrations at 11 am and 4 pm ($p < 0.05$). Plasma Zn concentrations did not differ at any of these time-points.

Table 4.2. GD 18 fetal and postnatal data from dams fed various dosages of ethanol throughout pregnancy

	Ethanol dosage (% ethanol derived calories)[a]			
	0 %[b]	20 %	27 %	35 %
GD 18 fetal data				
No. litters	5	5	3	2
No. fetuses	35	30	21	16
Average litter-size[d]	7.0 ± 1.2	6.0 ± 1.3	7.0 ± 1.2	8 ± 0.0
No. resorptions	4	7	3	4
% resorptions/per litter[d]	12.2 ± 5.6	15.4 ± 5.4	11.6 ± 5.8	19.2 ± 8.1
Fetal weight (mg)[c]	1116.0 ± 18.3	970.3 ± 9.6 *	904.7 ± 16.5 *†	865.0 ± 29.2 *†
Placental weight (mg)[c]	74.0 ± 2.9	53.2 ± 2.6*	56.9 ± 3.2*	63.8 ± 2.2
Postnatal data				
No. litters	-	5	4	4
No. offspring	-	31	26	13
Average litter-size at birth[d]	-	6.2 ± 0.7	6.5 ± 0.9	3.3 ± 0.8 †#
No. dead/total offspring (%) (PD 0- 40)	-	25	15	100
Offspring weight (g)[e] - PD 3	-	1.7 ± 0.09	1.8 ± 0.15	**No surviving pups**
- PD 9	-	4.4 ± 0.25	4.2 ± 0.35	-
- PD 15	-	7.0 ± 0.21	6.5 ± 0.52	-
- PD 40 (Female)	-	16.8 ± 0.7	16.7 ± 0.5	-
(Male)	-	20.6 ± 0.5	18.4 ± 0.6	-

[a]All groups were fed a control diet (0 % ethanol derived calories (EDC)) from GD 0-5, then placed on their allocated ethanol liquid diets (0, 20, 27 or 35 % EDC) from GD 6-18.

[b]Control treatment group data (i.e. 0 % EDC) was obtained from the previous preliminary study (8mgZn/L control liquid diet) for comparisons for GD 18 data.

Values represent the means ± SEM were applicable.

[c]Data analysed by one-way ANOVA, with Tukeys post-hoc test.

[d]Non-parametric data analysed by Mann-Whitney U test.

[e]Data analysed by GLM, with Tukeys post hoc test.

*Significantly different from 0 % EDC group, $p < 0.05$; †Significantly different from 20 % EDC group, $p < 0.05$; # Significantly different from 27 % EDC group, $p < 0.05$

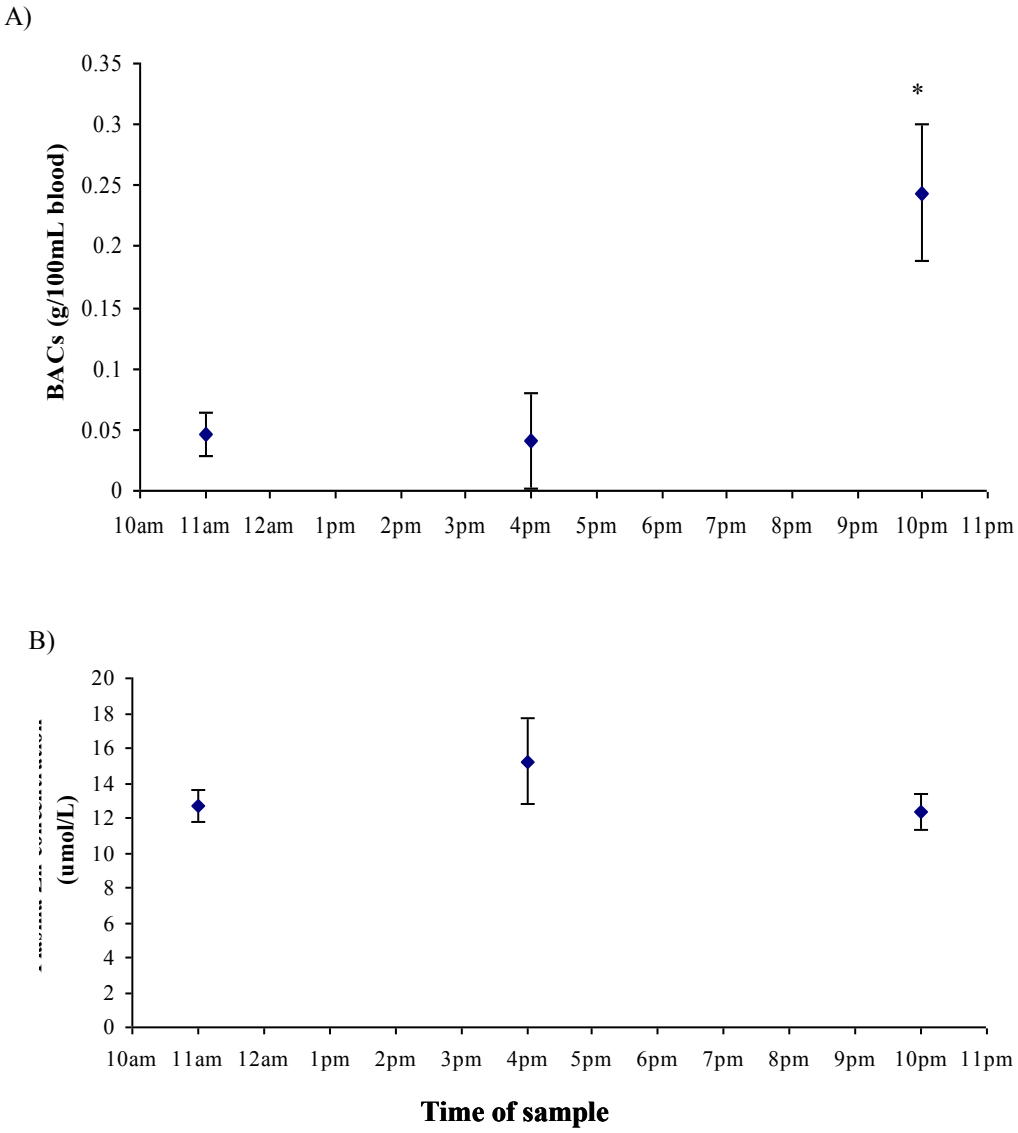

Figure 4.2. A) Blood alcohol concentrations (BAC‟s) and B) Plasma Zn concentrations of pregnant mice fed a 27 % EDC liquid diet from GD 0-14. Blood was collected at 3 time-points on GD 14. Points represent the means ± SEM (n = 3). *BAC is significantly different from other time-points, $p < 0.05$).

4.3.4 Chronic ethanol behaviour study: diets, postnatal growth and survival

The average daily volume of diet consumed by mice from each treatment group throughout pregnancy is shown in Figure 4.3. There were no differences between groups in the average consumption of the control liquid diet from GD 0-5. However, pregnant dams allocated the 27 % EDC liquid diet from GD 6-17 consumed significantly less diet during this period (average 15.5 ± 0.3 ml/ day) compared to dams on the other diets (control: 18.5 ± 0.4, control + Zn: 18.7 ± 0.4, ethanol + Zn: 17.5 ± 0.5 ml/ day) ($p < 0.05$). Maternal weight gains during pregnancy were similar between treatment groups (i.e. average 21 ± 2.2 % increase from GD 0 weight at GD 10).

There was no effect of ethanol on gestational length, with all treatment groups having an average gestational period of 19 days. The litter-size at birth and postnatal body weights of offspring are shown in Table 4.3. Dams fed the ethanol liquid diet alone throughout pregnancy had a smaller litter-size at birth compared to those fed the control + Zn supplemented diet ($p < 0.05$). There were no differences between treatment groups in postnatal growth of offspring, with offspring from all treatment groups displaying similar body weights at PD 3, 14 and 21 (Table 4.3). The cumulative survival probability (from birth until PND 40) of offspring from each treatment group is presented in Figure 4.4.

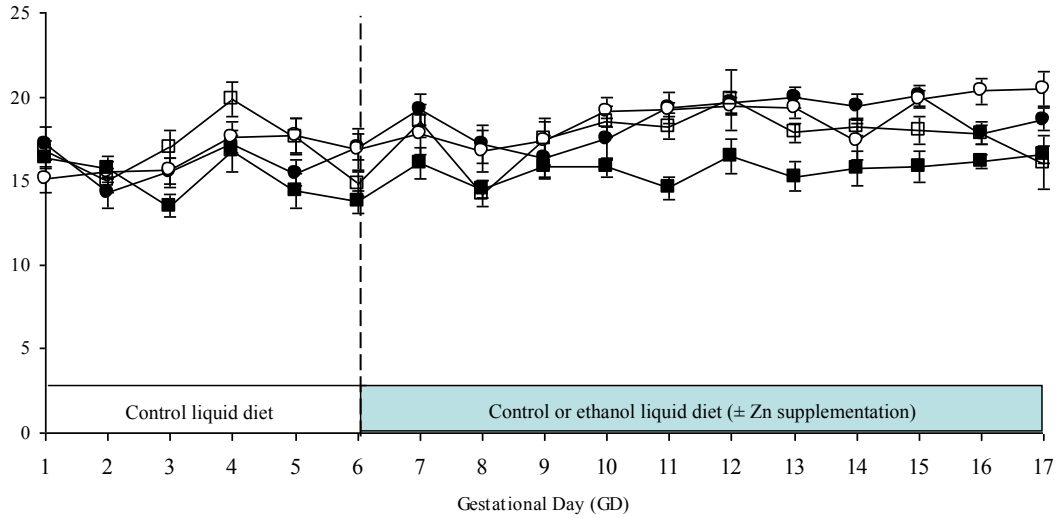

Figure 4.3. Average daily consumption of liquid diets by pregnant dams throughout pregnancy. All groups were fed a control liquid diet (8 mg Zn/ L) from GD 0-5, then fed their allocated control or ethanol liquid diets, with or without Zn supplementation, from GD 6-17 (Control (●), ethanol (■), control + Zn (○) or ethanol + Zn (□)). Data is present as means ± SEM.

Table 4.3. Postnatal measures of offspring exposed to ethanol ± dietary zinc supplementation throughout pregnancy

	Diet			
	control	**control + Zn**	**ethanol**	**ethanol + Zn**
No. of pregnant dams placed on diet	33	35	44	28
No. of dams with litters (n =)	15	17	22	13
Litter–size at birth[a]	5.0 ± 0.5	6.0 ± 0.3	4.8 ± 0.3*	4.8 ± 0.6
Body weight (g) [b] - PD 3	1.7 ± 0.09	1.7 ± 0.07	2.0 ± 0.06	1.9 ± 0.2
- PD 14	6.4 ± 0.2	6.0 ± 0.2	6.4 ± 0.2	6.6 ± 0.5
- PD 21	8.4 ± 0.4	8.0 ± 0.3	8.2 ± 0.4	9.0 ± 0.6

All mice were fed a control liquid diet (0 % ethanol derived calories (EDC), 8 mg Zn/L) from GD 0-5, then placed on their allocated diets from GD 6-18 (ethanol diets = 27 % EDC; Zn supplemented diets = 50 mg Zn/L)

Values represent the means ± SEM where applicable.

[a]Non-parametric data analysed by Mann-Whitney U test.

[b]Data analysed by GLM, with Tukeys post-hoc test.

*Significantly different from control + Zn supplemented group, $p < 0.05$.

There was a greater risk of stillbirths in the ethanol-treated groups (13 %), compared with 8 and 1 % for the control and control + Zn groups respectively. It is important to note that the pups found dead at birth in the control group were all from the same litter (possibly as a result of maternal complications). Following birth, the period with the greatest risk of mortality for all groups occurred in the first three days of life, with 5 % of offspring from each of the control groups and 10 % of offspring from the ethanol groups dying between PD 0 and 3 (Figure 4.4). By PD 40, the cumulative postnatal mortality was greater in the ethanol groups, with 28 % (ethanol) and 24 % (ethanol + Zn) of offspring surviving over this period, compared to 15 % (control) and 10 % (control + Zn) in mice fed the control diets. The number of litters affected by neonatal mortality (i.e. the cumulative percentage of litters with one or more dead pups) for all groups were at birth (control: 6, control + Zn: 6, ethanol: 33, ethanol + Zn: 30 %) and by PD 40 (control: 33, control + Zn 24, ethanol: 58, ethanol + Zn: 38 %).

Following the examination of all offspring for physical abnormalities, a small number of mice were eliminated from behavioural testing due to eye defects such as microphthalmia and anophthalmia (ethanol 11 %, control 6 %, Zn supplemented groups 0 %). These abnormalities had the potential to impair their ability to perform in the spatial learning and memory task which require intact vision. No other obvious physical abnormalities were observed

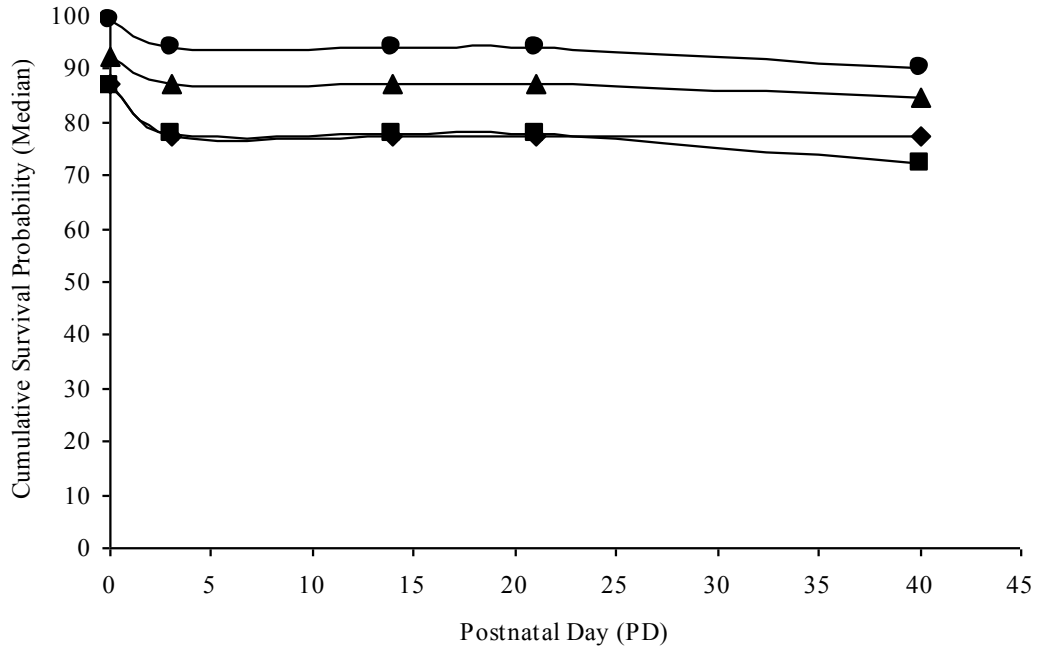

Figure 4.4. The effect of chronic ethanol exposure and/or dietary Zn supplementation throughout pregnancy on the postnatal survival probability of offspring. Control (8 mg Zn/L) (▲), control + Zn supplementation (50 mg Zn/L) (●), ethanol (27 % ethanol derived calories (EDC), 8 mg Zn/L (■), ethanol + Zn supplementation (27 % EDC, 50 mg Zn/L) (♦). N= 62-112 offspring per treatment at birth.

The mice randomly selected for the maze study were weighed on PD 50, prior to behavioural testing. Offspring exposed to the ethanol diet throughout pregnancy had significantly lower body weights than control offspring, but did not differ from either of the Zn-supplemented groups (control: 21. 9 ± 0.6, control + Zn: 21.1 ± 0.5, ethanol: 19.6 ± 0.6, ethanol + Zn: 21.1 ± 0.6, $p < 0.05$).

4.3.5 Chronic ethanol behaviour study: Spatial learning and memory

The escape latency and correct trials scores for each of the 5 d of spatial learning are shown in Figure 4.5. Mice from all treatment groups learnt the cross-maze escape task, indicated by a reduction in the time taken to find the platform position (Fig 4.5.B) and an increase in the number of correct trials across the period of training (Fig 4.5.A). There were no statistically significant differences between treatment groups in the escape latency scores or the number of correct trials over the 5 d of training (i.e. no significant treatment × day interaction), indicating that mice from all treatment groups learnt the task to the same level by the final day of testing.

The correct trials and escape latency scores for spatial memory (tested at 12 and at 28 d after mice learnt the initial platform position) are shown in Figure 4.6. Analysis of correct trial data using logistic regression models demonstrated that there were no significant effects of treatment on spatial memory performance at 12 d (Figure 4.6.A).

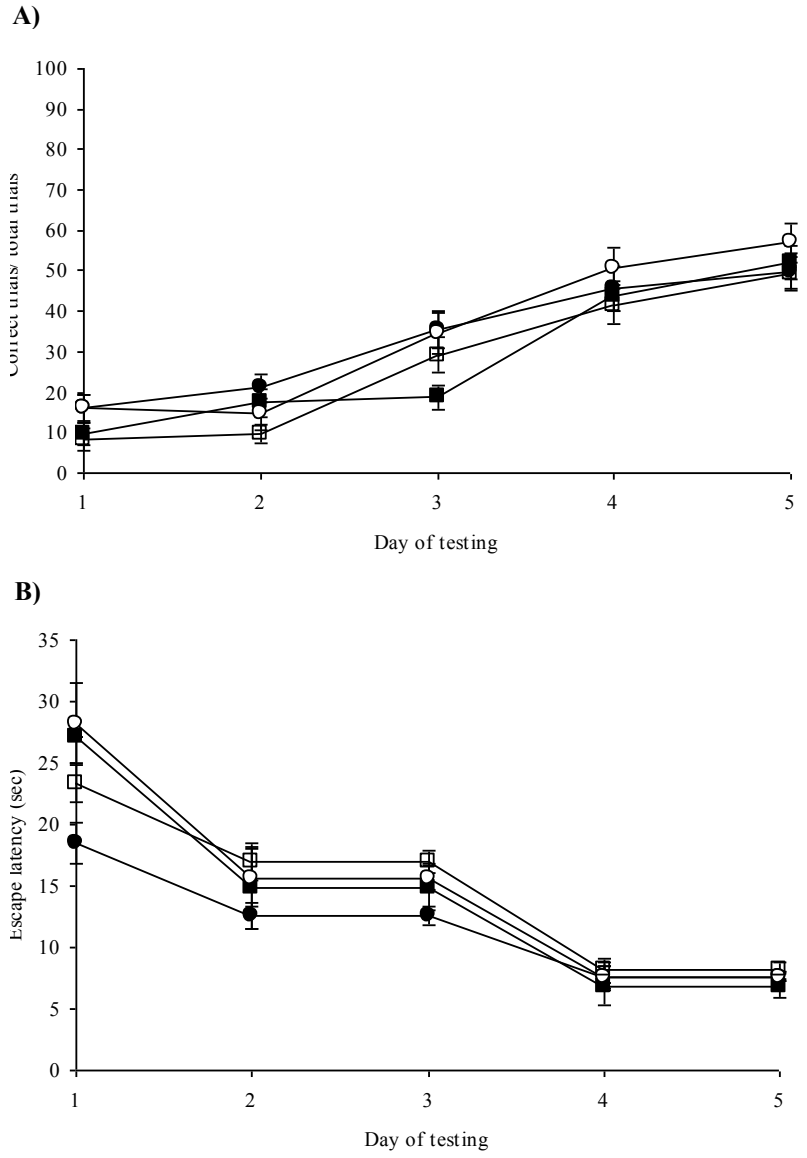

Figure 4.5. Spatial learning: The effect of treatment on correct trials and escape latency. Correct trials (A) and escape latency (B) data over 5 d of spatial learning testing for offspring exposed to a control or ethanol (27 % EDC) liquid diet from GD 6-18, with or without dietary Zn supplementation (Control (●), ethanol (■), control + Zn (○) or ethanol + Zn (□)) on GD 8. Dietary Zn supplementation was given from GD0-18. Correct trials are presented as predicted proportions (i.e. the number of correct trials/total observations for each treatment) ± SEM. Escape latency is presented as means ± SEM. Each point is the treatment groups mean of the sum of six daily trials for each mouse (n=24/treatment).

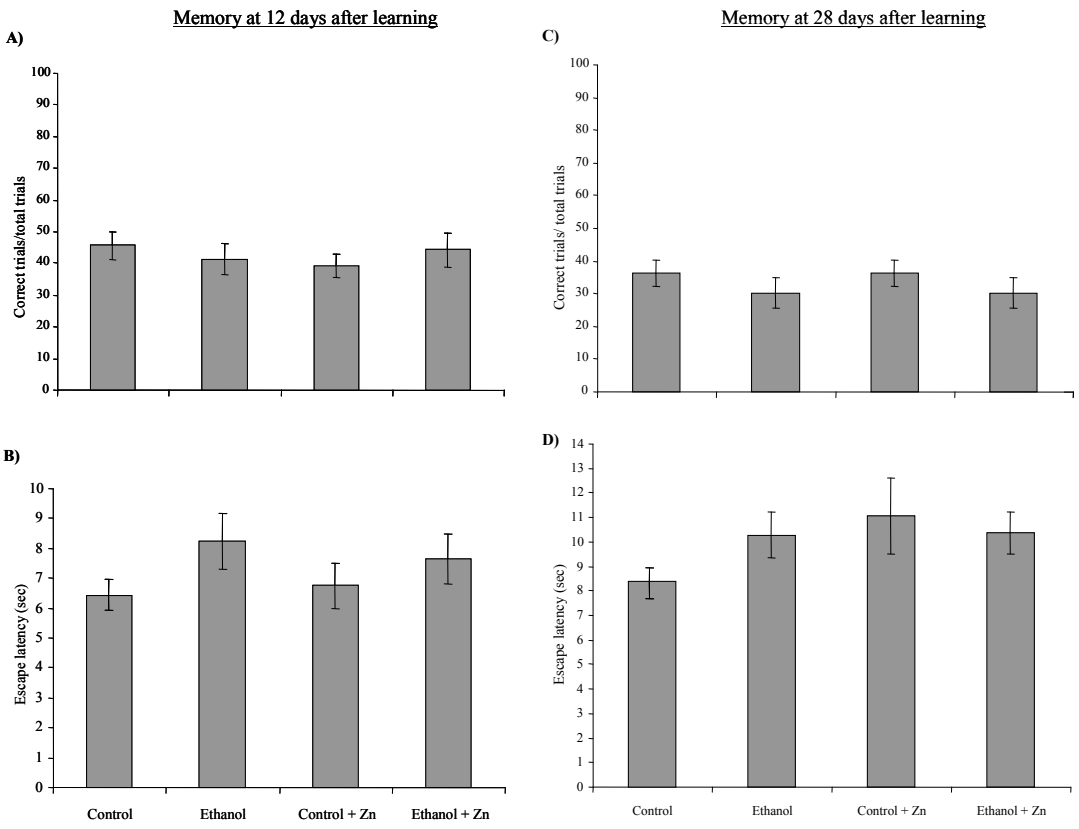

Figure 4.6. The effect of diet on spatial memory tested at 12 d and at 28 d after mice learnt an escape task. Correct trial (A and C) and escape latency (B and D) data for spatial memory testing of offspring exposed to a control or ethanol (27 % EDC) liquid diet from gestational day 6-18, with or without dietary Zn supplementation. Correct trials are presented as predicted proportions (i.e. the number of correct trials/total observations for each treatment) ± SEM. Escape latency is presented as means ± SEM. N=23-24/treatment.

In addition, there were no differences in the escape latency between treatment groups (Figure 6B).

When spatial memory was tested 28 d after training, there was also no effect of treatment on the number of correct trials or escape latency, with offspring from all treatment groups performing to the same level in the cross-maze escape task (Fig 6C, D).

Following the conclusion of this study, brain weights were obtained from all mice tested in the cross-maze task (control: 0.42 ± 0.007, ethanol: 0.43 ± 0.005, control + Zn: 0.40 ± 0.007, ethanol + Zn: 0.41 ± 0.009 g) and expressed as a ratio of their body weight. There were no significant differences in brain weight/ body weight between treatment groups (control: 0.016 ± 0.0005, ethanol: 0.017 ± 0.0006, control + Zn: 0.017 ± 0.0005, ethanol + Zn: 0.017 ± 0.0006).

4.4 DISCUSSION

The objective of this chapter was to examine whether variable chronic ethanol exposure from GD 6-17 could affect postnatal outcomes in mice offspring, in particular spatial learning and memory, and to determine whether dietary Zn supplementation could prevent adverse outcomes caused by ethanol. However, in the present study ethanol did not impair spatial behaviour in adult offspring, as demonstrated by the fact that offspring from dams exposed to a liquid ethanol diet

throughout gestation performed to the same level as control offspring for all parameters measured in the cross-maze escape task. Therefore, the potential influence of dietary Zn supplementation on this particular behavioural measure could not be examined.

The absence of an effect of ethanol on spatial behaviour in this study is difficult to compare with previous findings, as all of the studies that have demonstrated chronic ethanol exposure during pregnancy impairs spatial learning and memory have been performed in rats, not mice (Reyes et al., 1989; Gianoulakis, 1990; Zimmerberg et al., 1991; Kim et al., 1997; Matthews and Simson, 1998; Gabriel et al., 2002; Christie et al., 2005; Popovic et al., 2006). Thus, the dosage of ethanol required in the diet to impair spatial behaviour in a mouse model has not been documented. In the majority of rat studies, the ethanol dosage most consistently shown to impair spatial behaviour in offspring is 35 % EDC (which can achieve average BAC‟s of 0.18 ± 0.05 g/ 100ml) (Christie et al., 2005). However, our preliminary studies demonstrated that when mice were fed a 35 % EDC diet throughout pregnancy, it was extremely toxic to both the mothers and offspring (i.e. BAC‟s ranging up to 0.4 g/100ml), resulting in poor maternal health, a high incidence of fetal abnormalities, small litter sizes at birth and a 100 % neonatal mortality rate. This difference in the ethanol response observed between species is most likely due to differences in ethanol metabolism (i.e. mice show a rapid rise and elimination rate and significantly higher BAC‟s, whereas rats show more gradual profiles but retain the ethanol in the blood stream for longer (Livy

et al., 2003)) and indicates that lower ethanol dosages need to be applied in mice to ensure sufficient offspring survival for behavioural testing. However, while employing a 27 % EDC diet in this study did ensure sufficient survival of offspring, it did not affect spatial learning and memory. Considering not all offspring in each litter are affected by prenatal ethanol exposure, it is possible but unlikely that the mice randomly selected for behavioural testing happened to be non-impaired. A more likely explanation however, is that the BAC‟s achieved in the pregnant dams were not elevated sufficiently to affect spatial behaviour (as demonstrated in our previous studies where spatial memory was impaired in offspring exposed to a binge of ethanol in pregnancy) (Summers et al., 2006, Chapter 2). The dosage of ethanol injected intraperitoneally in these studies increase BAC‟s by 0.35 g/100ml 0.5 h after each injection and decline 0.1 g/100ml/h thereafter (Carey et al., 2000a). Although BAC‟s were not measured in the current behavioural study, dams fed the control ethanol diet throughout pregnancy consumed approximately 11-16 % less daily than the other treatment groups and therefore consumed less ethanol than could potentially be attained on this diet. Despite the fact the cross-maze escape task was not able to detect spatial impairments at these levels, other behavioural impairments have been demonstrated in C57 mice at lower ethanol dosages (i.e. 20-25 % EDC) throughout pregnancy, including increased locomotor activity (hyperactivity), deficits in different appetitive and aversive learning and memory paradigms (e.g. impairments in passive and shuttle avoidance and operant performance) and operant conditioning tasks (Randall et al., 1985; Gentry and Middaugh, 1988; Becker and Randall, 1989;

Middaugh et al., 1992; Middaugh and Gentry, 1992). In addition, there are a wide range of behavioural processes that are also known to be affected by prenatal ethanol exposure, such as attention, motor control and forms of learning and memory (e.g. object recognition memory) (Riley, 1990; Streissguth et al., 1994; Mattson and Riley, 1998; Mattson et al., 2001; Garcia-Moreno et al., 2002; Popovic et al., 2006). Thus, it would be beneficial to repeat and extend this research with the addition of a wider range of behavioural testing procedures, in order to increase the likelihood of demonstrating ethanol-related behavioural impairments in our mouse model and to then demonstrate whether dietary Zn supplementation throughout pregnancy is protective.

In addition to spatial learning and memory, the present study also examined the effect of chronic ethanol exposure throughout pregnancy on postnatal weights (i.e. growth) and survival. While exposure to the 27 % EDC diet throughout pregnancy decreased fetal weight in the preliminary studies, the body weights of offspring from mice maintained on this diet in the behavioural study did not differ from control or Zn-supplemented offspring near birth (i.e. PD 3). In addition, there was no effect of prenatal ethanol exposure on postnatal growth between birth and PD 21 (weaning). This would indicate that offspring were not affected by the fact that the ethanol group consumed less diet (and consequently less nutrients) between GD 6 and 18 than the other groups. Our findings are consistent with previous studies which used a similar ethanol dosage (25 or 27.5 % EDC) in C57BL/6J mice during pregnancy (Middaugh

et al., 1988; Middaugh and Boggan, 1995). An interesting finding from these earlier studies was that although birth-weight was not affected when ethanol exposure was terminated one day before birth (as was done in the present study), continuing ethanol exposure until birth resulted in a 10 % decrease in birth-weight compared to controls, indicating that recent ethanol exposure might be a necessary condition for birth weight reduction (Middaugh and Boggan, 1995). In addition, despite normal growth after birth, they found that ethanol attenuated peri-adolescent growth, with reduced body weights observed in offspring on PD 35 that persisted into adulthood. This phenomenon is reflected in the present study, where a cohort of offspring from the ethanol group that were tested for spatial impairments had significantly lower body weights than control offspring on PD 50 (although they were not different from Zn-supplemented offspring). Similarly, growth deficits have been noted in prenatal ethanol-exposed 4 year-old children with normal birth-weight (Day et al., 1990). Reductions in body weight beginning after weaning have been suggested to indicate that ethanol-exposed offspring are not physically mature enough to engage in behaviours required for food regulation or ethanol may affect systems involved with food and weight regulation (Middaugh et al., 1988).

Chronic ethanol exposure during pregnancy was demonstrated in this study to increase the risk of postnatal mortality. The percentage of pups found dead at birth in the ethanol groups (i.e. 13 %) was identical to those demonstrated by another research group that used 25 % EDC in C57BL/6J mice. However, the cumulative

percentage of offspring mortality after birth in our study (28%) was not as high as previously reported (47 %) (Middaugh et al., 1988). The increase in postnatal mortality for the ethanol group appears to be unrelated to poor postnatal nurturance. Firstly, the fact that mice from all treatment groups had similar weights after birth would suggest that maternal nurturance was consistent across all litters. Secondly, the relationship of total offspring mortality to the number of litters affected also suggests that the increased mortality was due to the ethanol effect on the pup, rather than the dam. For example, on PD 3, 22 and 23 % of the offspring exposed to ethanol (i.e. ethanol and ethanol + Zn groups respectively) were dead compared with 50 and 38 % of the litters affected in these groups. This difference suggests that ethanol only affected some mice within a litter rather than entire litters, which would be more indicative of poor maternal care. In comparison to our findings in Chapter 2 which demonstrated that Zn supplementation prevents postnatal mortality caused by binge ethanol exposure in early pregnancy, Zn supplementation did not appear to prevent mortality caused by chronic ethanol exposure. While the effects of having ethanol in the maternal blood circulation for the majority of pregnancy may be too severe for Zn supplementation to be beneficial, this could also suggest that the mortality caused by chronic ethanol exposure is not mediated by a Zn-related mechanism.

Findings from our preliminary study indicated that on GD 14, peak BAC‟s observed at 10:00 pm were not accompanied by changes in plasma Zn concentrations in pregnant mice fed a liquid ethanol diet. However, only 3 time-points were measured

over 24 hours and therefore alterations in Zn may have occurred between these time-points (most notably after the peak BAC). In addition, we only examined this relationship between BAC‟s and plasma Zn on a single day of pregnancy. As the basal level of plasma Zn and liver MT naturally change throughout the course of pregnancy, it is possible that MT-induced alterations in Zn metabolism may only be affected by ethanol exposure at specific stages of gestation, for example organogenesis, at which binge ethanol exposure has been shown to significantly induce MT and decrease plasma Zn (Carey et al., 2000b). MT is lowest at GD 8, increases significantly (peaking at GD 14-15) and then decreases during late gestation and therefore the effect of induction of MT may be greatest at GD 8. In support of this concept, we have previously shown that hepatic MT concentrations at GD 12 are naturally higher than those attained following ethanol treatment and therefore ethanol may not have the capacity to further induce liver MT in the dam and alter Zn homeostasis as in GD 8 (Carey et al., 2000a). In addition, an earlier study demonstrated that ethanol consumption did not affect maternal-fetal Zn transfer on GD 14 (Zidenberg-Cherr et al., 1988). Further research is therefore required to examine the effect of ethanol at various stages of pregnancy on Zn distribution and MT expression.

While we were unable to demonstrate any influence of dietary Zn supplementation on ethanol-related outcomes in this study, we were able to demonstrate the effect of various dietary Zn levels (including Zn supplementation) throughout pregnancy on

tissue Zn distribution, fetal and postnatal outcomes in the normal setting (i.e. without ethanol exposure). An interesting finding was that while the level of Zn in the diet did not affect plasma Zn concentrations in non-pregnant mice, in pregnant mice on GD 18, plasma Zn concentrations were increased in accordance with the level of dietary Zn (i.e. 4 mg Zn/L < 8 mg Zn/L < 25 mg Zn/L <50 mg Zn/L). This difference in response most likely reflects an increase in Zn absorption (i.e. Zn transporters) in pregnant mice which have been demonstrated to occur late in gestation/early lactation to cope with the increased requirement of Zn for the fetus (Davies and Williams, 1977a; Fung et al., 1997; Liuzzi et al., 2003), and is important to consider when comparing Zn levels between pregnant and non-pregnant mice. Pregnancy status and dietary Zn levels also affected liver Zn concentrations, as liver Zn were increased at the highest dietary Zn level (50 mg Zn/L) from normal in the pregnant but not non-pregnant mice. These findings confirm that Zn supplementation using the liquid diet is sufficient to enhance the uptake of Zn into the maternal circulation as required for Zn treatment. However, the 50 mg Zn/L diet was only shown to increase fetal Zn concentrations in comparison to the 4 mg Zn/L diet (not the control 8 mg Zn/L or 25 mg Zn/L diets), indicating that in normal pregnancy (i.e. without insults that influence maternal/fetal Zn distribution) fetal Zn transfer is reasonably well regulated despite changes in dietary Zn intake. While the consumption of a high Zn diet throughout pregnancy decreased placental and fetal weight compared to controls, it

did not demonstrate any negative effects on the mother, litter-size, physical abnormalities[5], birth weight, postnatal growth, mortality or spatial impairments in the main behavioural study. Thus, it appears the level of Zn used in the Zn-supplemented diet was not toxic to the mother or fetus or have any long-lasting effects on development. However, it also should be noted that there were also no further benefits of Zn supplementation in a normal pregnancy setting, as Zn-supplemented offspring did not differ from control offspring in any of the parameters measured. Thus, a healthy pregnancy can be achieved as long as the recommended daily Zn intake is maintained during pregnancy.

4.5 SUMMARY

In summary, the present study demonstrated that chronic ethanol exposure (27 % EDC liquid diet) from GD 6-17 in C57BL/6J mice elevated postnatal mortality. While surviving offspring from dams fed the ethanol diet displayed normal birth-weights and postnatal growth until weaning, they had reduced body weights in adulthood (PD 50). However, spatial learning and memory were not affected by prenatal ethanol exposure in these offspring. Most importantly, dietary Zn supplementation did not appear to prevent the adverse postnatal outcomes caused by long-term ethanol consumption during pregnancy (i.e. mortality, PD 50 weight).

[5] While the incidence of physical abnormalities observed in fetuses exposed to the 4, 25 and 50 mg Zn/L throughout pregnancy were higher than those observed in the 8 mg Zn/L group (which did not cause any abnormalities), they were similar to the basal incidence of abnormalities observed in C57BL/67 mice.

Further studies are required to demonstrate a wider range of postnatal impairments caused by chronic ethanol exposure during pregnancy than were assessed in this present study (i.e. behaviour, brain, heart, skeletal, urogenital systems) so that the influence of Zn supplementation on ethanol-related outcomes can be more broadly examined. In addition, studies are necessary to increase our understanding of the effect of chronic ethanol consumption on maternal and Zn distribution and the pathways involved in mediating teratogenicity.

CHAPTER *5*

5.1 SUMMARY

The consumption of ethanol during pregnancy is associated with a range of costly negative outcomes including pre- and post-natal mortality, growth retardation, physical abnormalities and neurobehavioural impairments. While earlier research in this area predominantly focussed on how chronic ethanol exposure throughout pregnancy affects the fetus, short-term or binge-like episodes of ethanol consumption can also have severe consequences if the insult occurs during a critical period of fetal development. Organogenesis, which encompasses weeks 3-9 in human gestation, is a particularly sensitive period to ethanol exposure. It is therefore possible that women can unknowingly expose the developing fetus to teratogenic insult, not knowing they are pregnant.

Considering the wide spectrum of organs and systems affected and abnormalities caused by prenatal ethanol exposure, it is likely that there are a number of causative factors underlying ethanol teratogenicity. However, a large number of studies including recent work in our laboratory have focussed on a mechanism whereby the effects of ethanol are mediated by alterations in maternal-fetal Zn homeostasis. Changes in Zn homeostasis following ethanol exposure in early pregnancy are caused by the inappropriate expression of MT in the maternal liver. This leads to a net movement of Zn from the plasma into the liver (where it is sequestered by MT),

resulting in a significant decrease in the maternal plasma Zn concentration. As Zn is an essential component for a multitude of proteins and enzymes involved in processes that underlie fetal growth and development, a restriction in the fetal Zn supply from the maternal plasma can result in a range of adverse fetal outcomes. The main evidence supporting the involvement of MT, maternal plasma Zn concentration and fetal Zn deficiency in mediating ethanol teratogenicity is that ethanol exposure in MT+/+ dams in early pregnancy significantly decreases the maternal plasma Zn concentration and fetal Zn content, resulting in increased fetal abnormalities. In comparison, in MT-/- dams, ethanol has the opposite effect, leading to an increase in plasma Zn levels (due to release of Zn from the muscle and skin stores) and the absence of fetal abnormalities (Carey et al., 2000a; Carey et al., 2000b). As the high plasma Zn levels appear to be the critical factor in protecting MT-/- fetuses from ethanol teratogenicity, then limiting the decrease in plasma Zn in MT+/+ dams has been postulated to reduce the teratogenic effect of ethanol. Our previous findings that enhancing plasma Zn levels via subcutaneous Zn treatment at the time of ethanol exposure on GD 8 prevents physical abnormalities and spatial memory impairments in offspring is supportive of this concept (Carey et al., 2003b; Summers et al., 2006). This thesis extended research on this hypothesis, by focussing on the potential influence of dietary Zn supplementation during pregnancy (a readily available and less invasive form of Zn treatment) on a range of ethanol-related outcomes in binge-drinking and chronic ethanol rodent models.

Chapter 2 of this thesis focussed on the most obvious adverse fetal outcomes caused by early ethanol exposure, which are the physical birth abnormalities (including craniofacial dysmorphology, growth deficiency and cardiovascular defects) and in extreme cases prenatal or postnatal mortality. These outcomes are generally perceived as the severe end of the spectrum of prenatal ethanol effects. However, our findings support previous studies demonstrating that even a single binge of ethanol on GD 8 in rodents can increase the incidence of physical abnormalities in offspring (including microphthalmia, anophthalmia, limb defects and haemorrhaging). While ethanol exposure on GD 8 did not affect birth weight or postnatal growth (growth deficiency being one of the features of FAS), it surprisingly increased the incidence of postnatal mortality in offspring, an outcome that is more commonly associated with chronic ethanol exposure (Lopez-Tejero et al., 1986; Middaugh et al., 1988; Middaugh and Boggan, 1995). Such a severe outcome highlights the harmful effects of binge drinking during pregnancy and suggests that ethanol exposure during the period of organogenesis can alter the development of other internal structures critical for postnatal development that were not investigated in this study (e.g. cardiovascular system, respiratory system). More importantly however, we demonstrated that dietary Zn supplementation throughout pregnancy prevented the physical abnormalities and postnatal mortality caused by early ethanol exposure.

With respect to our hypothesis, averting the decrease in maternal plasma Zn concentrations caused by acute ethanol exposure has been indicated as the means for

reducing ethanol teratogenicity (Seyoum and Persaud, 1995; Carey et al., 2003b; Summers et al., 2006). However, plasma Zn concentrations are generally well regulated despite changes in dietary Zn intake and it has previously been suggested in a publication that dietary Zn supplementation is unlikely to raise maternal plasma Zn concentrations to an extent comparable to the non-physiological levels attained following subcutaneous Zn treatment (Carey et al., 2003b). While this suggestion was confirmed in our findings in Chapter 2, dietary Zn supplementation throughout pregnancy did significantly increase basal plasma Zn levels and plasma Zn levels following ethanol exposure on GD 8 to a physiological level that was sufficient to prevent the effects of ethanol on fetal development (i.e. by preventing the decrease in plasma Zn concentrations caused by ethanol and/or activating other molecular pathways that could reverse or inhibit ethanol damage).

While physical impairments are a key feature of FAS, behavioural impairments are more commonly associated with prenatal ethanol exposure (i.e. FAS and other ethanol-related disorders without the appearance of physical defects). Ethanol-related behavioural impairments, such as learning, memory, attention, hyperactivity and motor coordination can be modelled in rodent models using various cognitive tasks. In Chapter 3, a major strength of our study was that we developed a comprehensive screening protocol so that all animals could be examined for physical and behavioural defects that could influence their performance in the major outcomes investigated, spatial learning and memory and object recognition memory. Thus, we

not only could determine the effect of ethanol on the outcomes measured during screening but in comparison to other studies, could also eliminate the potential influence of underlying impairments in the learning and memory tests. We did not observe any effect of GD 8 ethanol exposure on growth, visual, sensory, anxiety, motor or exploratory function during screening. However, similar to our previous study (Summers et al., 2006), we demonstrated that ethanol caused spatial memory impairments in adult offspring. In addition, we are the first group to demonstrate in mice that binge ethanol exposure causes impairments in object recognition memory (as previous studies used chronic ethanol exposure) (Garcia-Moreno et al., 2002; Popovic et al., 2006). These findings reflect that while the major brain growth spurt occurs later in pregnancy, ethanol exposure in early pregnancy can impair neurodevelopmental processes in the fetus that manifest as long-term impairments. Furthermore, both spatial memory and object recognition memory impairments caused by ethanol were prevented by dietary Zn supplementation. These findings in the binge drinking model support the idea that fetal Zn deficiency is a mediator of ethanol-related outcomes assessed in this study and highlight the importance of Zn for healthy fetal development.

While dietary Zn supplementation is protective against the effects of binge-drinking in early pregnancy, its effect on chronic ethanol exposure was not as beneficial. The studies in Chapter 4 were the first occasion that we applied the chronic ethanol model during pregnancy as well as the first occasion that spatial memory has been

investigated in a chronic ethanol mouse model. Therefore the ethanol dosage and feeding routines required to impair spatial behaviour in offspring were not established and was examined as part of our studies. We were unable to demonstrate any effects on spatial learning and memory in offspring at the ethanol dosage tested (27 % EDC diet). It is possible that despite the continuous consumption of ethanol during pregnancy, the optimal blood alcohol levels obtained were not sufficient to impair brain development (as in the binge-drinking model (Dumas and Rabe, 1994; Minetti et al., 1996; Dunty et al., 2001; Summers et al., 2006)) or alternatively, the physiological mechanisms involved in binge vs chronic ethanol damage could be different. While we did demonstrate that chronic ethanol exposure throughout pregnancy increased postnatal mortality and decreased adult body weights (while birth and neonatal weight were not affected), Zn supplementation did not appear to be beneficial in preventing these outcomes. However, further studies investigating a broader spectrum of outcomes in this model will perhaps produce a better picture of the influence of dietary Zn supplementation.

5.2 CLINICAL APPLICATIONS

Abstaining from ethanol during pregnancy is the only means to completely prevent the occurrence of FASD and other ethanol-associated disorders. However, despite this message and the dangers of prenatal ethanol exposure being increasingly publicised in the media over the past few years, a disturbing number of women are continuing to consume alcoholic beverages during pregnancy (Centers for Disease

Control and Prevention, 2002a; Goransson et al., 2003; Kesmodel et al., 2003; Colvin et al., 2007). While it has long been perceived that problems only occur with chronic alcoholism, it is clearly becoming evident from animal studies that episodes of binge-drinking are also harmful to fetal development. The findings from this thesis could support public warnings that even a single binge-drinking episode at a time when women often are unaware of their pregnancy can have adverse consequences to the fetus. As there is no known safe level of ethanol, it is important that all methods of drinking should be avoided during pregnancy.

At present, dealing with the effects of FASD and other ethanol-related disorders only occurs once symptoms have been identified in children prenatally exposed to ethanol and programs only help affected individuals cope with the existing problems. Researchers have been working for over 20 years to understand the mechanisms of fetal ethanol teratogenicity so that pharmaceutical interventions may be developed. While there are currently no maternal therapies to prevent ethanol damage, studies have evaluated a number of therapies for the prevention of specific pathways involved in ethanol damage in model systems (including neuroprotective peptides (Spong et al., 2001; Zhou et al., 2004; Endres et al., 2005), long-chain alcohol 1-octanol (Chen et al., 2001), nicotinamide (Ieraci and Herrera, 2006) and prevention of ethanol-induced apoptosis (Heaton et al., 2004; Kilburn et al., 2006)), which support our findings that the fetus is amenable to treatment. In this thesis, we demonstrated in mice that dietary Zn supplementation during pregnancy ameliorated

all the adverse outcomes that were observed to result from binge drinking in early pregnancy, including physical birth defects, postnatal mortality and spatial memory and object recognition memory in adult offspring. In addition to its protective influence against a range of ethanol-related outcomes, an advantage of dietary Zn supplementation (in comparison to the other agents investigated) is that it is readily available and relatively easy to administer in the human setting. The results of this study could therefore have a positive impact on the future, with the potential for Zn supplementation as a therapy for lowering the rates of FASD and preventing developmental disabilities in many individuals. However, one would have to be careful in promoting any strategy such as this, as women may see it as an opportunity to continue drinking during pregnancy when they may have otherwise abstained.

Another potential benefit of dietary Zn supplementation is that it protects against other prenatal insults in addition to ethanol exposure in early pregnancy. In addition to the obvious benefit of sufficient Zn nutrition in preventing the effects of Zn deficiency on fetal development, we have also demonstrated that Zn supplementation can reduce birth abnormalities and behavioural alterations caused by infection in early pregnancy (Carey et al., 2003a; Chua et al., 2006). In these studies, mice were injected with LPS (lipopolysaccharide) on GD 8, which has been shown to mediate its effects via an MT-induced Zn deficiency in a similar manner to ethanol (Carey et al., 2003a). Many low socioeconomic communities in Australia are at risk of alcohol abuse, poor diet and health (infection) (Kildea and Bowden, 2000; Panaretto et al.,

2006), and while each insult is teratogenic, the occurrence of these together could further potentiate their effects on fetal development (examples (Ruth and Goldsmith, 1981; Dreosti and Fraser, 1984)). Thus, Zn supplementation would prove beneficial in these communities to minimise the effects of these insults on their offspring. Possible suggestions for applications in the wider community (to indirectly target women at risk) could be to fortify foods with Zn (as with omega-3 fatty acids and folic acid) or even alcoholic beverages. While we did not find any positive benefit of excess Zn in the diet in the normal setting, adequate Zn nutrition is required in all individuals for normal growth and development (King and Turnland, 1989; Vallee and Falchuk, 1993; Coyle et al., 1998). The fact that excess Zn has not generally been found to be harmful would suggest that it is safe to apply to all individuals. However its influence in combination with other metabolic factors is not known and further research is necessary. FASD leads to a lifetime of high economic and emotional costs and thus, the possibility of even reducing a fraction of these cases will have a significant impact on the individual and community.

5.3 FUTURE DIRECTIONS

There are a number of potential studies that could be performed in light of our hypothesis and the findings in this thesis.

In relation to the potential application of Zn supplementation as an intervention strategy, a question that is of great interest is whether supplementing the diet of dams

with Zn at later time after exposure to ethanol (or infection) during pregnancy can protect the fetus against teratogenicity. The possibility that Zn treatment could be beneficial even hours after the initial insult (possibly by repair mechanisms) would enhance the application of this treatment in the human setting. However, the fact that subcutaneous Zn treatment at the time of ethanol exposure prevents ethanol teratogenicity (Carey et al., 2003b; Summers et al., 2006), although not conclusive, would suggest that treatment may need to be applied at or near the time of the insult to prevent or limit the alterations in Zn homeostasis. While this is also likely for dietary Zn supplementation, in this thesis, mice were fed a Zn supplemented diet throughout all stages of pregnancy making it difficult to ascertain at what stage treatment prevented the adverse effects of ethanol. Our binge-drinking mouse model could be used to determine the time frame within which Zn supplementation exerts a positive effect.

In addition to the timing of Zn treatment, further research is necessary to determine at what stages during pregnancy ethanol alters maternal-fetal Zn homeostasis (which is also an important determinant of when Zn treatment will be beneficial). We have demonstrated that ethanol exposure during early pregnancy can induce liver MT, leading to fetal Zn deficiency and negative outcomes in the offspring (Carey et al., 2000a; Carey et al., 2000b; Carey et al., 2003b; Summers et al., 2006). However, whether this mechanism occurs at times later in pregnancy has not been investigated. The application of the binge-drinking mouse model at various stages throughout

gestation could therefore be used to address this issue. Studies using the chronic ethanol model are difficult to examine the effects of ethanol on the MT-induced Zn deficiency mechanism in late pregnancy, as there is the possibility that maternal Zn homeostasis adapts to continuous ethanol exposure, or alternatively it could affect other means of Zn regulation independent of our hypothesis (e.g. changes in intestinal Zn absorption, Zn release from tissues, urinary Zn excretion, placental transfer). These potential effects of long-term ethanol exposure could also be examined.

As discussed previously in this thesis, another potential study is to determine at what level dietary Zn is beneficial in protecting against ethanol exposure. In our studies, we used Zn supplementation at a level more than five times greater than that of the control diet. While we did not observe any negative outcomes caused by excess dietary Zn, it could potentially affect other systems that were not examined in this study. Considering that we have previously shown that supplementation with Zn at only three times the control diet can significantly increase the maternal plasma Zn concentrations (Chapter 4) and ameliorate infection-mediated teratogenicity (Chua et al., 2006), this indicates the potential for a lower dosage of Zn to be used in the supplemented diet. The model could also be used to look at the interaction between ethanol and infection and to determine whether a lower BAC causes teratogenicity in an infection model.

This thesis examined the effects of prenatal binge drinking and dietary Zn supplementation on physical birth defects, mortality, growth and cognitive functioning in offspring. Thus, the outcomes that were examined in these studies were predominantly severe abnormalities that could be determined by simple observation of their external phenotype and behaviour. However, ethanol exposure during pregnancy can cause a wider range of less subtle external, internal and behavioural abnormalities that were not investigated due to a limitation in expertise in diagnosing these outcomes. In addition, we did not reach the point of investigating the effects of ethanol or dietary Zn supplementation on molecular processes (apoptosis, enzymes, proteins, gene expression) that are involved in fetal growth and development. Future work could therefore extend our research and incorporate additional techniques (microarrays, histological, morphological, behavioural) to determine the specific developmental pathways that are disturbed by ethanol-induced Zn deficiency and limited by Zn treatment.

5.4 CONCLUDING STATEMENT

This thesis presents evidence to support that ethanol-induced alterations in maternal-fetal homeostasis, leading to fetal Zn deficiency, is an important factor in mediating teratogenicity. Dietary Zn supplementation proved to be protective in preventing a range of adverse outcomes in mice caused by binge drinking in early pregnancy (including physical birth defects, post-natal mortality and spatial and object recognition memory impairments), indicating that the fetus is amenable to treatment.

Thus, therapeutical intervention in early pregnancy in the human setting may be beneficial in decreasing the incidence of FASD and preventing developmental disabilities in many individuals.

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