Ethanol teratogenicity: the aetiological importance of zinc and metallothionein

A thesis submitted for the award of Doctor of Philosophy

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TABLE OF CONTENTS

ABSTRACT

Ethanol-related teratogenicity is a major health concern, yet the underlying mechanisms are not fully understood. Changes in maternal-foetal zinc homeostasis resulting in a foetal deficiency may be an important contributing factor. Zinc is an essential trace metal, fundamental for many metabolic processes, including those associated with growth and development. Ethanol induces expression of hepatic metallothionein (MT), a zinc-binding protein, causing zinc transfer from the plasma to the liver. The ethanol-related reduction in the maternal plasma zinc concentration is critical in mediating a teratogenic outcome. In normal (MT+/+) mice, ethanol causes a greater than 50% decrease in plasma zinc levels, while the reverse occurs in mice that cannot express MT (MT-/- mice). These changes in plasma zinc correlate with a high (24%) and normal (3%) incidence of abnormal foetuses in MT+/+ and MT-/- mice respectively. The importance of zinc and MT in mediating the teratogenic effects of ethanol was further examined.

The increased plasma zinc concentrations in MT-/- mice following ethanol treatment were found to be a consequence of zinc release from the muscle and skin as determined using ^{65}Zn labelling studies. In MT+/+ mice this zinc is rapidly sequestered by the liver as zinc-MT, leading to a decreased plasma zinc concentration.

Zinc treatment in MT+/+ dams at the time of ethanol insult (gestation day, GD, 8) prevented teratogenicity, with only 4.5% of the foetuses being abnormal, compared to 14.3% of foetuses from dams not supplemented with zinc.

Examination of the relationship between ethanol treatment and maternal and foetal genotype revealed that teratogenicity in foetuses from MT+/- dams was intermediate (8% abnormal foetuses) between MT+/+ (24%) and MT-/- (3%), while foetal genotype did not have a major impact on outcome.

The teratogenic effect of lipopolysaccharide (LPS), a potent MT inducer, was also assessed, using the same protocol as the ethanol studies. Foetuses from LPS-treated MT+/+ dams exhibited significantly more abnormalities, and were smaller than foetuses from MT-/- dams. Zinc treatment on GD8 prevented the deleterious effects of LPS.

This work demonstrates a clear link between maternal hepatic MT induction, decreased foetal zinc supply, and teratogenicity. This has major implications for binge alcohol consumption in early pregnancy, as well as infection during pregnancy, as indicated by the LPS studies. The demonstration that zinc treatment is effective in preventing teratogenicity in both situations indicates potential treatment strategies.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Luke Charles Carey

Signature Date 3/3/2003

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CHAPTER 1

Background

1.1 ETHANOL TERATOGENICITY

Alcohol (ethanol) is the most widely consumed and socially accepted drug in western society. Perhaps because of this, the public awareness of alcohol as a teratogen is poorly recognised when compared to other less commonly used drugs such as cocaine, and in the past thalidomide. Underlying this statement is the observation that 15% of women in the United States consume alcohol at some stage during pregnancy (Ebrahim et al, 1998). Furthermore, in a recent Danish study it was found that of a cohort of pregnant women who consumed alcohol, 27% had at least one episode of binge[1] drinking during the gestational period (Andersen et al, 2001). The significance of these figures is emphasised by the fact that prenatal exposure to alcohol is considered to be the leading cause of congenital mental retardation in the US (Abel and Sokol, 1987).

1.1.2 Foetal alcohol syndrome

Foetal alcohol syndrome (FAS) was first described by Jones and Smith (1973) as "a pattern of altered growth and morphogenesis" in children born to alcoholic mothers. Since then there have been numerous human and animal studies published, clearly demonstrating the teratogenic nature of ethanol (see reviews by Hannigan and Armant, 2000; Becker et al, 1996; Streissguth et al, 1980). Furthermore, it is not

[1] A binge is defined as the consumption of 5 or more standard drinks in one session, where a standard drink contains 14 g of absolute ethanol.

exclusively the offspring of alcoholic mothers who are affected. It has been shown in animal studies that binge drinking episodes can have equally severe ramifications for the foetus as does chronic ethanol exposure, suggesting that the timing of alcohol consumption is also a key teratogenic factor (Sulik and Johnston, 1983; Fernandez et al, 1983). The period when the foetus is most susceptible to the disruptive impact of ethanol is during organogenesis, a time when a multitude of critical morphological events are taking place. In humans, organogenesis takes place between weeks 3 and 9 of gestation, while in rodents it begins on gestation day (GD) 7, and is complete by GD12.

1.1.3 Manifestations of foetal alcohol exposure

The severity and type of abnormalities associated with foetal alcohol exposure are highly varied, hence afflicted individuals are often phenotypically dissimilar. However, there are 3 distinct components to the FAS, and in terms of diagnostic criteria patients must display:

1) some degree of postnatal growth retardation
2) characteristic facial dysmorphologies
3) central nervous system (CNS) deficits

One of the major manifestations of foetal alcohol exposure is a low birth weight (<2.5 kg, where 3.5 kg is normal), which is indicative of intra-uterine growth restriction. Furthermore in the postnatal environment there is no evidence of the 'catch-up growth' often associated with low birth weight infants (Tenbrink and Buchin, 1975; Kyllerman et al, 1985).

The facial components of FAS include having a thin upper lip, wide nasal bridge, indistinct philtrum (the groove between the upper lip and nose), prominent epicanthic folds (the skin over the inner corner of the eye), clefts of the palate and lip, and microphthalmia, most of which are evident in Figure 1. Other abnormalities commonly seen in individuals with FAS include heart (ventricular and atrial septal defects), hearing, and urogenital defects.

Although the physical abnormalities are the most salient aspect of FAS, the brain related problems are by far the more debilitating and costly both to the individual concerned and the community in general. Recent investigations, undertaken in the laboratory of John Olney, have demonstrated that ethanol has a significant neurodegenerative effect in rodent brains (Ikonimidou et al, 2000; Olney et al, 2002). In humans, magnetic resonance imaging of individuals with FAS has revealed frequent underdevelopment of the brain (microcephaly), which may account for some or all of the CNS related abnormalities (Sowell et al, 2001; Mattson et al, 1996). In terms of CNS deficits resulting from these underlying effects, individuals with FAS are often classified as mildly mentally retarded (IQ < 75), have problems existing socially, and may exhibit a number of learning and behavioural disorders such as attention deficit hyperactivity disorder and attention deficit disorder (Streissguth and O'Malley, 2000).

Not all alcohol-exposed individuals meet the criteria necessary for the diagnosis of FAS. Some may have only one or several components and hence are classified as suffering alcohol related birth defects (ARBD). Similarly, alcohol induced brain dysfunction without other aspects of FAS is termed alcohol related neurodevelopmental disorder (ARND).

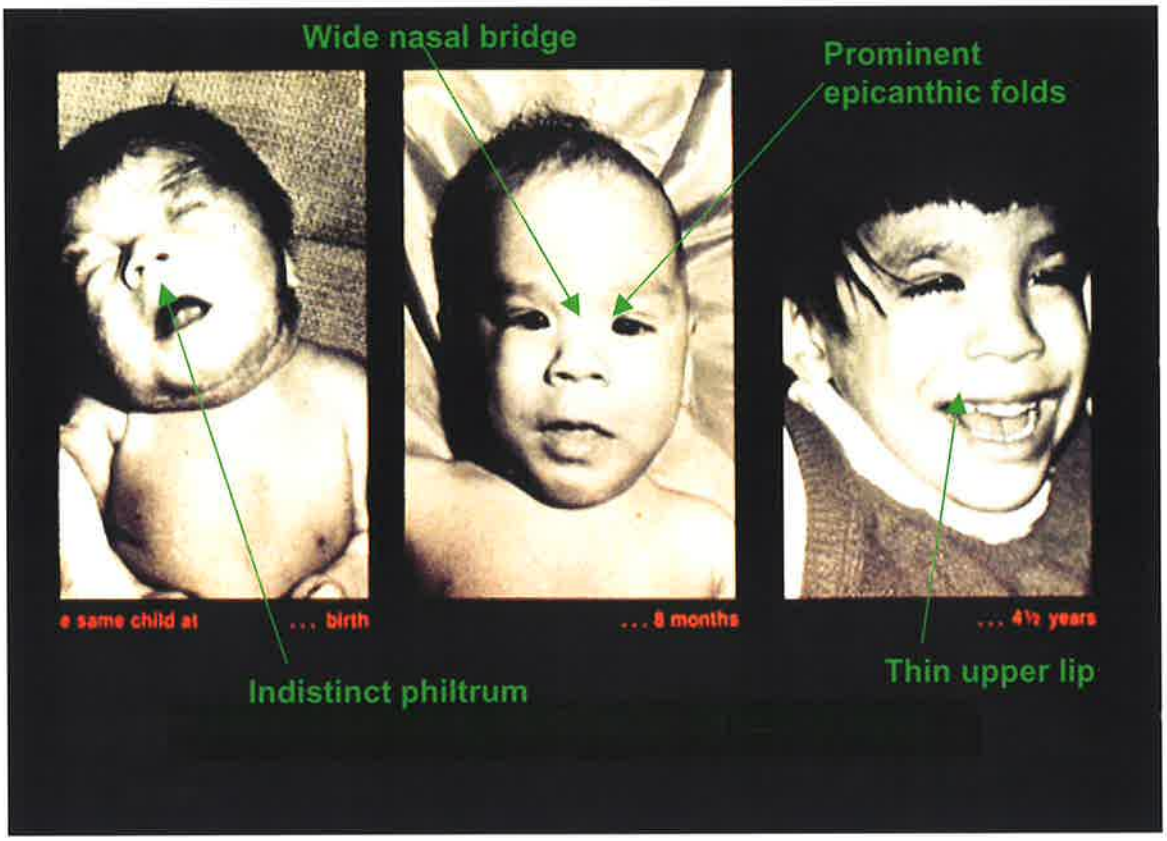


Figure 1. Pictures of a child taken at birth, 8 months and 4.5 years, displaying the characteristic facial features of FAS.

1.1.4 Incidence of FAS

Recent estimates in the USA suggest that approximately 3 out of every 1000 children born will suffer from FAS (Clarren et al, 2001), however, over the years there have been estimates ranging from 0.5 up to 5 per 1000 births (reviewed by May and Gossage, 2001). There are also marked differences in occurrence within different sub populations in the community, with indigenous, low socioeconomic and minority groups overrepresented (Burd and Moffatt, 1994; Abel, 1995; Viljoen et al, 2002). Assuming an annual birth rate of 4 million in the USA, there are then approximately 2000 to 12000 babies born each year with FAS (National Academy of Sciences report, 1996). This figure ignores individuals born with some, but not all, aspects of FAS (i.e. ARBD and ARND) the incidence of which is much higher and has been conservatively estimated to be in the vicinity of 1 in 100 births (Sampson et al, 1997). The actual frequencies of FAS observed through general/passive observation are far lower that what is seen in the specialised/active studies aforementioned (1 in 10,000 is typical), and this reflects the problematic nature of diagnosing FAS. Some features of foetal alcohol exposure may be caused by other contributing factors such as drug use and smoking, hence it can be difficult to single out alcohol as the offending teratogen. Problems in recognising FAS at birth mean that it's prevalence in the general community is almost certainly underreported, particularly in poorer countries and indeed some western countries where the general awareness of FAS is less prominent than in countries such as the USA and Canada. For example, in the state of South Australia there were only 9 recorded cases of FAS between 1986 and 1998, in which time there were approximately 240,000 births, thus giving an incidence far below even the most conservative estimates in previous studies (South Australian Birth Defects Register report, 2000). There are also difficulties in ascertaining whether a mother has consumed alcohol during gestation, as women are often

reluctant to discuss their drinking habits with health authorities. Furthermore, there is also a broad spectrum in the severity of abnormalities, from slight to extreme, making definitive diagnosis problematic. However steps are being taken to standardise the assessment of FAS (Astley and Clarren, 1999), and this will likely lead to more comprehensive identification of afflicted individuals, and hence an increased ability to deal with the problem, and prevent the development of secondary disorders.

1.1.5 Cost to the community

The most recent cost estimates for dealing with FAS alone in the USA come from a National Institute on Drug Abuse report regarding alcohol abuse and alcoholism which puts the total at around $1.9 billion per annum (National Institute on Drug Abuse, 1992). By far the bulk of this amount is associated with the ongoing costs of institutionalisation for mentally retarded individuals with FAS, and special education programs for mildly retarded individuals. There are also the more immediate costs of caring for low birth weight infants, and surgically repairing FAS related physical defects. Something that has not previously been discussed is the cost of drugs to treat behavioural disorders such as attention deficit disorder and the like, which are now known to be common in FAS. It should be noted that these estimates are for full FAS, and do not include the costs associated with ARBD and ARND. Hence one would expect the total cost of the teratogenic effects of alcohol to be far in excess of the estimates thus quoted.

1.1.6 Rodent studies

Ethanol teratogenicity has also been investigated quite extensively using rodent models of experimentation (reviewed by Becker et al; 1996). These studies have demonstrated the full spectrum of effects seen in humans, ranging from an increased

incidence of spontaneous abortion to the facial dysmorphologies characteristic of FAS (Chernoff, 1977; Sulik and Johnston, 1983). Both chronic and acute models of ethanol administration have been developed and utilised to good effect.

Chronic models, designed to mimic alcoholism in humans, are typically manifested in the form of a liquid diet containing ethanol (DeCarli and Lieber, 1967). Experimental dams are maintained on the diet during gestation, while control dams are also fed a liquid diet containing an isocaloric substitute for ethanol. There is also often a second control group where dams are pair fed the amount of diet consumed by the ethanol group.

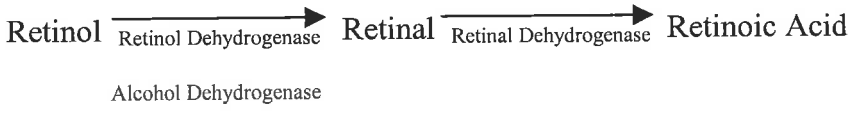
Acute, or binge-type models of ethanol exposure usually involve two intraperitoneal (ip) injections of ethanol, administered on a specific day during the organogenic period (Webster et al, 1980; Webster et al, 1983). While chronic treatment is associated with moderate blood alcohol concentrations (BAC), acute injection results in significantly higher levels, and is analogous to what occurs in a human binge drinking episode. Although ethanol administration by oral gavage is more comparable to human consumption, it is difficult to attain relevant BACs by this method without compromising the health of the animal. In mice, the stomach has a capacity to hold 0.2 mL of liquid, meaning that the solution gavaged has to contain approximately 60% ethanol to reach BACs comparable to those achieved following ip injection. This dose is know to damage the mucosa in both the stomach and small intestine (Kawashima et al, 1975; Takano et al, 2000).

1.1.7 Aetiology

We do not yet fully understand the mechanisms that underlie ethanol teratogenicity. There is little doubt that there are multiple factors involved, including both direct effects of ethanol on the foetus, and indirect effects mediated by associated changes in maternal-foetal homeostasis. There is also the argument that a significant proportion of ethanol teratogenicity is associated with the effects of its intermediate metabolite, acetaldehyde. As yet it has not been determined if there is one dominant factor responsible. Some of the more prominent indirect effects proposed include; interference with retinoic acid synthesis, altered prostaglandin metabolism, hypoxaemia, and impaired nutrient delivery.

1.1.7.1 Inhibition of retinoic acid synthesis

The binding of retinoic acid (RA) to specific retinoic acid receptors initiates a cascade of events that ultimately impinge on gene expression, and consequent embryogenesis and cellular differentiation (Kastner et al, 1994; Chytil and Haq, 1990; Maden et al, 1989). RA is a metabolite of retinol (or vitamin A), and is formed by two oxidation steps shown below.



The rate limiting step in this process is the conversion of retinol to retinal.

It seems likely that there is some degree of functional overlap in the ability of retinol and alcohol dehydrogenase to catalyse the precursor alcohols to aldehydes (Connor and Smit, 1987; Yang et al, 1994), leading to the suggestion that competition between

ethanol and retinol for enzyme catalysis leads to a deficit in RA accumulation, and consequent foetal dysmorphology.

For more detail on this topic see reviews by Napoli, 1999 and Zachman and Grummer, 1998.

1.1.7.2 Altered Prostaglandin metabolism

Prostaglandins are known to play a role in stimulating parturition, however in excess they are teratogenic (Persaud, 1975; Collins and Mahoney, 1982). There is evidence to suggest that ethanol causes an increase in endogenous levels of prostaglandins as a result of increased cellular release and/or decreased catabolism (see reviews by Smith et al, 1991; Randall et al, 1987).

1.1.7.3 Altered blood flow

As the foetus is wholly reliant on maternal blood for supply of nutrients and oxygen, any impediment to delivery is likely to have significant implications for growth and development. It has been reported that maternal ethanol exposure is associated with foetal hypoxaemia (Mukherjee and Hodgen, 1982), as well as decreased transfer of amino acids (Lin, 1981) and glucose (Singh et al, 1989) to the foetus. All of these effects could be accounted for by changes in foetal blood flow. Other investigations have failed to find an effect of ethanol on these physiological processes (Patrick et al, 1985; Schenker et al, 1989).

1.1.7.4 Direct effects of ethanol on the foetus

In vitro experiments have demonstrated that ethanol, which freely crosses the placenta (Wilkening et al, 1982; Clarke, 1986), has direct deleterious effects on foetal

tissue. These effects, most likely mediated by reactive oxygen species (ROS) generated during the metabolism of ethanol, include the inhibition of DNA methylation and thymidine incorporation (and consequent decreased DNA and protein synthesis) (Garro et al, 1991; Dreosti et al, 1981), and alterations in membrane integrity (as a result of lipid peroxidation) leading to inevitable changes in transport mechanisms and receptor dynamics (Kotch et al, 1995; Chen and Sulik, 1996).

For more detailed reviews on this topic see Henderson et al., 1999, and Brooks, 1997.

1.1.7.5 Acetaldehyde

Acetaldehyde has demonstrated teratogenic effects when administered as an independent agent (O'Shea and Kaufman, 1979; Sreenathan et al, 1982). This has led to the proposition that acetaldehyde, formed by the alcohol dehydrogenase-mediated oxidation of ethanol, is one of the underlying mediators of ethanol teratogenicity (Dreosti et al, 1981).

Although this is an attractive and logical mechanism, there is a scarcity of data from *in vivo* experiments supporting this assertion. It is unlikely that concentrations of acetaldehyde in this setting would reach a level comparable to the experiments where acetaldehyde was administered as a sole agent. Furthermore it has been reported that the peak concentrations of acetaldehyde in maternal and foetal blood following ethanol administration are some 1000-fold less than corresponding ethanol concentrations (Brien et al, 1983). In addition, investigations utilising the acetaldehyde dehydrogenase inhibitor disulfiram have failed to demonstrate any increase in teratology following ethanol treatment (Webster et al, 1983), while use of

the alcohol dehydrogenase inhibitor 4-methylpyrazole further increases the teratogenic outcome as compared to ethanol treatment alone (Blakely and Scott, 1984).

It should be stressed that none of the mechanisms discussed have been proven, and therefore remain viable hypotheses rather than fact. Hence this thesis focuses on another potential mechanism: ethanol-induced foetal zinc deficiency. To understand why this could be a significant factor in the underlying teratology it is first necessary examine in some detail the general characteristics of zinc and why it is so important in all aspects of life, not the least being early foetal development.

1.2 ZINC

Behind iron, zinc is the most abundant trace element in the body, and is essential for a whole range of processes underlying general functioning in both humans and animals. The importance of zinc is perhaps best exemplified by its presence in over 300 enzymes, where it conveys both catalytic activity and structural stability. Structurally, zinc atoms are also present in cell membranes and DNA binding domains. It is primarily through these enzymes and binding domains that zinc plays a critical role, not only in general metabolic processes, but also in growth and development.

1.2.1 Biochemistry

In the physiological setting zinc exists almost exclusively as a divalent cation (Zn^{2+}). There are several basic properties of zinc that make it extremely prominent in biochemical systems. Firstly, zinc does not undergo either oxidation or reduction, and thereby confers a high degree of stability to complexes in environments where there

is a continual flux between the oxidised and reduced state. Secondly, zinc ion complexes can rapidly exchange ligands (this being an essential characteristic for catalycity). Thirdly, zinc can assume multiple coordination geometry's, thus allowing for a high degree of flexibility with respect to structural conformation. In short, the stability and versatility of zinc enables it to interact with a wide range of ligands, and play a key role in normal functioning.

1.2.2 Zinc enzymes

Over 60 years ago, carbonic anhydrase was the first zinc-containing enzyme to be discovered (Kielin and Mann, 1940). Since that time there have been hundreds of other zinc enzymes reported in all 6 enzymatic classification groups. For the vast majority of zinc containing enzymes, the zinc atom itself (with its highly localised charge and electron affinity) plays a direct catalytic role whereby the metal ion binds to specific ligands. Prominent examples of catalytic zinc enzymes include angiotensin converting enzyme, collagenase, carboxypeptidases, phospholipase C, and dipeptidase. There are several enzymes directly involved in regulating the mechanics of gene expression, which also fall in this category. These include RNA polymerase, reverse transcriptase, DNA topoisomerase, and tRNA synthetase. In other enzymes zinc plays a structural role, conveying stability to quaternary structural formations. Protein kinase C, aspartate transcarbamylase, and alcohol dehydrogenase (note: alcohol dehydrogenase also contains catalytic zinc atoms) are examples of enzymes in this category. Zinc is also known to play coactive roles in some enzymes, where its presence, although not absolutely essential, may facilitate or depress catalytic function.

1.2.3 Zinc proteins

As well as in enzymes, zinc plays a critical role in proteins modulating gene expression. Nearly 20 years ago it was discovered that transcription factor IIIA (TFIIIA) from *Xenopus* oocytes requires zinc to enable binding to RNA (Hanas et al, 1983). Several years later, TFIIIA was found to contain characteristic zinc-binding motifs, which were later named zinc fingers (Miller et al, 1985). Structurally TFIIIA consists of a pattern of amino acids, with conserved cysteine and histidine residues to which zinc binds, forming a tetrahedral arrangement (Diakun et al, 1986). The binding of zinc to the amino acid sequence of TFIIIA conveys stability to the underlying structure, and allows binding to RNA and consequent transcription in the presence of RNA polymerase III. In simple terms, a zinc finger can be described as 'a polypeptide loop folded back on itself with the aid of a zinc ion' (Klug and Schwabe, 1995). Since the structural elucidation of TFIIIA, numerous other transcription factors have been discovered to contain, what is now considered, the 'classic' zinc finger motif. Prominent examples include; in humans Sp1 (Kadonaga et al, 1987), in mice Zif268 (Pavletich and Pabo, 1991), and in drosophila Kruppel (Rosenberg et al, 1986).

Shortly after the discovery of zinc finger proteins, other proteins were also found to contain similar, yet conformationally different zinc binding motifs. The proteins, also involved in initiating transcription, contain zinc atoms tetrahedrally bound to cysteine residues. Differences in zinc interatomic distances, and surrounding amino acid conformation, have led these motifs to be termed zinc clusters, and twists (Vallee et al, 1991). The steroid hormone receptors for glucocorticoids and oestrogen have several binding domains; one each for the hormone itself, DNA, and RNA polymerase. The DNA binding domain contains 2 zinc atoms, encaptured in a twist

formation. The removal of these zinc atoms by chelation prevents DNA from binding, and halts transcription (Freedman et al, 1988). The GAL4 protein in yeast, which is involved in initiating galactose metabolism, exhibits a prototypic zinc cluster formation. As with the oestrogen and glucocorticoid receptors, the removal of zinc stops transcription at the DNA binding step (Johnston, 1987).

1.2.4 Antioxidant properties of zinc

It well accepted that zinc deficiency leads to increased production of free radicals, and susceptibility to oxidative damage. It follows that supplementation with zinc (both acute and chronic) leads to significant antioxidant effects. Zinc, with only one valence state, is not a traditional antioxidant, in that it is not directly involved in negating hydroxyl radical ($^{\bullet}OH$) formation. Zinc is known to induce metallothionein (MT), a cysteine rich protein which has antioxidant properties (discussed further on page 29). Furthermore, superoxide dismutase, a zinc containing enzyme/free radical scavenger, displays increased activity on relief of the deficient state (Shaheen and el Fattah, 1995).

There are two mechanisms through which zinc exerts acute antioxidant influence. These are firstly by stabilising sulphydryl groups, and secondly by inhibiting the production of $^{\bullet}OH$ from H_2O_2 and O_2^{-}, as catalysed by redox-active transition metals (Powell, 2000). As previously mentioned, the chemical stability of zinc is a key reason for its presence in numerous enzymes and proteins. Therefore it is not surprising that the binding of zinc to sulphydryl groups, or nearby proteins, is thought to reduce sulphydryl reactivity, and hence decrease susceptibility to oxidation. Both copper and iron have been demonstrated to catalyse the formation of $^{\bullet}OH$ radicals,

which in turn can promote lipid peroxidation. Zinc acts as a competitive antagonist with these other transition metals, thus negating the process (Korbashi et al, 1989).

A more in depth discussion on zinc and antioxidation appears in the review by Powell (2000).

1.2.5 Zinc homeostasis

In humans, the major sources of dietary zinc are (in decreasing order) lean red meat, pork, legumes, cheese, and whole grain products. Absorption of zinc occurs in the upper portion of the small intestine, however the mechanisms underlying this process are not fully understood. Transport from the lumen to the vasculature is thought to be mostly transcellular, and both a passive and/or facilitated process (Hoadley et al, 1988a; Hoadley et al, 1988b; Steel et al, 1985). A number of specific zinc transporters (ZnT-1 to ZnT-5) have been identified in the last decade, and it appears that these proteins are involved in cellular absorption, processing, and secretion of zinc in the small intestine and other tissues (Palmiter and Findley, 1995; Palmiter et al, 1996a; Palmiter et al, 1996b; Huang and Gitschier, 1997; McMahon and Cousins, 1998; Kambe et al, 2002).

Excretion, rather than absorption, is the major physiological mediator of zinc balance, although in times of deficiency intestinal absorption is upregulated (review: Hambidge and Krebs, 2001). Cells in both the intestinal serosa and the pancreas secrete zinc into the lumen, with the content in the faeces changing with respect to dietary intake.

There is approximately 2.5 g of zinc in the body, most of which is concentrated in the muscle, bone, skin and liver (Table 1). Only a small percentage zinc is contained within the blood plasma, rendering this compartment exquisitely sensitive to rapid changes in zinc distribution between tissues and organs.

1.2.6 Zinc deficiency in humans

The importance of zinc is perhaps best demonstrated by examining the plethora of effects deficiency has on normal body functioning. It is generally accepted that zinc deficiency is endemic in many third world countries, and even (albeit with a lower incidence and to a milder degree) in western nations. Pioneering work in this area was conducted by Prasad and co-workers in the middle east, where severe dietary zinc deficiency was found to be associated with dwarfism, hypogonadism and poor skin condition (Prasad et al, 1961; Prasad et al, 1963). Supplementing the diet with zinc alleviated these problems.

Since these early studies, zinc deficiency has been implicated in causing and exacerbating a variety of conditions, the more prominent of which will be mentioned here.

The skin appears to be the first tissue affected, with development of dermatitis around the nose and mouth. With continued deficiency the dermatitis spreads, and hyperkeratotic lesions may develop (Aggett, 1989). Gastrointestinal functioning is also altered, with resultant diarrhoea. Long-term zinc deficiency can have serious inhibitory effects on growth and development, as noted above. Zinc deficiency is also associated with compromised CNS functioning, specifically mood changes,

Table 1. Percentage distribution of total body zinc in human tissues. Data taken from Jackson (1989).

Tissue	% of total zinc (2.5 g)
Muscle	57
Bone	29
Skin	6
Liver	5
Brain	1.5
Heart	0.4
Other	1.0
Plasma	0.1

learning and behavioural difficulties, as well as emotional instability (Hambidge and Walravens, 1982; Wallwork, 1987).

Much of what we now know about the effects of zinc deficiency has come from observing individuals with acrodermatitis enteropathica, a congenital disorder of zinc metabolism where intestinal absorption is inadequate for bodily requirements. As one would predict, the classic symptoms of this affliction include: severe dermatitis, alopecia, conjunctivitis, diarrhoea and other gastrointestinal disorders, retarded growth, and cognitive deficits. Treatment with oral $ZnSO_4$ is completely successful in relieving these symptoms, without which individuals would not survive past infancy.

1.2.7 Zinc deficiency during pregnancy

As previously discussed, zinc plays integral roles in growth and development. As such it should come as no surprise that maternal zinc deficiency during pregnancy has profound effects on foetal development. Studies utilising animal models have been prolific in this area and provide undeniable evidence of the link between zinc deficiency and teratology. Hurley et al. (1971), found that when rats were fed a diet containing 0.3 parts per million (ppm) of zinc (vs. a control of 100 ppm) for differing lengths of time during pregnancy there was significant foetal resorption, as well as developmental abnormalities, particularly involving the brain, eyes, tail, urogenital system and limbs. Similarly, Hickory et al. (1979), using a less severe zinc restriction (1.3 ppm), demonstrated that rat foetuses in the zinc deficient group weighed some 15% less than controls, and also displayed evidence of retarded bone development as indicated by decreased calcification and ossification.

Gross abnormalities of the brain such as microcephaly and exencephaly are traditional observed teratogenic outcomes of maternal/foetal zinc deficiency. More recently however, it has become apparent that in addition to these gross deformities, zinc deficiency may exert a more subtle, but nevertheless significant, influence on brain development. Wang and colleagues (2001) fed mice a zinc deficient diet (1 ppm) from the beginning of pregnancy onwards, and then examined foetal brains at various times from GD10.5 to postnatal day 10 for the expression of nestin, a marker of proliferation in neuroepithelial stem cells. At all time points nestin levels were found to be significantly lower in the zinc deficient compared to the control groups (fed either 30 or 100 ppm zinc), prompting the authors to conclude that zinc deficiency impedes the normal development of neural stem cells, and that this in turn may become manifest in later life in the form of 'neuroanatomical and behavioural abnormalities'.

In humans, mild maternal zinc deficiency (as assessed by serum zinc or hair zinc concentrations) has been associated with below average birth weight, and an increased frequency of neural tube defects (Neggers et al, 1990; Srinivas et al, 2001). However the more telling findings again come from studying the pregnancy outcomes of women with acrodermatitis enteropathica. To this extent, Hambidge and colleagues (1975) found that out of the seven pregnancies assessed, three were abnormal as evidenced by instances of spontaneous abortion, anencephaly, low birthweight and perinatal death.

1.2.8 Teratogenicity: zinc deficiency vs. ethanol exposure

The teratogenic outcomes of prenatal ethanol exposure and zinc deficiency as separate events are very similar (Table 2). Not only do both interfere with general

Table 2. Similarities in rodent foetal outcome following maternal zinc deficiency, or ethanol exposure during gestation.

Foetal Defect	Reference	
	Zinc Deficiency	Ethanol
Resorptions	Dreosti et al., 1986	Randall and Taylor, 1979
Low birth weight	Hickory et al., 1979	Sauerbier, 1987
Exencephaly	Keen and Hurley, 1989	Randall and Taylor, 1979
Anopthalmia	Dreosti et al., 1986	Sauerbier, 1987
Cleft Lip/palate	Hurley et al., 1971	Sulik et al., 1981
Neurobehavioral	Halas et al., 1977	Krsiak et al., 1977

growth and development of the foetus, but they also initiate abortion, and significant malformations, most notably in the cranio-facial region. Furthermore, the simultaneous insult of ethanol and zinc is synergistic, and results in an increased incidence of foetal abnormalities compared to either agent alone (Keppen et al, 1985). This fact and the similarities of individual teratogenic effects on the foetus are highly suggestive of a common underlying zinc-related aetiology. It is proposed that metallothionein (MT), a zinc binding protein, plays a key role in this process.

1.3 METALLOTHIONEIN

MT is a low molecular weight (<7 kDa) intracellular metalloprotein, first isolated in the equine renal cortex (Margoshes and Vallee, 1957). The 61-68 amino acid sequence of MT is characterised by a predominance of cysteine residues (Figure 2), imbuing the protein with a unique metal binding capacity. In the physiological setting zinc, and to a lesser extent copper, are bound by MT, however a multitude of other exogenous metals, most notably cadmium, are also MT ligands (Nielson et al, 1985). In terms of affinity, copper has the highest stability constant, followed by cadmium and then zinc (Waalkes et al, 1984a). A maximum of 7 divalent zinc or 12 monovalent copper atoms may bind to MT in tetrahedral and trigonal arrangements respectively (Nielson et al, 1985).

As can be seen in Figure 2, MT has two distinct subunits, the α and β-domains. The carboxy terminal α-domain has the capacity to bind 4 divalent metal atoms, while the amino terminal β-domain binds 3. These domains also differ in binding stability, with the more stable α-domain exchanging ligands at a slower rate than the less stable and hence more reactive β-domain (Kägi and Kojima, 1987).

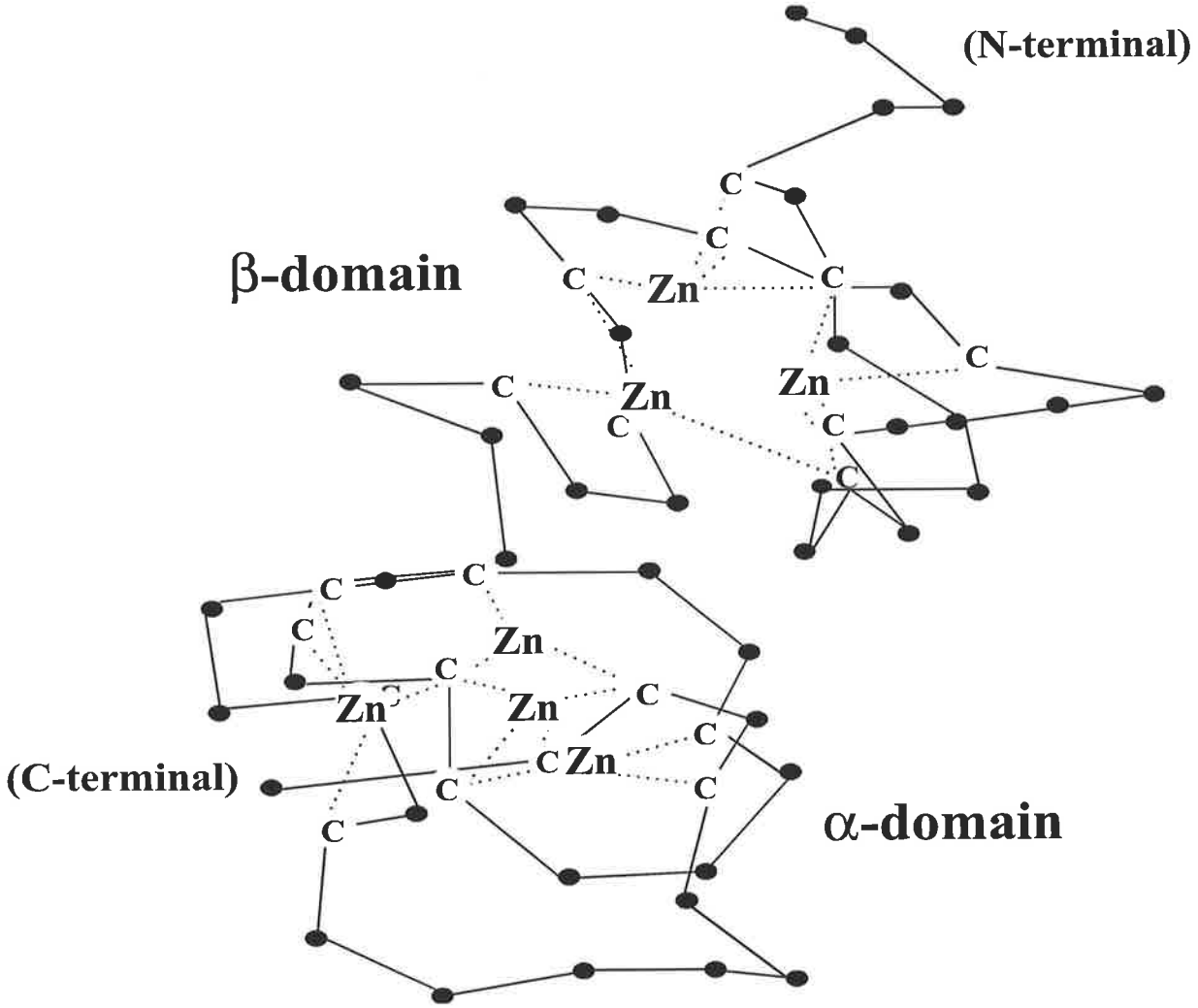


Figure 2. Tertiary structure of MT. Note the two distinct domains, predominance of cysteine residues, and tetrahedral binding of zinc.

1.3.1 Isoforms

There are four currently known major isoforms of MT, termed MT-1 to –4. Although all are expressed in humans and rodents alike, what follows focuses on the rodent (or more specifically the mouse), as the vast majority of findings in this area pertain to animal experimentation. The MT isoforms differ slightly with respect to amino acid sequence, areas of expression and inducibility. MT-1 and –2 are the most abundant isoforms, and are found in most tissues, with the highest areas of expression being in the liver, pancreas, kidneys and intestine. MT-3 is predominantly expressed in the brain (Palmiter et al, 1992), however MT-3 mRNA has been detected in a number of other tissues (Hoey et al, 1997; Moffatt and Séguin, 1998). Message from MT-4, the most recently cloned isoform, has been detected in stratisfied sqamous epithelia, and the maternal deciduum (Quaife et al, 1994; Liang et al, 1996).

1.3.2 Induction

MT-1 and –2 are the only inducible isoforms of MT[2]. Expression of MT is most dynamic in the liver, where a range of compounds have demonstrated MT inductive properties. Inducers of MT can roughly be divided into 3 categories; metals, acute phase or stress proteins, and xenobiotics.

1.3.2.1 Metals

Of the metals, zinc and cadmium have been the focus of many investigations, and both are potent inducers. Copper, mercury, bismuth, and silver are also known to induce hepatic MT to differing extents (Farr and Hunt, 1989; Piotrowski et al, 1974; Naruse and Hayashi, 1989; Sugawara and Sugawara, 1984). As zinc is the major physiological metal inducer of MT, there has been extensive investigation regarding

[2] Note: from this point onwards, the term 'MT' refers collectively to the MT-1 and –2 isoforms.

the molecular mechanisms leading to its expression. Zinc has been shown to bind to a metal transcription factor (termed MTF-1), which in turn then associates with metal response elements (MREs) on the MT gene itself, leading to expression (Westin and Shaffner, 1988). It is not known precisely how other metals initiate MT gene expression, although the MRE step in the process appears to be a consistent one (Palmiter, 1994).

1.3.2.2 Acute phase proteins

A number of cytokines and hormones released in large concentrations as a consequence of inflammation or infection have significant MT inductive properties. Cytokines such as interleukin (IL)-6, IL-1 and tumour necrosis factor alpha (TNF-α) are particularly prominent in this respect, while glucocorticoids and catecholamines are also effective inducers of hepatic MT (Coyle et al, 1993; Sato et al, 1992; Karin and Herschman, 1980; Brady and Helvig, 1984; Bremner, 1987). These effectors initiate transcription via interaction with response elements in the promoter region of the MT gene, which are distinct from MREs. Environmental stressors such as cold, heat and restraint also increase levels of hepatic MT, most likely via inflammatory intermediates (Oh et al, 1978; Hidalgo et al, 1986; Hernandez et al, 2000).

1.3.2.3 Xenobiotics

A significant number of xenobiotics including paracetamol, ethanol, urethane, α-hederin, lipopolysaccharide (LPS), and carbon tetrachloride have been shown to induce MT (Wormser and Calp, 1988; Waalkes et al, 1984b; Brzeznicka et al, 1987; Liu et al, 1993; Min et al, 1991). It is not known exactly how these substances facilitate MT expression, although it would seem that there is no direct interaction with the promoter region of the gene. Most likely it is as a result of associated

increases in the release of inflammogens which would then promote transcription as previously discussed.

In all cases, the induction of hepatic MT precedes a consequent movement of zinc from the plasma into the liver where it is subsequently bound by MT. While the liver zinc concentration rises, there is a significant decline in the plasma zinc concentration, which can result in levels dropping from normal (approximately 13 μmol/L in mice) to 2 μmol/L within 10 hr (Chapter 6). This is an extreme example, and obviously the extent of the changes depend on the degree and duration MT induction, which is in turn influenced by the concentration and type of inducer. A single episode of induction may lead to hepatic MT and zinc levels being elevated for up to 48 hr, while plasma zinc concentrations tend to return to basal within 12-20 hr.

As mentioned on page 16, only 0.1% of total body zinc is contained within the plasma compartment. Therefore it is emphasised that even small changes in the overall distribution of zinc can have a major effect on plasma zinc levels, while not necessarily impacting on concentrations in other tissues such as the liver and muscle.

1.3.3 Transgenic Mice

A number of genetically modified mice have been generated, and subsequently advanced our knowledge in the MT field considerably.

1.3.3.1 MT-null mice

MT-null mice (MT-/-) were produced by two independent laboratories in the early 90's, by disrupting the MT-1 and –2 gene sequences (Michalska and Choo, 1993; Masters et al, 1994). Late gestation MT-/- foetuses and newborns have lower basal

levels of zinc in the liver than their wildtype (MT+/+) counterparts (Kelly et al, 1996; Carey et al, 2000a), however, the observation that these mice grow, develop and reproduce quite normally refutes the argument that MT has an essential function. The only other difference between MT-/- and MT+/+ mice in the normal setting is that older MT-/- mice tend to be more susceptible to obesity (Beattie et al, 1998). In situations where the normal environment is compromised in some way, MT-/- mice are often disadvantaged as will become evident in later discussion.

1.3.3.2 MT-overexpressing mice

Mice with multiple copies of the MT-1 gene (MT-TG mice) were engineered in 1995 (Iszard et al, 1995). MT-TG mice have quite significantly elevated basal levels of MT expression in the liver, pancreas and stomach, and as one would predict liver zinc concentrations which are 50% higher than in MT+/+ mice. These mice also have pancreatic Zinc levels that are 300% greater than normal.

1.3.3.3 MT-3 knockout mice

Although not of specific relevance to this thesis, mice which lack expression of the MT-3 gene have also been generated (Erickson et al, 1997). As previously mentioned, MT-3 is expressed predominantly in the brain. MT-3 knockout mice have decreased zinc levels in the hippocampal and other regions of the brain, and are more susceptible to kainic acid induced seizures than are MT+/+ mice.

1.3.4 Function

There has been extensive debate as to what the primary function of MT is. Although the zinc binding capacity of MT suggests involvement in the homeostatic regulation of this metal, the unique chemical structure and amino acid composition of MT makes

it a suitable candidate for a number of roles, none of which appear to be obviously more integral than the next.

1.3.4.1 Zinc homeostasis

The interaction between MT and zinc implies a likely role for MT in mediating (at least to some degree) zinc homeostasis. The observation that MT-/- mice are unhindered in their development and functioning in the normal setting deems any role to be of negligible importance. However, in situations of extreme zinc challenge (excess or deficiency) MT+/+ mice have an advantage in dealing with the adversity. Coyle et al. (2000a) found that in the zinc deficient state MT+/+ mice absorb a greater proportion of an orally administered zinc solution than do MT-/- mice. This is thought to be due to increased levels of MT expression in non-gut tissues, particularly the liver. During pregnancy, the feeding of zinc deficient diets to dams severely restricts zinc delivery to MT-/- foetuses, resulting in a higher rate of abnormal development than that seen in MT+/+ foetuses (Andrews and Geiser, 1999; Rofe et al 1999a). Conversely in a state of excess zinc, MT+/+ mice are better able to restrict zinc uptake and enhance its excretion than are MT-/- mice. High levels of oral zinc induce MT in the intestine, leading to zinc binding. This decreases absorption, and it is thought that the zinc-MT in the intestinal mucosa is later transported back into the lumen and excreted (Hoadley et al, 1988b). In addition, MT appears to play a further role in the removal of zinc via pancreatic secretions. De Lisle et al. (1996) found elevated MT concentrations in pancreatic juice from MT+/+ mice treated with high concentrations of $ZnCl_2$. In short, MT-/- mice lack a fully functional mechanism to prevent the accumulation of zinc, which if extreme can result in pancreatic damage (Kelly et al, 1996).

1.3.4.2 Heavy metal detoxicant

MTs capacity for binding metals has led researchers to focus on its involvement in heavy metal detoxification, in particular with respect to cadmium, for which MT has a high binding affinity. In both the initial studies where MT-/- mice were generated, the susceptibility of these mice to $CdSO_4$ toxicity was also investigated (Michalska and Choo, 1993; Masters et al, 1994). While Michalska and Choo (1993) observed that MT-/- mice were physically more effected by cadmium treatment compared to MT+/+ mice, Masters et al. (1994) demonstrated increased lethality in MT-/- mice, and also found evidence of severe liver damage in the form of necrosis and haemorrhage. Park et al. (2001) demonstrated that the lethal dose 50 of cadmium was some 6.2 times higher in MT+/+ mice than in MT-/- mice. Furthermore, the increased level of MT expression seen in MT-TG mice offers an additional level of protection from that seen in MT+/+ compared to MT-/- mice. In the *in vitro* setting MT-/- cells also exhibit greater cytotoxic effects following cadmium exposure (Zheng et al, 1996; Coyle et al, 2000b). MT also negates the severity of cadmium associated renal damage. Liu et al. (2000a) found that MT-/- mice exposed to cadmium through drinking water over a 6-month period developed significant nephrotoxicity as indicated by increased levels of renal caspase-3, a marker of apoptosis.

Although the bulk of work in this particular area has focussed on cadmium, toxicity associated with other heavy metals including arsenic and mercury appears to be MT dependent (Liu et al, 2000b; Satoh et al, 1997).

1.3.4.3 Antioxidant

The predominance of cysteine residues in MT not only provide the basis for metal binding, but also facilitate the binding and neutralisation of free radical molecules to an extent which (in the liver) is greater than that associated with glutathione (Miura et al, 1997). Hence the suggestion that MTs primary function is that of an antioxidant. A number of studies have addressed this issue by exposing normal and MT-/- mice to different free radical producing agents. Liu et al. (1999) demonstrated that MT-/- mice exposed to paracetamol presented with increased evidence of hepatotoxicity compared to MT+/+ mice. Lipid peroxidation was more prominent in these mice, as indicated by the high prevalence of 4-hydroxynonenal and malondialdehyde protein adducts. The findings of a study by Rofe et al. (1998) were similar both *in vivo*, and *in vitro*. Other agents including carbon tetrachloride, ultra violet B radiation, various antineoplastic drugs, and gamma irradiation known to generate free radicals are also more damaging in MT-/- mice (Klaasen and Liu, 1998; Hanada, 2000; Kondo et al, 1995; Deng et al, 1999).

1.3.4.4 Inflammation

The induction of hepatic MT during inflammation/infection is pronounced, and is mediated by acute phase proteins, in particular IL-6. It is not clear whether the associated zinc redistribution plays a part in the inflammatory response, or whether it is just coincidental. Coyle et al. (2002) suggested three possible beneficial effects of this process:

1) The lowering of plasma zinc modulates leukocyte function and cytokine production.
2) The increased intracellular zinc pool (as bound by MT) facilitates enhanced metabolism during the acute phase response.

3) The binding of zinc by MT prevents zinc related inhibition of enzymes.

It is also possible that MT itself plays an important antioxidant role during inflammation.

1.3.4.5 Redox sensitive zinc chaperone

The most recent theory links zinc binding and release from MT to the redox state of the cellular environment, and hence designates MT a critical role in regulating zinc homeostasis and delivery. Research has focussed on the interactions between MT and the glutathione redox couple and the associated effects on MT bound zinc. Maret (1994) found that all 7 radiolabelled zinc atoms were displaced from MT binding sites in the presence of glutathione disulfide (GSSG). Reduced glutathione (GSH) on the other hand inhibits the release of zinc and transfer to apo-sorbitol dehydrogenase (a zinc requiring enzyme) from MT (Jiang et al, 1998a). However the binding of GSH to MT (Brouwer et al, 1993) induces a conformational change in structure, such that in the presence of GSSG the rate of zinc liberation is enhanced as compared GSSG alone (Jiang et al, 1998a). Jiang and co-workers (1998b) demonstrated that ATP also binds to MT, facilitating zinc release and donation to apo-sorbitol dehydrogenase, prompting the suggestion that 'both the redox and energy states of the cell seem to control zinc distribution from MT'.

1.3.5 Inappropriate MT induction and teratology

It is clear that MT+/+ mice maintain a survival advantage over MT-/- mice in certain situations. However, there is evidence that inappropriate induction of MT during pregnancy may have a negative impact on foetal development. Daston and colleagues (1991) were the first to suggest this after examining the relationship between urethane-induced teratology and changes in maternal-foetal zinc homeostasis.

Urethane was found to significantly induce liver MT in GD11 rats, decrease maternal plasma zinc concentrations by 30%, and inhibit the transfer of ^{65}Zinc to foetal tissues by 50% compared to controls. Foetuses examined on GD18 following maternal urethane exposure on GD11 exhibited decreased weight, and delayed skeletal ossification. This led the authors to conclude that the induction of liver MT by urethane, and the associated decrease in plasma zinc, impaired foetal zinc transfer and contributed to the abnormalities observed on GD18. A number of other teratogens including α-hederin, TNF-α, and 2-ethylhexanoic acid have since been found to exert similar effects on maternal MT and zinc homeostasis, and foetal outcome to those observed with urethane (Daston et al, 1994; Taubeneck et al, 1994; Taubeneck et al, 1995; Bui et al, 1998). Although the findings of these studies strongly support the concept that foetal zinc deficiency is a key factor in causing teratology, there is only circumstantial evidence for the involvement of MT in regulating the changes in zinc distribution. Controlling for the influence of MT, via the use of MT-/- mice, would without doubt address the importance of MT in this regard.

1.4 SUMMARY

Both chronic and acute (binge) consumption of ethanol can severely impair foetal development, resulting in spontaneous abortion and a variety of birth defects. The birth defects associated with prenatal exposure to ethanol, collectively known as the foetal alcohol syndrome, are low birth weight, central nervous system deficits, and characteristic facial dysmorphologies. Despite the relatively high prevalence and cost to the community of ethanol related birth abnormalities, the aetiology underlying this teratology is not fully understood. Zinc is an essential trace element which is not only critical for life, but plays an integral role in foetal (and adult) growth and

development via its presence in a number of enzymes and proteins that regulate gene expression. The significance of maintaining an adequate foetal zinc supply during pregnancy is best exemplified by the observation that maternal zinc deficiency impairs foetal development, evidenced by malformations at birth. Similarities between the teratogenic effects of ethanol and zinc deficiency imply a degree of aetiological overlap. This thesis focuses on the impact of ethanol on maternal-foetal zinc homeostasis, with the rationale being that ethanol induced foetal zinc deficiency is one of the underlying mediators of teratology. Metallothionein is a cysteine rich, intracellular zinc (and other metal) binding protein ascribed roles in the regulation of zinc homeostasis, cellular metabolism, antioxidation, and heavy metal detoxification. Expression of MT is most dynamic in the liver, an organ of high and labile zinc content. Hepatic induction of MT in the liver is triggered by a multitude of endogenous and exogenous mediators, which precedes movement of zinc from the plasma into the liver (for incorporation into MT), thus depleting the plasma of zinc. The observation that ethanol, along with a number of other teratogens, is a potent inducer of MT has led to the suggestion that 'the deleterious effects of ethanol on the foetus are in part due to a metallothionein mediated decrease in maternal plasma zinc which compromises foetal zinc supply'. Recent work in this laboratory using MT-/- mice has provided strong support for this hypothesis. It was found that GD18 MT+/+ foetuses from dams exposed to ethanol on GD8 had significantly more external abnormalities than did MT-/- foetuses (Carey et al, 2000a). Furthermore, the incidence of abnormalities in MT-/- foetuses was no different from saline treated controls of both genotypes. In another experiment conducted on GD12, ethanol was found to severely impede the transfer of ^{65}zinc to MT+/+ foetal tissue, while having no effect on the delivery to MT-/- foetuses (Carey et al, 2000b).

1.5 AIMS

With these findings, and the original hypothesis in mind, investigations in this thesis were directed towards clarifying and extending the findings of earlier experiments (Carey et al, 2000a,b). More specifically the major aims were to:

Determine whether zinc treatment at the time of ethanol exposure reduces teratogenicity.

Assess the teratogenic effects of ethanol when administered later in gestation.

Explore the possibility that foetal MT genotype is an important factor in modulating the teratogenic effect of ethanol.

Examine the how the maternal feeding of zinc deficient diets in combination with ethanol treatment modulates foetal outcome (keeping in mind that MT-/- foetuses are more susceptible to zinc deficiency induced teratology than are MT+/+ foetuses).

Investigate other potential mechanisms by which ethanol may interfere with foetal development.

Assess whether MT-/- foetuses are afforded protection from lipopolysaccharide, a known inducer of MT and teratogen.

CHAPTER 2

Maternal metallothionein expression during pregnancy.

Amelioration of ethanol teratogenicity by zinc supplementation.

INTRODUCTION

Although the MT-1 and –2 isoforms are not critical for foetal development in the normal setting, the observation that there is coordinated expression of these genes in both maternal and foetal tissue during gestation implies a role for MT in the regulation of zinc distribution. In the preimplantation mouse embryo, GD<4.5, MT-1 mRNA is expressed from the time of fertilisation (one cell), and is responsive to metal induction by the blastocyst stage (Andrews et al, 1991). MT-1 and –2 proteins have also been demonstrated in the preimplantation embryo (Vidal and Hidalgo, 1993). Post implantation, all 4 isoforms of MT mRNA have been detected in the decidua, with increases in expression to maximum levels attained by GD10 (Liang et al, 1996). At this point, levels of MT-1 and –2 mRNA in the placenta begin to rise, peaking at GD16, as decidual levels decrease concomitantly (De et al, 1989; Andrews et al, 1984). At the same time (GD12-16), there are high levels of MT-1 and MT-2 mRNA in both maternal and foetal liver, and the visceral yolk sac (Liang et al, 1996; Andrews et al, 1984). MT mRNA is expressed in foetal liver shortly after formation (around GD11), but it is maximal during latter gestation (GD16-17), after which there is a gradual decline, such that by 12 days post partum basal adult levels are attained (Andrews et al, 1984; Oullette, 1982; Quaife et al, 1986). Although MT mRNA expression in some foetal and maternal tissues is well described during gestation,

little is known about the ontogeny of MT protein expression in the maternal liver. To this extent, it was found in an earlier study conducted in this laboratory that ethanol did not have any inductive effect on hepatic MT at GD12 due to already raised levels of expression (Carey et al, 2000b). Hence it is of critical importance with respect to studies examining MT induction via external agents (such as ethanol), that the ontogeny of pregnancy related hepatic MT expression is defined. Experimentation in the first part of this Chapter examines the changes in maternal hepatic MT protein content during gestation.

It has been suggested that ethanol related teratogenicity is partly a result of impaired foetal zinc supply, however the actual mechanisms underlying this remain unexplained. The induction of liver MT by ethanol leads to decreased plasma zinc levels in MT+/+ mice (Taubeneck et al, 1994; Carey et al, 2000a). This decrease is sustained, and can result in zinc concentrations dropping by over 60%, 8 hr after treatment (Carey et al, 2000a). MT-/- mice, that cannot express MT, do not exhibit a decrease in plasma zinc concentration after ethanol injection. In fact the reverse is the case, with plasma zinc levels increasing in these mice by approximately 50%. As the teratogenic effects of ethanol are markedly reduced in the MT-/- setting, it is suggested that a raised plasma zinc limits, and a decreased plasma zinc increases the incidence of foetal abnormalities. If this is the case, then one would expect that supplementing MT+/+ dams with zinc at the time of ethanol exposure, and hence preventing the decrease in plasma zinc levels, would protect the foetuses from incurring a zinc deficit, and reduce resultant teratology. The second part of this Chapter aims to address this issue.

MATERIALS AND METHODS

Animals and mating procedure

MT+/+ mice were of the C57BL6 strain, purchased from the Animal Resources Centre, Canning Vale, Western Australia. Mice were maintained in an animal house at 22°C, subject to a 14 hr light/10 hr dark cycle and given unrestricted access to water and a commercial non-purified diet (Milling Industries, Adelaide, Australia), except where indicated.

Mating was carried out by pairing females (aged 10-14 weeks) with a proven male and examining the female every morning for the presence of a vaginal plug. Confirmation of a plug was designated GD1, at which time females were removed from males and housed individually until the time of experimentation.

Maternal MT expression during pregnancy

Pregnant mice at different stages of gestation (GD9-18) were anaesthetised with halothane and blood taken via cardiac puncture prior to cervical luxation. Livers were immediately removed, stored on ice, and then analysed for MT and zinc content. Plasma zinc levels were also assessed.

Ethanol and zinc supplementation in non-pregnant mice: timecourse

Female mice were injected ip at 0 and 4 hr with 25% ethanol in 0.85% saline (v/v) at a dose of 2.9 g/kg (0.015 mL/g). Immediately after the first injection, mice were given an additional 250 μL subcutaneous (sc) injection in the nape of the neck of $ZnSO_4$ in 0.85% saline, where the solution contained 50 mg/kg of zinc. Food was removed following the first injection and replaced approximately 3 hr after the

second. Over the following 16 hr mice were killed, as described on the previous page, and plasma zinc concentrations quantified.

GD8 ethanol, zinc supplementation and teratogenicity

GD8 mice were treated with ethanol and $ZnSO_4$ as previously described. Control mice were treated in an identical manner, but with 0.85% saline, while an additional group of pregnant mice were injected with only ethanol on GD8.

Mice were killed on GD18 by cervical luxation following blood collection. Uteri were immediately excised, examined for resorption sites, and the number of foetuses noted. Individual foetuses and placenti were then separated, weighed and the foetal crown to rump length measured. Foetuses were examined under low power magnification to determine the incidence of anophthalmia, microphthalmia, microcephaly, micrognathia and any other obvious external malformations. Multiple comparisons were made to assess abnormalities. These were within litter comparisons, between litter comparisons (i.e. ethanol exposed foetus vs. control foetus), and bilateral and proportional comparisons of individual foetuses.

Metallothionein analysis

Maternal livers were diluted 1:5 (w/v) with TRIS-HCl (10 mM, 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 water), and then centrifuged for 4 min at 14 000 × g. The resultant supernatant was then diluted 1:25 with de-ionised water and assayed for MT using the method of Eaton and Toal (1982).

The key steps in this process are illustrated in Figure 1.

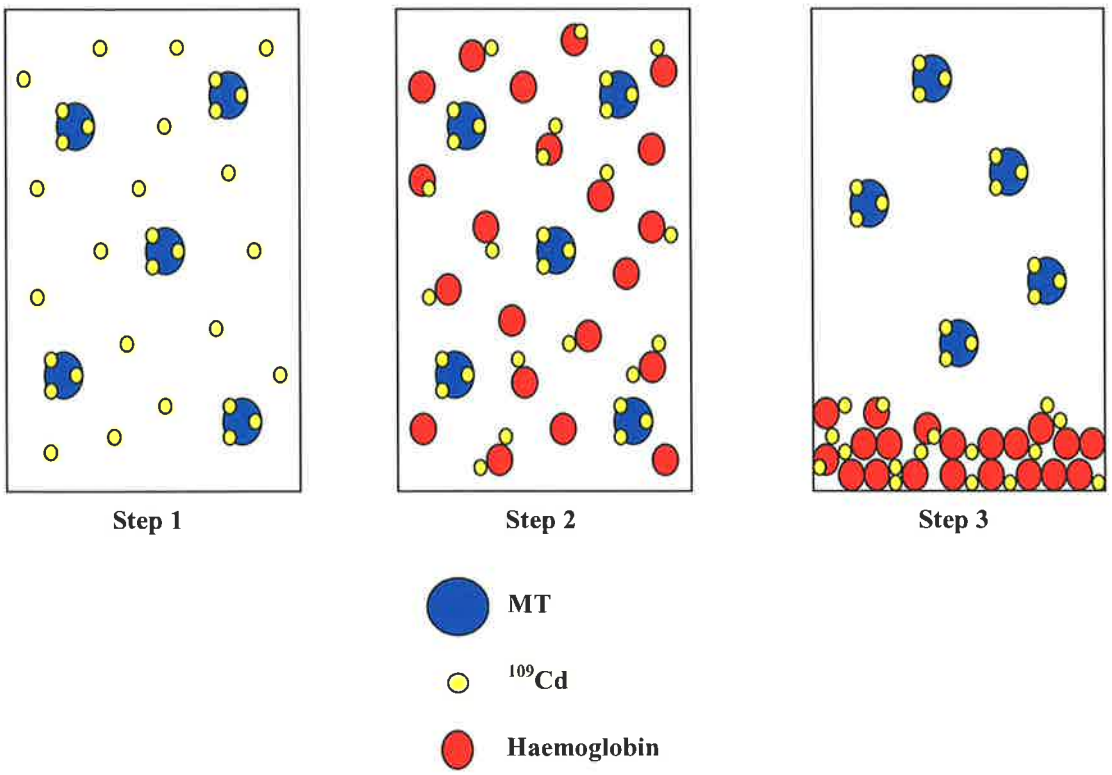


Figure 1. Key steps in the quantification of metallothionein.

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.

Zinc analysis

Liver samples were diluted 1:5 (w/v) with TRIS HCl (pH 8.2), and homogenised as per the MT method. The homogenate was dried at approximately 65°C for 48 hr, and digested with concentrated nitric acid whilst heating to evaporate the liquid. 1M HCl was then added to dissolve the remaining solid material and the resultant solution was analysed for zinc content by flame atomic absorption spectroscopy (Perkin Elmer Analyst 300, Überlingen, Germany). Blood samples were centrifuged at 14 000 × g for 4 min, and zinc concentrations in the plasma were determined by flame atomic absorption spectroscopy.

Statistical analysis

Data were compared by two-way analysis of variance (ANOVA). A binomial transformation was performed on data pertaining to foetal abnormalities before analysis. Significance was determined using Tukey's post hoc test.

All statistical analysis was performed using Minitab statistics software (Minitab Inc., State College, PA). Results are presented as mean ± SEM. Differences were considered to be significant at $p < 0.05$.

RESULTS

Maternal MT expression during pregnancy

Measurements were made on GD9, GD12, GD15 and GD18.

By GD9 liver MT concentrations were approximately 3-fold higher than the basal levels of 4 nmol Cd bound/g wet weight (Figure 2). From GD9 to GD15 MT levels increased dramatically, peaking between GD12 and GD15 at concentrations above 80

nmol Cd bound/g wet weight, before decreasing to half peak values at GD18.

Liver and plasma zinc levels in these mice increased and decreased respectively in accordance with the level of hepatic MT expression (Figures 3, 4). Plasma zinc concentrations were lowest between GD12 and GD15, while the reverse was true for liver zinc concentrations. Plasma zinc levels had returned to basal (GD0) levels by GD18. At the same time liver zinc levels were still slightly elevated but tending towards normal.

Ethanol and zinc supplementation in non-pregnant mice: timecourse

Following ethanol and zinc treatment, plasma zinc concentrations increased dramatically, peaking within 2 hr at 61 ± 5 μmol/L (Figure 5). Over the next 2 hr plasma zinc levels declined by over 50% (to 26 ± 3 μmol/L), before gradually returning towards basal levels over the succeeding 12 hr.

GD8 ethanol, zinc supplementation and teratogenicity

GD18 litters from dams treated with ethanol on GD8 had significantly fewer foetuses and contained more resorption sites compared to litters from both ethanol and saline injected dams that were supplemented with zinc on GD8 (Table 1).

There were no abnormal foetuses observed in the saline + zinc group (although 2 foetuses were extremely underdeveloped and would not have survived in the postnatal environment), while the percentage of abnormal foetuses was 3-times higher in the ethanol treatment group compared to ethanol + zinc (Table 1). The specific abnormalities were: microphthalmia × 3 in the ethanol + zinc group, and microphthalmia × 3, anophthalmia and micrognathia in the straight ethanol group.

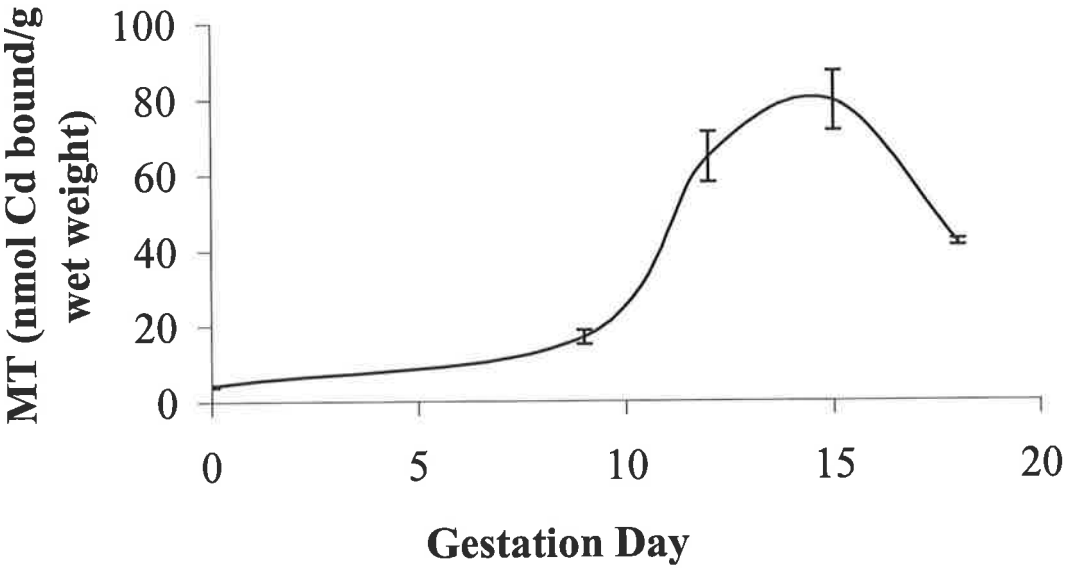


Figure 2. Liver MT concentrations in MT+/+ mice killed at different times during gestation. Points represent the mean ± SEM, n = 4-5.

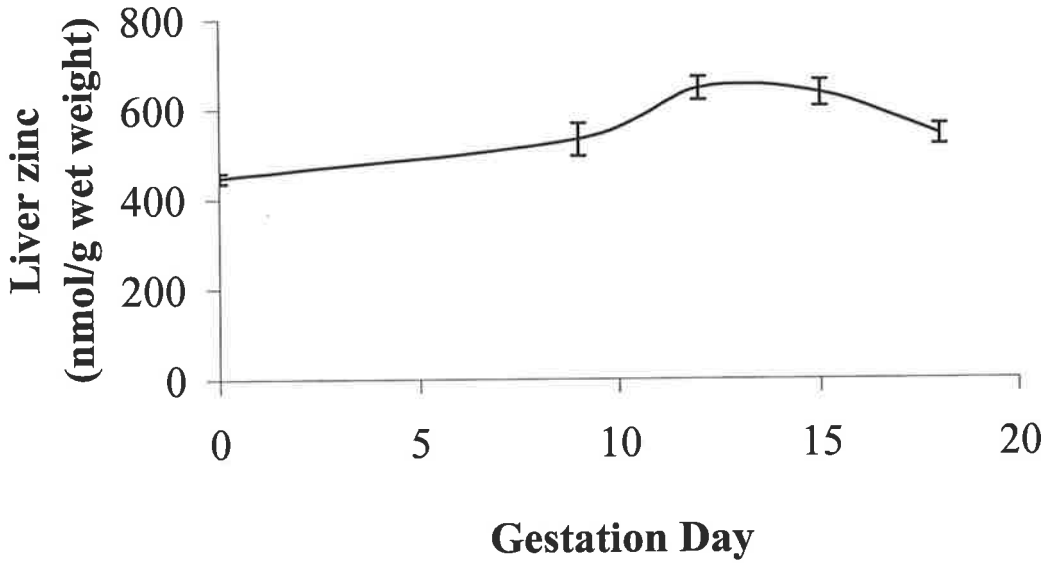


Figure 3. Liver zinc concentrations in MT+/+ mice killed at different times during gestation. Points represent the mean ± SEM, n = 4-5.

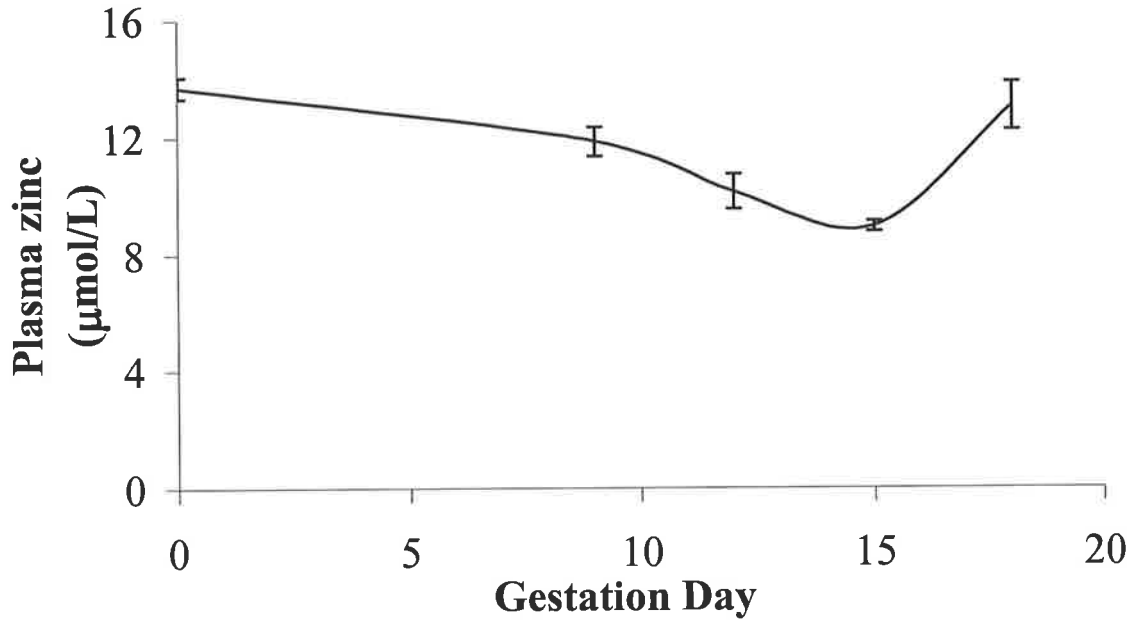


Figure 4. Plasma zinc concentrations in MT+/+ mice killed at different times during gestation. Points represent the mean ± SEM, n = 4-5.

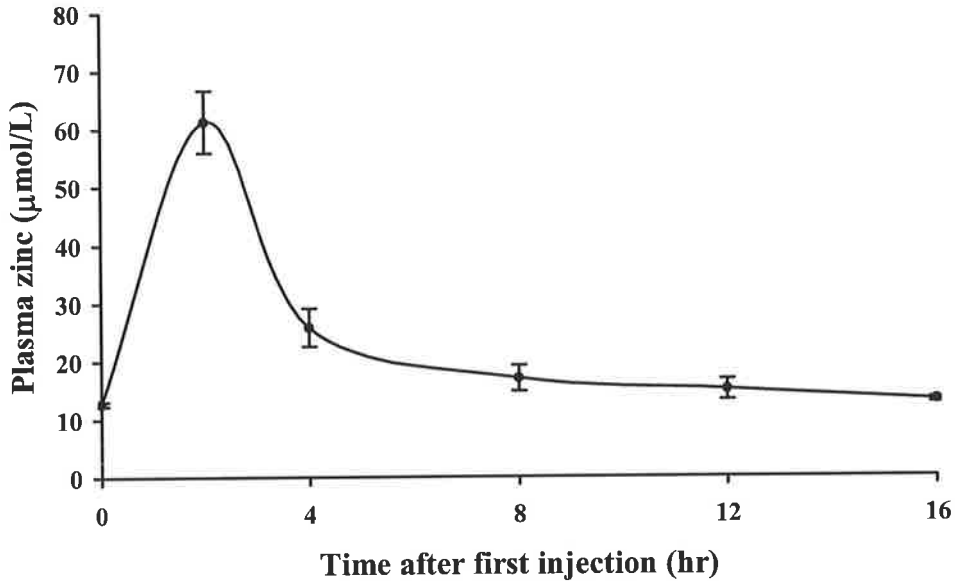


Figure 5. Effect of ethanol and zinc supplementation on plasma zinc concentrations in non-pregnant mice over 16 hr. Mice were ip injected with 25% ethanol (0.015 mL/g) on one or two occasions (0 and 4 hr) depending on kill time, and injected sc with 250 μL of $ZnSO_4$ (50 μg/g) after the first ethanol injection. Points represent the means ± SEM, n = 3-4. Plasma Zn concentrations were significantly higher than basal levels at 2 and 4 hr ($p < 0.05$).

Table 1. GD18 foetal data from MT+/+ dams treated with saline + $ZnSO_4$ or ethanol ± $ZnSO_4$ on GD8

	Treatment		
	Saline + Zinc	Ethanol + Zinc	Ethanol
No. litters	6	7	6
No. foetuses	52	66	35
Litter size	8.8 ± 0.9	9.4 ± 0.7	5.8 ± 1.2[ab]
Resorptions sites per litter	0.4 ± 0.3	0.8 ± 0.4	2.2 ± 0.7[ab]
No. abnormal foetuses	0	3	5
% Abnormal foetuses	-	4.5	14.3
Weight (mg)	842 ± 13	852 ± 12	781 ± 13[ab]
Crown-rump length (mm)	19.3 ± 0.2	19.2 ± 0.1	18.4 ± 0.2[ab]
Placental weight (mg)	101 ± 2	96 ± 1[a]	95 ± 1[a]

Values represent the means ± SEM where applicable.

Data was analysed by two-way ANOVA, and Tukey's post-hoc test.

[a] Significantly different from saline + zinc group, $p < 0.05$.

[b] Significantly different from ethanol + zinc group, $p < 0.05$.

GD18 foetuses exposed to ethanol on GD8 were smaller in terms of weight and length compared to foetuses from zinc supplemented dams, regardless of treatment (Table 1). Placenti from saline injected dams were heavier than those from ethanol exposed dams.

DISCUSSION

During the gestational period MT mRNA concentrations in maternal and foetal tissues fluctuate markedly. Experiments in this Chapter demonstrate that the expression of MT protein in the maternal liver also changes dramatically during gestation, the major difference from the mRNA being that the increases are more sustained. MT levels in the maternal liver begin to rise shortly after implantation, reaching 4-fold basal by GD9, and peaking at concentrations 20-fold basal near GD14. Maternal liver mass also doubles over gestation. Over the last stage of gestation, maternal hepatic MT begins a gradual decline, which although not assessed here, is known to continue after parturition. Evidence from studies in the rat indicate that there may be species differences with regard to the magnitude and timing of changes in maternal liver MT, with those in the rat possibly being delayed and not as marked compared to the mouse (Piletz et al, 1983; Hidalgo et al, 1988).

It seems highly likely that this induction of maternal MT is initiated at least in part by circulating glucocorticoids and IL-6 released from the uterus, the concentrations of which rise and fall over a similar time course during gestation (Quaife et al, 1986; De et al, 1992). The probable reason behind the staged maternal hepatic MT induction (and resulting zinc accretion) is at first to provide for the extreme metabolic and growth demands of the dam and after, in late gestation when the foetal liver is

developed and able to play a role in regulating zinc homeostasis, to release stored zinc for placental transfer. This decline in maternal hepatic MT begins at a time when the foetal liver appears to be anatomically fully developed and becoming functional. MT concentrations in foetal liver are extremely high at GD18, 4-5 times higher than the already elevated maternal levels (Carey et al, 2000a) but after birth fall to adult levels by 4 weeks post-partum (Panemangalore et al, 1983; Bell, 1979). It seems logical that placental MT would also play an important role, peaking early in gestation (when maternal MT is low) and declining as maternal and foetal hepatic MT concentrations increase.

These fluctuations in maternal and foetal liver, and placental MT are not critical for successful pregnancy, as evidenced by the unhindered development of MT-/- foetuses in a normal setting. However when the dam is exposed to a low zinc environment, the importance of MT is manifested by a significant survival advantage in MT+/+ foetuses (Andrew and Geiser, 1999; Rofe et al, 1999a).

The key findings that ethanol causes a large increase in the plasma zinc levels in MT-/- mice, and that foetuses born to these mice exhibit decreased teratogenicity compared to MT+/+ foetuses, designates the maternal plasma zinc concentration as a major determinant of foetal outcome following ethanol exposure (Carey et al, 2000a). Results from experiments conducted in this Chapter are supportive of this concept.

Co-administration of sc zinc with ethanol yielded a highly reproducible plasma zinc response, maintaining concentrations above basal concentrations for up to 16 hr in non-pregnant mice. This is in contrast to the normal situation where ethanol treatment is associated with a significant reduction in the plasma zinc concentration (Carey et

al, 2000a). The early 'spike' in the plasma zinc concentration observed at 2 hr may be viewed as being non-physiological, and perhaps even deleterious. However there is little evidence to suggest that zinc at this level in the blood is toxic, and indeed there was no evidence of increased teratogenicity following zinc treatment (with saline injection) in the present investigation. To the contrary, no abnormal foetuses were seen in the control zinc treated group, thus implying a beneficial effect of zinc supplementation on foetal development in the normal pregnancy setting.

Supplementing MT+/+ dams with $ZnSO_4$ immediately after the first of two ethanol injections on GD8 decreased the percentage of abnormal foetuses seen on GD18 as compared to foetuses from dams that were not supplemented. Although there were more abnormal foetuses observed in the ethanol supplemented vs. the saline supplemented group, this incidence is scarcely different from what would be considered normal. Previously it was found that approximately 6% of foetuses from dams exposed to saline on GD8 were abnormal, compared with 4.5% in the ethanol supplemented group here (Carey et al, 2000a).

It is well recognised that one of the major outcomes of foetal ethanol exposure in humans (and indeed animals) is low birth weight, and that this is indicative of impaired intrauterine growth. Zinc supplementation in this study was found to prevent ethanol-related deficits in foetal growth. The foetal weight and crown-rump lengths in the supplemented groups were similar and comparable to previous findings where dams were exposed to saline only on GD8 (Carey et al, 2000a), while foetuses from the straight ethanol group exhibited delayed development.

The general consensus of previous studies where zinc supplementation has been used in an attempt to ameliorate the teratogenic effect of ethanol has been that supplementation is of no benefit. Keppen et al. (1990) found that pregnant mice fed a liquid diet containing ethanol with supplemental zinc (4-times the recommended daily allowance) had foetuses that were not different from the non-supplemented group in terms of developmental defects. While Tanaka and co-workers found both positive and no effect when pregnant rats were given excess zinc in the diet (Tanaka et al, 1982; Tanaka et al, 1988; Tanaka, 1998).

The major difference between the present study and those discussed is the method of administration. Here zinc was administered sc, while in the other investigations zinc was delivered orally as part of a liquid or solid diet. Bypassing the gastrointestinal processing step, as is achieved by the sc route, is critical in terms of resultant plasma zinc concentration. There is good evidence demonstrating that mice fed diets with a high zinc content do not exhibit particularly large increases in intestinal absorption (Coyle et al, 2000a). In keeping with this, Tran et al. (1998) found that plasma zinc concentrations in mice fed a diet containing 400 ppm zinc for one week increased by only 1 μmol/L. It is evident that there are strong homeostatic mechanisms controlling body zinc balance. Indeed, as mentioned on page 27, MT in the gastrointestinal tract is thought to play a role in restricting zinc absorption in times of excess. It is therefore unlikely that dietary zinc supplementation would raise maternal plasma zinc concentrations to an extent that is comparable to the levels attained when zinc is administered sc. It is important to realise that the purpose of this experiment was to raise maternal plasma zinc to levels similar to those observed in MT-/- mice following ethanol injection, with the rationale being that hyperzincaemia offers significant protection from ethanol related teratogenicity.

In conclusion, the results from this Chapter demonstrate that there is natural induction of maternal hepatic MT during pregnancy resulting in associated changes in plasma and liver zinc concentrations. It is suggested that this induction, mediated by endogenous factors, is important for marshalling zinc stores to meet the heightened metabolic demands associated with pregnancy. In addition it has been demonstrated that supplementing pregnant MT+/+ mice with zinc at the time of ethanol exposure on GD8 significantly decreases foetal teratogenicity and growth impediments.

CHAPTER 3

Ethanol-induced zinc release from tissues.

Effect of ethanol on zinc transfer to the foetus on gestation day 8.

Teratogenic effects of gestation day 12 ethanol treatment.

INTRODUCTION

Earlier work in this laboratory examined the effects of ethanol on maternal-foetal zinc distribution and consequent teratogenicity in MT+/+ and MT-/- mice. Following maternal ip exposure to 25% ethanol on GD8, it was found that on GD18, MT+/+ foetuses had a far greater incidence of external abnormalities than did MT-/- foetuses (Carey et al, 2000a). In the same study, non-pregnant MT+/+ and MT-/- mice were injected with ethanol in order to examine changes in zinc homeostasis. Predictably, hepatic MT levels in MT+/+ mice increased significantly, causing plasma zinc concentrations to drop from 12.6 μmol/L to 4.6 μmol/L. One would assume that since there is no possibility of a MT mediated zinc response in MT-/- mice, there would have been no change in the plasma zinc concentrations. It was therefore surprising to note that ethanol exposure was associated with increased plasma zinc levels in these mice. Concentrations peaked at 20.8 μmol/L 4 hr after ethanol treatment, and were sustained above normal for 12 hr. This finding has important implications for the MT-/- foetus. In the MT+/+ setting the low maternal plasma zinc would favour decreased foetal transfer, while the increase observed in MT-/- dams would maintain or provide an excess of zinc for foetal transfer. In light of these findings it was

deemed important to further investigate how ethanol alters zinc homeostasis in mice, and more specifically to assess whether the raised plasma zinc concentrations in MT-/- mice are due to release of zinc from other tissues.

Previous experiments in this laboratory have demonstrated that ethanol impairs the transfer of zinc to GD12 MT+/+, but not MT-/- foetuses (Carey et al, 2000b). In the study, pregnant mice were injected with 25% ethanol, in addition to a ^{65}Zn solution, and the distribution of radioactivity in foetal tissues was assessed. Over twice as much ^{65}Zn accumulated in MT-/- than MT+/+ foetuses, and MT-/- foetal tissue zinc concentrations were also higher. Although these are impressive results, the timing of the insult in this instance may be somewhat late, as organogenesis is largely complete by GD11-12. Hence the relevance of linking the effect of ethanol on zinc transfer at GD12 to abnormalities seen in GD18 foetuses exposed to ethanol at GD8, is questionable. Furthermore it is known that the timing of ethanol exposure during organogenesis has a definite bearing on the type of abnormalities induced. Webster and colleagues (1983) examined the teratogenic effects of ethanol administered on GD7-GD10, and found that early exposure (GD7-8) was associated with craniofacial anomalies such as micrognathia, and exencephaly, while treatment in later organogenesis (GD9-10) caused a predominance of limb defects. Other studies are also supportive of a distinctive difference in foetal outcome depending on whether exposure is early or late (Sauerbier, 1987; Kronick, 1976).

The two further experiments in this Chapter were designed to mirror certain aspects of previous work conducted in this laboratory (Carey et al, 2000a,b[3]). The first

[3] It should be noted that this investigation was originally conducted on GD12 for reasons of foetal size. It was thought that there would be insufficient foetal tissue at GD8 to adequately assess zinc transfer.

examined the effect of ethanol on the distribution of ^{65}Zn in GD8 MT+/+ and MT-/- mice. The second assessed the teratogenic impact of exposure to ethanol on GD12.

MATERIALS AND METHODS

Animals and mating procedure

MT-/- mice were originally produced at the Murdoch Institute, Royal Children's Hospital, Victoria and were from a mixed genetic background of OLA129 and C57BL6 strains (Michalska and Choo, 1993). They were subsequently backcrossed with C57BL6 mice for five generations to minimise genetic differences between the MT-/- and MT+/+ (C57BL6) populations.

See Chapter 2 (page 36) for information regarding MT+/+ mice, housing and mating.

GD8 ethanol and maternal-foetal zinc distribution

GD8 MT+/+ and MT-/- mice were injected with ethanol or saline using the previously described dosing regimen (page 36). Immediately after the second injection, mice were injected sc with 300 μL of 0.85% saline solution containing 37 kBq of ^{65}Zn tracer (specific radioactivity >37 GBq/g of zinc in 0.5MHCl; NEN Life Science Products, Boston, MA). Three hours later, the mice were anaesthetised with halothane and blood taken via cardiac puncture prior to cervical luxation. Concepti and maternal organs were immediately dissected, weighed and counted for ^{65}Zn activity using a cassette fed Packard Auto-Gamma Counter (model 5650, Canberra-Packard, Melbourne, Australia). Radioactivity in maternal skin and muscle was ascertained by taking a 4 cm^2 sample of skin and 200 mg of abdominal wall muscle.

On completion of counting, the maternal liver and concepti were processed for zinc and MT analysis where applicable.

Ethanol and tissue zinc release

To examine the effect of ethanol on tissue zinc release, non-pregnant mice of both genotypes were injected sc with ^{65}Zn tracer as described above. 72 hr later, mice were injected on one occasion with 25% ethanol, and the differences in distribution of ^{65}Zn in different organs and tissues were assessed at 2 and 4 hr post treatment. The end point timing (after ethanol exposure) corresponds with the extremes of change in plasma zinc levels.

GD12 ethanol and teratogenicity

Mice treated on GD12 (first injection 12:00 PM) were killed on GD18 by cervical luxation following blood collection. Foetal tissues were processed as described in Chapter 2 (page 37).

Metallothionein analysis

Maternal and foetal liver samples were diluted 1:5 (w/v) and 1:10 with TRIS-HCl, and subsequently analysed for MT (see Chapter 2, page 37).

Zinc analysis

Tissue and plasma zinc concentrations were determined as described in Chapter 2, page 39.

Metabolite analysis: glucose, lactate & glycogen

Upon maternal blood collection, a 0.1 mL aliquot was immediately added to 0.4 mL of ice cold 0.8 mol/L perchloric acid. Similarly, a 0.25 g portion of liver was excised and homogenised in 1 mL of the perchloric acid solution. Both acidified liver and blood samples were then centrifuged at 14,000 × g for 4 min, the supernatant removed and neutralised with saturated potassium bicarbonate. Glucose and lactate levels in blood and liver, and glycogen concentrations in liver were assessed using an Abbott bichromatic analyser 100 (Abbott Diagnostics, Pasedena, CA), as per the method of Rofe and Williamson (1983).

Statistical analysis

Concentrations of plasma, liver and whole conceptus zinc, tests of metabolic function, and all data pertaining to the distribution of ^{65}Zn were compared by two-way analysis of variance (ANOVA). Liver MT concentrations at GD8 were compared by Student's t-test. Significance was determined using Tukey's post hoc test.

RESULTS

GD8 ethanol and maternal-foetal zinc distribution

There were no differences in the amount of ^{65}Zn transferred to concepti within genotypes, however for both treatments MT+/+ concepti accumulated more radioactivity than their MT-/- counterparts (Table 1). Similarly, MT+/+ whole conceptus zinc concentrations were higher than MT-/-, while again there were no within genotype effects of treatment.

Approximately two times as much ^{65}Zn was found in the livers of MT+/+ compared

Table 1. Distribution of ^{65}Zn radioactivity in GD8 MT+/+ and MT-/- maternal tissues and concepti, 3 hr after subcutaneous injection of ^{65}Zn and 7 hr after initial ethanol or saline injection

		Treatment	
		Saline	Ethanol
Tissue	Gt*	% Total ^{65}Zn Counts/g tissue	
Liver	+/+	17.2 ± 0.72[b]	21.2 ± 1.03[a,b]
	-/-	9.9 ± 1.05	10.7 ± 0.39
Concepti	+/+	9.84 ± 0.17[b]	9.74 ± 0.14[b]
	-/-	5.90 ± 0.37	7.66 ± 0.40
Pancreas	+/+	21.0 ± 1.33[b]	23.1 ± 3.81
	-/-	13.3 ± 1.31	21.0 ± 1.32[a]
Kidneys	+/+	14.5 ± 0.62[b]	16.4 ± 0.53[b]
	-/-	9.9 ± 0.83	12.0 ± 0.22

Values represent the means ± SEM.

*Gt = genotype.

Data was analyzed by two-way ANOVA, with Tukey's post hoc test.

Mice: n = 6 (MT+/+ Saline), 6 (MT+/+ Ethanol), 5 (MT-/- Saline), 6 (MT-/- Ethanol). Concepti: n = 47 (MT+/+ Saline), 48 (MT+/+ Ethanol), 37 (MT-/- Saline), 59 (MT-/- Ethanol).

[a] Significantly different from saline exposed tissue of the same genotype, $p < 0.05$.

[b] Significantly different from MT-/- tissue of the same treatment, $p < 0.05$.

with MT-/- dams. Genotypic difference were also seen in the kidneys, and the pancreas (although only the saline comparison was significant, Table 1). Effects of treatment were observed in the livers of MT+/+ and in the pancreas of MT-/- mice, where ethanol was found to increase the retention of ^{65}Zn.

Although liver MT concentrations were higher in ethanol injected MT+/+ compared to MT+/+ saline injected mice, this failed to influence plasma zinc levels to any noticeable extent (Table 2). Both MT+/+ saline and ethanol exposed dams had plasma zinc concentrations which were lower than those seen in the corresponding MT-/- treatment groups, while MT-/- mice injected with ethanol had plasma zinc concentrations far in excess of MT-/- saline injected mice. Liver zinc levels were lower in MT-/- ethanol treated mice as compared to MT+/+ ethanol and MT-/- saline treated mice respectively.

Ethanol treatment resulted in marked decreases in liver and blood glucose levels, liver lactate concentrations, and liver glycogen stores in both MT+/+ and MT-/- mice (although the difference in liver glycogen levels was only significant in the MT+/+ comparison, Table 2). MT+/+ saline injected mice had higher liver glucose and glycogen levels than did MT-/- saline injected mice.

Ethanol and tissue zinc release

Tissue levels of ^{65}Zn were examined in non-pregnant MT+/+ and MT-/- mice that had been injected with ^{65}Zn 3 days prior to ethanol administration. Prior experience with this approach has shown that steady state labelling is achieved within 3 days. The pancreata of MT+/+ mice demonstrated increased retention of ^{65}Zn as previously reported (Rofe et al, 1999b), with ethanol having no effect on this retention in either

Table 2. Maternal metabolites, liver MT and zinc, plasma zinc and concepti zinc concentrations from GD8 MT+/+ and MT-/- mice, 7 hr after initial ethanol injection.

	Genotype and treatment			
	+/+ Saline	+/+ Ethanol	-/- Saline	-/- Ethanol
Liver MT (nmol Cd bound/g wet weight)	35 ± 13	53 ± 4[a]	NA	NA
Liver zinc (nmol/g wet weight)	551 ± 12	534 ± 26[b]	532 ± 23	440 ± 18[a]
Plasma zinc (μmol/L)	7.9 ± 0.3[b]	8.3 ± 0.3[b]	11.4 ± 0.6	22.8 ± 1.4[a]
Concepti zinc (nmol/g wet weight)	543 ± 24[b]	587 ± 22[b]	338 ± 6	364 ± 21
Liver glucose (mM)	50.2 ± 6.6[b]	4.1 ± 0.2[a]	21.2 ± 0.9	3.7 ± 0.3[a]
Blood glucose (mM)	8.7 ± 1.0	4.1 ± 0.2[a]	6.6 ± 0.3	3.8 ± 0.3[a]
Liver lactate (mM)	12.0 ± 1.5	3.3 ± 0.3[a]	11.9 ± 0.8	2.4 ± 0.1[a]
Liver glycogen (mM)	38.4 ± 9.8[b]	1.2 ± 0.6[a]	8.1 ± 4.2	0.1 ± 0.4

Values represent the means ± SEM

Data was analysed by two-way ANOVA (except for liver MT where Student's t test was used), with Tukey's post hoc test.

Mice: n = 6 (MT+/+ saline), 6 (MT+/+ ethanol), 5 (MT-/- saline), 6 (MT-/- ethanol).

Foetuses: n = 47 (MT+/+ saline), 48 (MT+/+ ethanol), 37 (MT-/- saline),

59 (MT-/- ethanol).

[a] Significantly different from saline treated mice of the same genotype, $p < 0.05$.

[b] Significantly different from MT-/- mice of the same treatment, $p < 0.05$.

genotype (Table 3). Liver also showed greater retention of ^{65}Zn in MT+/+ compared to MT-/- mice. Muscle and skin showed a significant decrease in ^{65}Zn over this time in both genotypes, whereas other tissues examined, including the liver and kidney were unaffected in this regard. While there were no significant differences between genotypes with respect to zinc release from skin and muscle, there was a trend towards greater ^{65}Zn release from skin and muscle at an earlier time in MT-/- mice i.e. 24% vs. 2% decrease (MT-/- vs. MT+/+) for muscle, and 28% vs. 15% decrease (MT-/- vs. MT+/+) for skin at 2 hr. The ^{65}Zn in plasma was not significantly affected, although the trend of increased (MT-/-) and decreased (MT+/+) plasma ^{65}Zn at 4 hr is consistent with previously observed findings for zinc concentration.

GD12 ethanol and teratogenicity

Only 1 MT+/+ foetus, from a dam treated with ethanol on GD12, exhibited an external abnormality (this was microphthalmia). Both MT+/+ and MT-/- foetuses exposed to ethanol had significantly lower body weights and crown-rump lengths compared to their respective saline controls (Table 4). In addition, placenti from MT+/+ ethanol foetuses were heavier than those from MT-/- ethanol foetuses. Although there were no between group differences in litter size, resorption sites were more prevalent in MT-/- ethanol pregnancies than in MT-/- saline.

DISCUSSION

Experimentation in this Chapter was directed towards clarifying and extending the findings of earlier work conducted in this laboratory (Carey et al, 2000a,b). Specifically, the following questions were addressed; does ethanol impair transfer of zinc to the foetus on GD8?, what is the source tissue of the excess zinc seen in the

Table 3. Distribution of radioactivity in non-pregnant MT+/+ and MT-/- mice prelabelled with ^{65}Zn and killed after injection of 25% ethanol (2, 4 hr only)

Tissue	Gt*	% Total counts/g tissue		
		0 hr	2 hr	4 hr
Liver	+/+	6.52 ± 0.54	6.68 ± 0.24[b]	6.56 ± 0.17
	-/-	6.03 ± 0.41	5.35 ± 0.38	5.69 ± 0.48
Skin	+/+	1.20 ± 0.14	1.03 ± 0.08	0.73 ± 0.04[a]
	-/-	1.23 ± 0.06	0.88 ± 0.14[a]	1.03 ± 0.20
Muscle	+/+	1.46 ± 0.06	1.43 ± 0.04	1.23 ± 0.04[a]
	-/-	1.70 ± 0.04	1.29 ± 0.02[a]	1.35 ± 0.12[a]
Pancreas	+/+	5.92 ± 0.48[b]	7.04 ± 0.23[b]	6.62 ± 0.22[b]
	-/-	3.75 ± 0.25	3.48 ± 0.11	4.34 ± 0.22
Kidneys	+/+	3.57 ± 0.52	4.05 ± 0.22	3.94 ± 0.26
	-/-	3.37 ± 0.05	3.44 ± 0.22	3.81 ± 0.23
Plasma	+/+	0.18 ± 0.01	0.21 ± 0.03	0.14 ± 0.01
	-/-	0.14 ± 0.01	0.16 ± 0.02	0.20 ± 0.02

Values represent the means ± SEM.

*Gt = genotype.

Data was analysed by two-way ANOVA, with Tukey's post hoc test.

There was a significant effect over time for both the skin and muscle ($p = 0.028$ and 0.004 respectively). There was a significant effect on genotype for the liver and pancreas values ($p = 0.01$ and 0.001 respectively).

Mice (at each time point): n = 4 (MT+/+), n = 3 (MT-/-).

[a] Significantly different from mice of the same genotype killed at 0 hr, $p < 0.05$.

[b] Significantly different from MT-/- mice at the same time point, $p < 0.05$.

Table 4. GD18 MT+/+ and MT-/- foetal parameters following GD12 maternal ethanol or saline treatment

	Genotype and treatment			
	+/+ Saline	+/+ Ethanol	-/- Saline	-/- Ethanol
No. litters	6	5	6	6
No. foetuses	39	29	40	30
Litter size	6.7 ± 0.5	6.0 ± 0.6	7.0 ± 1.2	5.3 ± 0.7
Resorptions/litter	1.5 ± 0.4	2.8 ± 0.7	1.0 ± 0.8	4.3 ± 1.1[a]
Weight (mg)	831 ± 14	778 ± 17[a]	863 ± 17	731 ± 17[a]
Crown-rump length (mm)	19.0 ± 0.1	18.5 ± 0.2[a]	19.2 ± 0.2	18.3 ± 0.2[a]
Placental weight (mg)	96 ± 2	108 ± 3[b]	99 ± 3	90 ± 2

Values represent the means ± SEM.

Data was analysed by two-way ANOVA, and Tukey's post-hoc test.

[a] Significantly different from saline treated cohort of the same genotype, $p < 0.05$.

[b] Significantly different from MT-/- cohort of the same treatment, $p < 0.05$.

plasma of MT-/- mice after ethanol?, does maternal ethanol given on GD12 exert a significant teratogenic effect?

In accordance with previous investigations, ethanol was found to induce expression of hepatic MT in GD8 mice (Carey et al, 2000a). Liver MT levels in saline treated mice were higher than expected for the stage of gestation (see Chapter 2, page 41), implying an inductive effect of the injection itself. Although there was still a significant difference in MT levels between saline and ethanol exposed mice, this did not impact on the respective plasma zinc concentrations, both of which were below normal values (approximately 13 μmol/L). It is possible that the higher liver MT concentrations in ethanol treated mice would have resulted in a further decrease in plasma zinc levels at a later time, and hence a difference when compared to saline injected mice. Indeed, it has previously been demonstrated that minimum plasma zinc levels are attained 8 hr after the initial ethanol injection (Carey et al, 2000a).

The accumulation of ^{65}Zn in maternal tissues following ethanol treatment was for the most part dependent on genotype. The increased flux of radioactivity to the liver, kidneys and pancreas of MT+/+ compared to MT-/- mice can be attributed to MT expression in these organs. Ethanol did not appear to have an additional inductive effect, except for in the livers of MT+/+ mice, where the increased expression of MT was associated with a further increase in retention of ^{65}Zn. The findings with respect to the maternal liver on GD8 are consistent with those obtained previously (Carey et al, 2000b), where it was found that transfer of ^{65}Zn was also directly related to MT expression. In that study however, genotype had no effect on accumulation of zinc in the kidneys and pancreas, which is contrary to what was seen here. One can only

assume that at GD12, the concentration of MT in the kidneys and pancreas is lower, and not a significant influence on zinc flux.

In contrast to the earlier work (Carey et al, 2000b), maternal ethanol administration on GD8 did not impair the transfer of ^{65}Zn to MT+/+ foetal tissue (in this case the whole conceptus). Interestingly, there was a definite genotypic influence at this stage of gestation, with MT+/+ concepti of both treatments retaining more radioactivity than corresponding MT-/- concepti. This MT-related effect is further underlined by the observed higher MT+/+ whole conceptus zinc concentrations, and can perhaps be explained by placental MT. The conceptus typically weighs approximately 30 mg on GD8, at which stage the placenta is by far the largest component. Placental tissue is likely to have a high level of MT expression around this time. Therefore one could argue that the higher MT+/+ conceptus zinc concentrations and retention of ^{65}Zn are a direct consequence of pervading placental MT concentrations, which are superimposed upon and may augment ethanol related changes in maternal zinc distribution. Ethanol may have had an effect on ^{65}Zn transfer to the foetus itself, however the small size of the foetus at this time makes dissection difficult and it is unlikely that there would be sufficient accumulation of radioactivity to enable meaningful measurement.

A finding consistent with previous experimentation is the marked increase in plasma zinc concentrations seen as a response to ethanol in MT-/- mice (Carey et al, 2000a). The results from the experiment where mice were prelabelled with ^{65}Zn, 3 days prior to ethanol challenge, indicate that ethanol causes zinc loss primarily from the muscle and skin. Direct effects of ethanol on skeletal muscle have been described (see Amaladevi et al., 1995), including the release of creatine kinase, so it might be

expected that zinc release may also occur in this setting. Indeed chronic ethanol exposure has been shown to reduce muscle zinc concentrations in rats (Gonzalez-Reimers et al, 1993). While it was initially thought that the absence of MT might facilitate zinc loss from muscle or skin, the present study did not show any difference between MT+/+ and MT-/- mice in the amount of zinc lost from these tissues following acute ethanol exposure. The major difference in plasma zinc concentrations between MT+/+ and MT-/- mice would therefore appear to reside in the ability of the MT+/+ dams to sequester zinc in the liver due to greatly elevated MT concentrations. Consequently, MT+/+ foetuses are exposed to much lower maternal plasma zinc concentrations.

Ethanol administered on GD12 did not have any externally observable teratogenic effect on MT+/+ or MT-/- foetuses as assessed on GD18. A study similar to this one conducted by Kronick (1976) also failed to demonstrate a teratogenic effect of ethanol at GD12 in mice. It is possible that the organogenic period was complete at the time of exposure in this instance, hence explaining a lack of effect, or indeed there may have been foetal abnormalities pertaining to the internal organs and central nervous system which were not detected. What these findings do imply however, is that the previously observed decrease in the transfer of ^{65}Zn to MT+/+ foetuses on GD12 (Carey et al, 2000b) is not an important determinant of foetal viability and external teratology. More subtle damage, escaping detection by the assessment criteria utilised here, cannot be ruled out.

Despite appearing morphologically normal, both ethanol exposed MT+/+ and MT-/- foetuses were significantly smaller in terms of weight and crown-rump length than saline controls. This is a well described, frequent outcome of prenatal ethanol

exposure, but one that has not previously been demonstrated in this laboratory. The smaller physical size of these foetuses is highly indicative of an ethanol induced nutrient deficiency underlying the delayed development. To this extent, the findings of the study conducted on GD8 show that ethanol has a significant effect on maternal glucose homeostasis. Ethanol is known to alter the redox state in the liver, interfering with gluconeogenesis and causing hypoglycaemia (Krebs et al, 1969; Madison et al, 1967). Both GD8 MT+/+ and MT-/- dams experienced pronounced hypoglycaemia as a response to ethanol treatment, with decreases of approximately 50% observed at 7 hr. As the foetus is wholly reliant on the maternal blood for nutrient supply, this drop in plasma glucose could well impair foetal supply, and be a contributing factor underlying the growth deficits seen on GD18 in this study.

There were no differences in average litter size between the treatment groups following GD12 ethanol, however compared to previous work where ethanol was injected on GD8 (Carey et al, 2000a) it would seem that all are below what is considered normal (control numbers were previously 8.9 and 8.2 in MT+/+ and MT-/- litters respectively vs. 6.7 and 7.0 in this instance). As the actual foetal size is significantly larger on GD12 compared to GD8, one can speculate that the injection at GD12 is directly causing damage, leading to fewer viable foetuses on GD18 regardless of the treatment.

As has previously been mentioned the timing of the teratogenic insult appears to be critical, not only with respect to the resultant abnormalities, but also in relation to the importance of the mediating influence of MT. Findings in this Chapter demonstrate that exposure to ethanol on GD12 does not exert an observable teratogenic effect, but does impair growth regardless of genotype. Previously, when administered on GD8,

ethanol was found to cause abnormal development in MT+/+, but not MT-/- foetuses (Carey et al, 2000a). Hence while MT expression in early organogenesis appears to be detrimental to the foetus, later expression exerts no external teratogenic effect. These observations are perhaps best explained by the natural induction of MT during pregnancy presented in Chapter 2. The level of innate MT expression on GD12 is such that ethanol does not cause significant additional induction (Carey et al, 2000b), while at GD8 there is further induction. Consequently one would predict the associated changes in maternal zinc distribution on GD8 to have a negative impact on the transfer of zinc to MT+/+ foetuses. The findings from labelling studies conducted in this Chapter did not demonstrate an effect to this extent. It may be that the method used is not suitable for the small size of foetal tissues on GD8.

The abnormalities resulting from GD8 ethanol treatment were previously attributed to changes in maternal plasma zinc concentrations, where in MT+/+ dams there was a decrease, while in MT-/- dams there was a significant increase from basal levels (Carey et al 2000a). The increase in plasma zinc in MT-/- dams was addressed in this Chapter and found to be as a result of zinc release from the muscle and skin in the absence of MT.

CHAPTER 4

Co-teratogenic effects of maternal dietary zinc deficiency and ethanol treatment.

Ethanol teratogenicity in metallothionein heterozygous mice.

The influence of foetal genotype in mediating teratogenicity.

INTRODUCTION

Zinc deficiency is a frequent phenomenon in both undeveloped and developed societies (Prasad, 1996; Sandstead, 1991). Manifestation, however, may be subtle and as a consequence remain undetected, especially in western populations where zinc deficiency is generally mild. Conversely, the effects of extreme dietary zinc deficiency have been documented almost exclusively in undeveloped countries and are most apparent in the form of growth, development and cognitive deficits (discussed in Chapter 1). Both human and animal studies have demonstrated that a state of maternal zinc deficiency during pregnancy is also associated with a multitude of negative outcomes that include foetal abortion, growth and development impediments, and teratogenicity (reviewed by King, 2000).

MT plays an important role in maximising zinc retention during the deficient state, where expression in non-gut tissues ultimately results in increased fractional absorption. MT-/- mice have a decreased ability to maintain homeostasis in zinc deficient environments. During pregnancy this is most evident in the observed increase in the frequency of abnormal foetuses from MT-/- mice fed zinc deficient

diets (Andrews and Geiser, 1999; Rofe et al 1999a). This finding is in direct contrast to those associated with ethanol teratogenicity, where as demonstrated previously (Carey et al, 2000a) and in Chapter 2, MT+/+ foetuses display an increased incidence of abnormalities compared to MT-/- foetuses. Hence depending on circumstances, the expression of MT can be either beneficial or deleterious to foetal development.

Current knowledge suggests that the foetal insult of maternal zinc deficiency (albeit mild) coupled with ethanol exposure is a relatively common occurrence in many pregnancies. In rodents this combined insult leads to further increased foetal teratology as compared to either agent alone (Keppen et al, 1985; Miller et al, 1983). It is not known how this effects foetuses from MT-/- dams. Individually these teratogenic insults exert what would appear to be opposite effects on zinc homeostasis in MT-/- mice. Ethanol causes zinc to be released from muscle and skin in MT-/- dams, and the resultant increase in the maternal plasma zinc concentration appears to be beneficial in maintaining foetal zinc supply which is otherwise compromised in the MT+/+ setting. While when MT-/- dams are maintained on a zinc deficient diet, the lack of MT results in exacerbated zinc deficiency and resultant foetal teratogenicity when compared to what occurs in the MT+/+ scenario. The first section of this Chapter investigates the synergistic action of maternal dietary zinc deficiency and ethanol exposure in mediating teratogenicity in MT+/+ and MT-/- foetuses. Both zinc deficiency and ethanol are known to interfere with bone development, hence in addition to the standard assessment of foetal teratology, bone development will be examined in this study.

Investigation in the second half of this chapter addresses several issues via the use of mice heterozygous for MT (MT+/- mice).

The question as to how foetuses from MT+/- dams fare in response to ethanol insult, and/or dietary zinc deficiency has not yet been addressed. Indeed there are few reports of experimentation involving MT+/- mice in the wider literature, hence it is not surprising that the MT response in these mice is poorly characterised. Experience with MT+/- mice in this laboratory suggests that induction of MT (both protein and mRNA) is approximately half that of MT+/+ mice. Changes in plasma and liver zinc concentrations are also less extreme than those that occur in MT+/+ mice following ethanol treatment. One would therefore expect that, in relation to the teratogenicity of ethanol, foetuses from MT+/- dams would be half as affected as MT+/+ foetuses, while in a zinc deficient setting foetuses from MT+/- dams would display a degree of teratology half of that seen in MT-/- foetuses.

Thus far investigation has revolved around the central hypothesis that ethanol-related teratogenicity, and the importance of MT, are solely as a consequence of changes in the maternal environment, which then impact deleteriously on the foetus. Experimental evidence supports this notion. Nevertheless, the question as to whether foetal genotype plays a role in mediating the outcome is one that should not be ignored. This issue is also examined in this Chapter by employing breeding combinations that result in differing maternal and foetal genotypes.

MATERIALS AND METHODS

Synthetic diets

Synthetic diets were egg white based, and composed as seen in Tables 1, 2 and 3. The constituents of the diet were combined in a large commercial dough mixer (OEM VE201, Italy) in the following order:

Cellulose and starch added, mixed for 20 min.

Rest of dry ingredients added, mixed for 30 min.

Corn oil added, mixed for 30 min.

Water containing $ZnSO_4$ added (see below), mixed until smooth consistency obtained.

The basic mixture (with no added zinc) was analysed by flame atomic absorption spectroscopy, and found to have a zinc content of approximately 1 mg/kg (1 ppm). Appropriate amounts of aqueous ZnSO4 were added to the mixture to produce deficient and normal feed with zinc levels of 5 and 50 ppm respectively. For each kg of dry mix, 600 mL of water (containing ZnSO4) was added. The mixture was placed in plastic trays up to a thickness of approximately 10 mm, and dried for 7-8 days at 37°C. Zinc concentrations were confirmed via flame atomic absorption spectroscopy.

Table 1. Composition of synthetic diet.

Ingredient	g or mg/kg
Egg white (spray dried)	180 g
Starch	430 g
Sucrose	200 g
Cellulose	30 g
Choline bitartrate	2 g
Corn oil	100 g
Biotin	20 mg
Mineral mixture (AIN-93G)	35 g
Vitamin mixture (AIN-93VX)	10 g

Table 2. Constituents of the mineral mixture used for the preparation of synthetic diets.

AIN-93G mineral mixture	
Ingredient	g or mg/kg mixture
Calcium carbonate	357 g
Monopotassium phosphate	196 g
Potassium citrate monohydrate	70.78 g
Sodium chloride	74 g
Potassium sulphate	46.6 g
Magnesium oxide	24 g
Manganese carbonate	0.63 g
Copper carbonate	0.3 g
Sodium metasilicate.$9H_2O$	1.45 g
Icing sugar	220.97 g
Potassium iodate	10 mg
Sodium selenate (anhydrous)	10.3 mg
Ammonium molybdate.$4H_2O$	7.95 mg
Chromium potassium sulphate.$12H_2O$	275 mg
Lithium carbonate	15.1 mg
Boric acid	81.45 mg
Sodium fluoride	63.5 mg
Nickel chloride.$6H_2O$	63.6 mg
Ammonium vanadate	6.6 mg
Ferric citrate.$6H_2O$	6.06 mg

Table 3. Constituents of the vitamin mixture used for the preparation of synthetic diets.

AIN-93-VX vitamin mixture	
Ingredient	g/kg mixture
Nicotinic acid	3.00
D-Calcium pantothenate	1.60
Pyridoxine HCl	0.70
Thiamine HCl	0.60
Riboflavin	0.60
Folic acid	0.20
D-Biotin	0.02
Vitamin B12 (0.1% triturated in mannitol)	2.50
Alpha tocopherol powder (250 U/g)	30.00
Vitamin A polmitate (250,000 U/g)	1.60
Vitamin D3 (4,000,000 U/g)	0.25
Phylloquinone	0.075
Powdered sucrose	959.66

Animals and mating procedure

See page 36 for animal information.

Mice heterozygous for MT were produced by mating female MT+/+ mice with male MT-/- mice.

Teratogenicity following maternal dietary zinc deficiency and ethanol treatment

Prior to mating, MT+/+ and MT-/- female mice were fed the normal synthetic diet containing 50 ppm of zinc and distilled water for an acclimatisation period of 3 weeks. During the mating period male and female mice were fed the normal synthetic diet, and distilled water. Following successful copulation, females were housed in cages with stainless steel wire bottoms (to prevent coprophagy) and fed the zinc deficient diet (5 ppm zinc) from GD1-GD9, and then the normal synthetic diet to GD18. Maternal treatment with ethanol or saline was on GD8 as in previous experiments (see page 36). Processing of maternal and foetal tissues on GD18 is described on pages 36, 37. Foetal bone development was also assessed.

Heterozygous cross studies

To determine whether foetal genotype is an important factor in mediating teratology, GD18 foetuses from MT+/- dams (mated with MT+/- sires) were genotyped, and examined following previously described maternal ethanol exposure on GD8, dietary zinc deficiency (from GD1-GD9), and dietary zinc deficiency in addition to ethanol treatment. Foetal liver MT and zinc levels were determined.

Note: foetal data was also pooled (irrespective of genotype) for comparisons of maternal effect.

Bone development

This staining method of Uriu-Adams et al. (2001) was slightly modified to assess the extent of bone and cartilage development in GD18 foetuses:

Following external examination and growth measurements, foetuses were immersed in 70°C water for 15 sec to facilitate the removal of skin. Eyes and viscera were also removed. Evisceration was achieved by making a small slit at the base of the abdomen, and then extruding internal organs, taking care not to cut or damage the ribs or vertebrae.

Foetuses were fixed in approximately 10 mL of 95% ethanol for a minimum of 1 week, then placed in acetone (10 mL) for 24 hr to remove fatty tissue from the carcass, and then in 1% KOH for 24 hr to facilitate soft tissue digestion.

Foetuses were then stained with Alizarin red and Alcian blue.

The staining solution (10 mL per foetus) contained the following:

0.3% filtered alcian blue in 70% ethanol:	1 volume
0.1% filtered alizarin red s in 95% ethanol:	1 volume
Acetic acid:	1 volume
70% ethanol:	17 volumes

Note: the stain solutions were saturated, and filtered through 5 μm Acrodisc syringe filters.

Alcian blue 8 GX was obtained from Sigma Chemical Co., St Louis , MO.

Alizarin red S was obtained from Searle Diagnostics, High Wycombe, England.

Duration of staining was 72 hr.

Following staining, de-colourisation and further maceration was achieved by placing foetuses in:

10 mL of 20% 1 % KOH, 80% glycerol for 72 hr

10 mL of 50% 1 % KOH, 50% glycerol for 72 hr

10 mL of 100% glycerol indefinitely

Foetuses were then examined under low power magnification using the method of Aliverti et al. (1979). This involved counting the number of ossification centres in specific regions (see Figure 1), and counting the number of ribs. Centres were scored as either 1 or 0, with 1 designated to centres containing any degree of ossification

Genotyping offspring of the heterozygote cross

Offspring of the MT+/- cross were genotyped using the method of Michalska and Choo (1993). Briefly, this involved initial digestion of approximately 200 mg of foetal tissue using proteinase K (Merck KgaA, Darmstadt, Germany), followed by DNA extraction using phenol chloroform, and purification using ethanol. Polymerase chain reaction (PCR) was then performed, using the mixture outlined in Table 4.

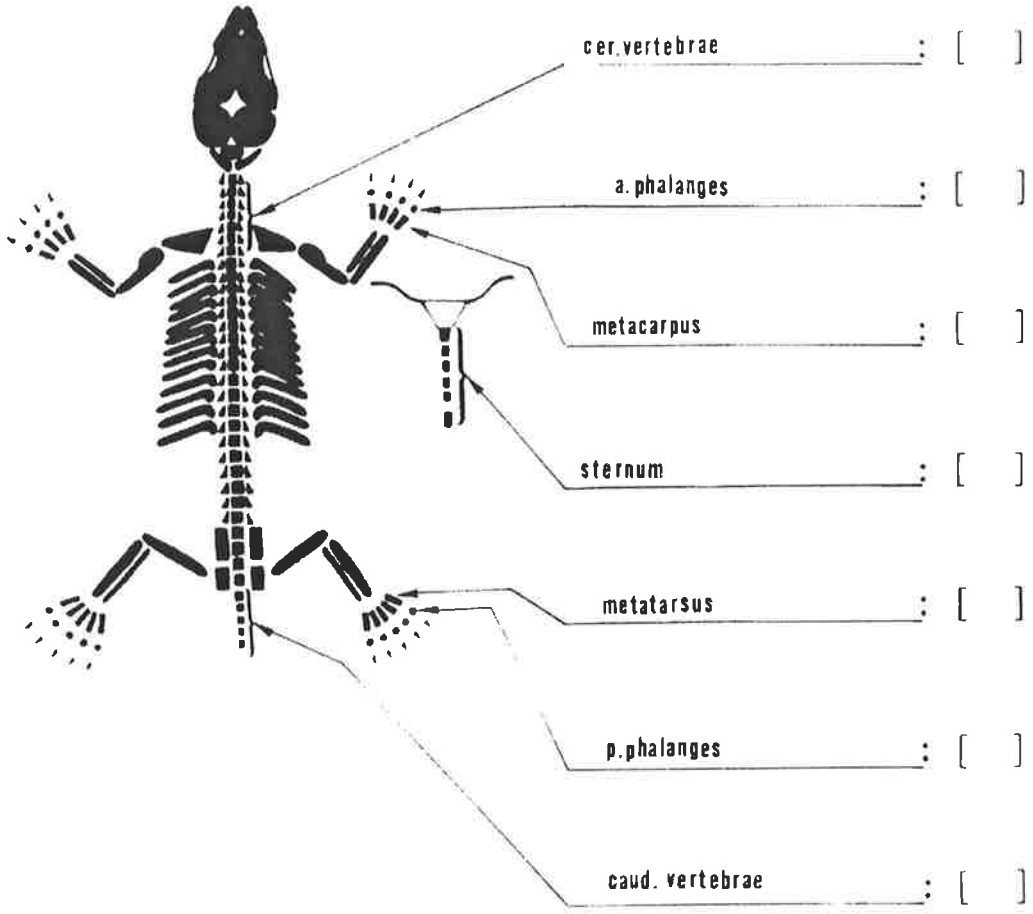


Figure 1. Location of centres examined for evidence of ossification in GD18 foetuses stained with Alizarin red and Alcian blue. Note: cervical vertebrae ossification was not assessed. Image taken from Aliverti et al, 1979.

Table 4. The PCR mixture for genotyping foetuses from MT heterozygous crosses.

Component	Volume (μL) per sample
Sterile water	30.5
10X PCR buffer	5
$MgCl_2$	3
DNTP's (2 mM mixture)	5
Primer 2 (100 ng/μL)	2.5
Primer 3 (100 ng/μL)	2.5
Gold TAQ polymerase (Perkin Elmer)	0.2

Primer sequences were as followed:

P2 = TCGTCCAACGACTATAAAGA

P3 = AAGAAACCAGAGTTAGACTC

Thermal cycling consisted of:

1. 12 min at 95°C

2. 2 min at 95°C

   2.5 min at 55°C

   3 min at 72°C

Step 2 was repeated 35 times.

3. 7 min at 68°C

PCR product was cleaved using *Xba* 1 (New England Biolabs inc., Beverly, MA), electrophoresed on a 2% agarose gel, and the resultant fragmentation visualised by fluorescent imaging. Fragments were present at 260 base pairs (bp), 126 and 154 bp, and 126, 154, and 260 bp for MT+/+, MT-/- and MT+/- foetuses respectively.

Metallothionein analysis

See page 37.

Zinc analysis

See page 39.

Statistical analysis

All data were compared by two-way ANOVA, except for that pertaining to the observed vs. expected comparison for foetal genotype following the heterozygote cross, where Chi-square analysis was used.

A binomial transformation was performed on data pertaining to foetal abnormalities before analysis.

For further details regarding statistics see page 39.

RESULTS

Teratogenicity following maternal dietary zinc deficiency and ethanol treatment

Comparisons were made with previously obtained foetal data from MT+/+ and MT-/- dams treated with ethanol or saline.

The feeding of a 5 ppm zinc diet from GD1 to GD9 did not significantly impact on the size or the number of resorption sites (Table 5) in a litter. However, for all comparisons (except for MT-/- ethanol vs. MT-/- ethanol + zinc deficiency) regardless of treatment, there was a trend for litter size to be lower and the number of resorptions sites increased as a consequence of gestational zinc deficiency.

MT+/+ foetuses from dams treated with ethanol on GD8 displayed increased teratology compared to MT+/+ saline injected controls, and corresponding MT-/- foetuses. There was no difference in the percentage of abnormal foetuses between the MT+/+ ethanol group and the MT+/+ ethanol group where maternal dietary zinc deficiency was an additional factor.

Table 5. GD18 foetal data from MT+/+ and MT-/- dams fed normal or zinc restricted diets from GD1-GD9, and treated with ethanol or saline on GD8.

	Genotype and Treatment							
	+/+ Saline	+/+ Ethanol	+/+ Saline, ZnD*	+/+ Ethanol, ZnD	-/- Saline	-/- Ethanol	-/- Saline, ZnD	-/- Ethanol, ZnD
No. litters	7	8	6	7	5	5	6	7
No. foetuses	62	50	46	38	41	37	42	55
Litter size	8.9 ± 0.5	6.3 ± 1.1	7.7 ± 0.6	5.4 ± 0.8	8.2 ± 0.2	7.4 ± 0.4	7.0 ± 1.1	7.9 ± 0.7
Resorptions/litter	0.57 ± 0.30	1.50 ± 0.85	1.2 ± 0.5	2.3 ± 0.7	1.0 ± 0.55	2.0 ± 0.45	2.0 ± 0.6	2.6 ± 0.8
Abnormalities	3	12	2	7	3	1	1	4
% Abnormal foetuses	4.8	24[ab]	4.3	18.4[ab]	7.3	2.7	2.4	7.3
Weight (mg)	837 ± 13	802 ± 13[a]	902 ± 17[a]	956 ± 27[a]	880 ± 23	869 ± 34	832 ± 15	788 ± 13
Crown-rump length (mm)	19.08 ± 0.10	18.65 ± 0.13[bc]	19.43 ± 0.13	20.35 ± 0.22[ab]	19.26 ± 0.17	19.06 ± 0.31[c]	18.97 ± 0.15	18.58 ± 0.12

Values represent the means ± SEM where applicable.

* ZnD = Zinc deficient diet

All data was analysed by two-way ANOVA, except for that pertaining to abnormalities, where a binomial GLM was used. Tukey's post-hoc test was used.

[a] Significantly different from MT-/- foetuses of the same treatment and diet, $p < 0.05$.

[b] Significantly different from MT+/+ foetuses of the same diet, where dams were treated with saline, $p < 0.05$.

[c] Significantly different from foetuses of the same genotype and treatment, where dams were fed 5 ppm zinc, $p < 0.05$.

MT+/+ foetuses from dams fed 5 ppm dietary zinc from GD1-GD9 were heavier than their MT-/- counterparts, regardless of maternal treatment on GD8. Foetuses in the MT+/+ ethanol/zinc deficient group had larger crown-rump lengths than did MT+/+ ethanol, MT+/+ saline + zinc deficient and MT-/- ethanol + zinc deficient foetuses.

MT-/- foetuses from dams fed 5 ppm zinc and treated with ethanol were shorter than MT-/- foetuses from dams exposed to ethanol and fed a diet containing normal zinc.

Bone development in GD18 foetuses following maternal dietary zinc deficiency in addition to ethanol or saline injection on GD8

Ossification in the metacarpal bones was more pronounced in MT-/- compared to MT+/+ foetuses (Table 6). This effect was independent of maternal treatment. MT-/- foetuses from dams treated with saline on GD8 displayed greater metatarsal ossification than their MT+/+ counterparts.

No effects of treatment or genotype were apparent in the other centres examined.

One foetus in each of the MT+/+ ethanol, MT-/- saline and MT-/- ethanol groups was found to have a rudimentary rib.

Teratogenicity following GD8 ethanol exposure in heterozygous dams

Foetal data from the heterozygous cross in which dams were injected with ethanol on GD8 was compared with previously obtained data concerning corresponding teratology in the MT+/+ and MT-/- setting. This comparison was made to assess the importance of maternal genotype, hence foetal genotype from the MT+/- cross was not taken into consideration.

Table 6. Extent of GD18 MT+/+ and MT-/- foetal bone ossification from dams fed a diet containing 5 ppm zinc from GD1-Gd9, and injected with ethanol or saline on GD8.

	Maternal genotype and treatment			
	MT+/+		MT-/-	
Ossification centre	Saline	Ethanol	Saline	Ethanol
Metacarpus[1]	6.35 ± 0.15	6.71 ± 0.24	7.61 ± 0.18[a]	7.52 ± 0.19[a]
Metatarsus[1]	8.00 ± 0	8.47 ± 0.21	8.94 ± 0.24[a]	8.24 ± 0.14
Anterior phalanges[1]	0.46 ± 0.27	1.65 ± 0.58	2.83 ± 0.60	0.95 ± 0.31
Posterior phalanges[1]	0	1.0 ± 0.58	0.17 ± 0.17	0
Sternum	5.12 ± 0.18	5.24 ± 0.18	4.78 ± 0.29	4.81 ± 0.23
Caudate vertebra	2.58 ± 0.16	3.18 ± 0.32	3.00 ± 0.26	2.71 ± 0.21
Ribs (number)[1]	25.92 ± 0.12	26.00 ± 0.12	26.00 ± 0	26.00 ± 0

Values represent the means ± SEM.

Data was analysed by two-way ANOVA, and Tukey's post-hoc test.

[1] Left and right ossification centres and individual ribs were counted independently and then added together.

[a] Significantly different from corresponding MT+/+ group ($p < 0.05$).

Following ethanol injection on GD8, maternal genotype did not significantly impact on the litter size, number of resorption sites or foetal weight as assessed on GD18 (Table 7). The percentage of abnormal foetuses in the MT+/+ group was 8 and 3 times higher than in the MT-/- and MT+/- groups respectively. Although there were more abnormal foetuses from MT+/- compared to MT-/- dams, the difference was not significant. Foetuses in the MT+/+ group were shorter than foetuses from both other groups, but significantly only from the MT+/- group.

Teratogenicity and foetal genotype following maternal ethanol treatment

In the control setting, where MT+/- dams were injected with saline on GD8, there was no bias towards foetuses of a particular genotype being less or more effected in terms of abnormalities, weight or crown-rump length as assessed at GD18 (Table 8). This was also found to be the case when dams were treated with 25% ethanol on GD8 (Table 9).

There was an insignificant tendency for MT-/- foetuses to be underrepresented and MT+/+ foetuses overrepresented when dams were injected with saline, while when dams were treated with ethanol MT-/- foetuses were overrepresented at the expense of MT+/- foetuses.

Liver MT concentrations in MT+/- foetuses were approximately half those of MT+/+ foetuses, while liver zinc levels were almost linearly related to MT expression.

Table 7. GD18 foetal data from MT+/+, MT-/- and MT+/- dams exposed to ethanol on GD8

	Maternal genotype		
	+/+	-/-	+/-
No. litters	8	5	5
No. foetuses	50	37	50
Litter size	6.3 ± 1.1	7.4 ± 0.4	8.0 ± 0.4
Resorptions/litter	1.50 ± 0.85	2.0 ± 0.45	1.6 ± 0.8
Abnormalities	12	1	4
% Abnormal foetuses	24	3[a]	8[a]
Weight (mg)	802 ± 13	869 ± 34	832 ± 20
Crown-rump length (mm)	18.65 ± 0.13	19.06 ± 0.31	19.3 ± 0.1[a]

Values represent the means ± SEM where applicable.

All data was analysed by two-way ANOVA, except for that pertaining to abnormalities, where a binomial GLM was used. Tukey's post-hoc test was used.

[a] Significantly different from MT+/+ group, $p < 0.05$.

Table 8. Measures of teratogenicity and development in GD18 foetuses from MT+/- dams mated with MT+/- sires, and injected with saline on GD8.

Foetal genotype	Foetal genotype		
	+/+	-/-	+/-
No. Foetuses	17	9	24
No. Foetuses expected	12.5	12.5	25
No. Abnormal foetuses	0	0	0
% Abnormal foetuses	-	-	-
Weight (mg)	810 ± 21	846 ± 26	807 ± 22
Crown-rump length (mm)	18.80 ± 0.22	19.37 ± 0.30	18.60 ± 0.24
Liver MT (nmol Cd bound/g wet weight)	126 ± 8[a]	NA	50 ± 3
Liver zinc (µmol/L)	789 ± 38[ab]	408 ± 17[a]	612 ± 24

Values represent the means ± SEM where applicable.

Data was analysed by two-way ANOVA, except for the observed vs. expected comparison for foetal numbers, where a Chi-square test was used. Tukey's post-hoc test was used.

[a] Significantly different from MT+/- group ($p < 0.05$).

[b] Significantly different from MT-/- group ($p < 0.05$).

Table 9. Measures of teratogenicity and development in GD18 foetuses from MT+/- dams mated with MT+/- sires, and injected with 25% ethanol GD8.

	Foetal genotype		
	+/+	-/-	+/-
No. Foetuses	11	15	14
No. Foetuses expected	10	10	20
No. Abnormal foetuses	2	2	0
% Abnormal foetuses	18	13	-
Weight (mg)	825 ± 51	818 ± 29	852 ± 32
Crown-rump length (mm)	19.43 ± 0.33	19.16 ± 0.10	19.37 ± 0.31
Liver MT (nmol Cd bound/g wet weight)	128 ± 14[a]	NA	50 ± 5
Liver zinc (μmol/L)	756 ± 46[b]	391 ± 23[a]	699 ± 68

Values represent the means ± SEM where applicable.

Data was analysed by two-way ANOVA, except for the observed vs. expected comparison for foetal numbers, where a Chi-square test was used. Tukey's post-hoc test was used.

[a] Significantly different from MT+/- group ($p < 0.05$).

[b] Significantly different from MT-/- group ($p < 0.05$).

Teratogenicity and foetal genotype following maternal ethanol treatment and dietary zinc deficiency

There were no teratogenic or developmental differences with respect to foetal genotype in response to dams being fed 5 ppm zinc diets from GD1-GD9, with or without ethanol treatment on GD8 (Tables 10, 11).

The number of MT-/- foetuses was slightly (but insignificantly) lower than expected, while MT+/- numbers were elevated.

As in the experiments where dams were fed normal diets and treated with ethanol or saline, liver MT concentrations in MT+/- foetuses were roughly half those of MT+/+ foetuses. However the hepatic MT levels in both MT+/+ and MT+/- foetuses from dams fed the 5 ppm zinc diet were almost double the levels in corresponding foetuses from dams fed the normal diet. Liver zinc concentrations were also altered with respect to the level of MT expression. These differences in hepatic MT and liver zinc were significant when compared by Student's t-test ($p < 0.05$). For these comparisons, as there were no treatment effects, maternal treatment on GD8 was disregarded and foetal MT and zinc data were pooled according to maternal dietary zinc content.

DISCUSSION

Mild zinc deficiency is without doubt common in modern society, as is the consumption of ethanol during pregnancy. Although figures are not readily available it seems logical that there are a significant number of zinc deficient females who also consume ethanol during pregnancy. The teratogenic effects of zinc deficiency and

Table 10. Measures of teratogenicity and development in GD18 foetuses from MT+/- dams mated with MT+/- sires, fed a synthetic diet containing 5 ppm zinc from GD1-GD9, and injected with saline on GD8.

	Foetal genotype		
	+/+	-/-	+/-
No. Foetuses	7	8	21
No. foetuses expected	9	9	18
No. Abnormal foetuses	0	0	0
Weight (mg)	865 ± 17	866 ± 44	842 ± 14
Crown-rump length (mm)	19.4 ± 0.16	19.23 ± .37	19.08 ± 0.11
Liver MT (nmol Cd bound/g wet weight)	202 ± 14[a]	NA	115 ± 9
Liver zinc (μmol/L)	887 ± 123[b]	328 ± 34[a]	739 ± 52

Values represent the means ± SEM where applicable.

Data was analysed by two-way ANOVA, except for the observed vs. expected comparison for foetal numbers, where a Chi-square test was used. Tukey's post-hoc test was used.

[a] Significantly different from MT+/- group ($p < 0.05$).

[b] Significantly different from MT-/- group ($p < 0.05$).

Table 11. Measures of teratogenicity and development in GD18 foetuses from MT+/- dams mated with MT+/- sires, fed a synthetic diet containing 5 ppm zinc from GD1-GD9, and injected with 25% ethanol on GD8.

	Foetal genotype		
	+/+	-/-	+/-
No. Foetuses	9	6	23
No. foetuses expected	9.5	9.5	19
No. abnormal foetuses	0	0	0
Weight (mg)	831 ± 25	790 ± 27	820 ± 17
Crown-rump length (mm)	19.05 ± 0.28	18.57 ± 0.21	18.88 ± 0.16
Liver MT (nmol Cd bound/g wet weight)	182 ± 17[a]	NA	86 ± 5
Liver zinc (μmol/L)	879 ± 115[ab]	483 ± 36[a]	647 ± 40

Values represent the means ± SEM where applicable.

Data was analysed by two-way ANOVA, except for the observed vs. expected comparison for foetal numbers, where a Chi-square test was used. Tukey's post-hoc test was used.

[a] Significantly different from MT+/- group ($p < 0.05$).

[b] Significantly different from MT-/- group ($p < 0.05$).

ethanol exposure as separate events have been described in previous Chapters. In animal experiments it has been demonstrated that the combined maternal insult of dietary zinc deficiency and ethanol exposure is associated with increased teratogenicity as compared to either alone (Keppen et al, 1985; Miller et al, 1983). Initial experiments in this Chapter examined the effects of maternal dietary zinc deficiency with or without ethanol treatment on foetal outcome in MT+/+ and MT-/- mice.

Pregnant MT+/+ and MT-/- mice were fed synthetic zinc diets containing 5 ppm zinc from GD1 to GD9. This regimen of zinc content and duration of feeding was designed to maintain pregnancy, whilst inducing a moderate deficient state in the dam and foetus around the time of early organogenesis, hence exerting a teratogenic effect. Foetuses from dams of both genotypes displayed no overt signs of teratogenicity following application of this protocol. One can conclude that the feeding regimen did not have a major impact on maternal and foetal zinc stores or homeostasis, and that only a relatively mild deficiency was attained. There is certainly evidence for a mild zinc deficient effect in the MT-/- setting. As previously mentioned, these mice are more susceptible to the deleterious effects of zinc deficient environments than are MT+/+ mice (Andrews and Geiser, 1999; Rofe et al 1999a). Here, although teratogenicity was not observed, MT-/- foetuses from dams fed the zinc deficient diet tended to be smaller in terms of weight and crown-rump length than corresponding MT+/+ foetuses, and MT-/- foetuses from dams fed the normal zinc diet.

Other studies of this nature have shown a definite teratogenic effect of maternal dietary zinc deficiency in both normal and MT-/- mice. Sato et al. (1985) investigated the teratogenic effects of feeding MT+/+ dams 0.5 ppm zinc from GD1-GD18 or

from GD5-GD14. A very high degree of teratology was observed on GD18, where 100 and 96% of foetuses in the two groups respectively were malformed in some manner (micromelia, exencephaly and oligocdactyly were most common). Similarly, Dreosti et al. (1986) found that dams fed a diet containing 10 ppm zinc (or less) from GD1 onwards gave rise to a high percentage of abnormal foetuses. In another study, Dalton and co-workers (1996) maintained normal pregnant mice on a 0.5-1.5 ppm zinc diet from GD1-GD14, examining foetuses on GD14. This protocol resulted in 50% foetal resorptions and significant dysmorphology, where neural tube, craniofacial and limb bud defects were observed in 55% of surviving foetuses. Andrews and Geiser (1999) fed MT+/+ and MT-/- dams diets containing 1 ppm or 5 ppm zinc from GD1-GD14, assessing foetal development on GD14. The 1 ppm diet was highly teratogenic in both genotypes, however more so for MT-/- foetuses, where dysmorphology was 53%, vs. 32% in MT+/+ foetuses. The 5 ppm regimen exerted a slight effect in the MT+/+ setting compared to normal (4.7% of foetuses were abnormal vs. 0% of controls), while again this effect was heightened in the MT-/- setting where 13.6% of foetuses were abnormal.

There are several critical methodological considerations in studies of this nature, which perhaps may explain why an effect was not observed in the present investigation. The zinc content of the diet is one of these. A balance must be achieved such that the deficiency induced is not so severe as to prevent pregnancy from proceeding, while also restricting zinc availability such that an effect can be observed without having to use excessive numbers of animals. Another important consideration is the duration of feeding the zinc deficient diet, with the aim again being to reach a balance between extreme deficiency and sufficiency. In all the studies mentioned previously either the dietary zinc content was lower, or the duration of feeding was

longer than what was used here. In short, the zinc deficient insult to the dam was more extreme. One final issue worth addressing is the manner in which the constituents of the basic diet affect zinc absorption. Experimentation utilising synthetic diets generally use either an egg white, soy protein or casein base mix. There are marked differences in the bioavailability of zinc depending on the base, with egg white providing the highest (Uenishi et al, 1993). This study and all those above, bar the work by Dreosti and colleagues (1986), used diets that were egg white based. This perhaps explains why Dreosti was able to demonstrate teratology following the feeding of soy based diet containing 10 ppm zinc, a level not considered to be overly restrictive.

Assuming that MT+/+ dams in these experiments were at most only mildly zinc deficient, it is hardly surprising to note that the double insult of ethanol injection and dietary zinc restriction did not exert a synergistic teratogenic effect. The abnormalities observed in this group can be attributed to the effects of ethanol alone.

A number of studies were conducted assessing the effects of maternal dietary zinc deficiency and/or ethanol exposure in the MT+/- setting. Here the aims were two-fold; firstly, to compare foetal outcome for all three maternal genotypes, and secondly to assess, by use of a heterozygote cross, whether foetal genotype is a consideration in resultant teratology.

Although ethanol did not exert a significant teratogenic effect on foetuses from MT+/- dams (mated with MT+/- sires), there was a definite trend for these foetuses to be more affected than those from MT-/- dams. Foetuses in the MT+/- group displayed intermediate effects in terms of abnormalities and weight when compared to foetuses

from MT+/+ and MT-/- dams as assessed on GD18. These findings are in turn consistent with an intermediate ethanol induced change in MT+/- maternal-foetal zinc homeostasis on GD8, in which liver MT induction and the subsequent decrease in plasma zinc concentration is half of that seen in MT+/+ mice.

The notion that genotype and hence MT expression in the foetus may play a part in mediating teratogenicity was examined with respect to maternal ethanol insult with or without concurrent dietary zinc deficiency. Following heterozygote mating, none of the four treatment paradigms was found to favour the survival of foetuses of a specific genotype, or have any differential genotypic effects with respect to the percentage of abnormal foetuses, weight or crown-rump length measures. It should be noted that the maternal feeding of a 5 ppm zinc diet did not appear to have an effect on the foetus. This is consistent with the already discussed findings in MT+/+ and MT-/- mice, where it was concluded that only a mild (MT-/-) or very mild (MT+/+) maternal zinc deficiency was achieved. Ethanol in concert with the maternal feeding of 5 ppm zinc did not have a teratogenic effect in the MT+/- setting. This is a somewhat puzzling outcome, and one that is difficult to explain. Perhaps subtle changes in liver MT and plasma zinc levels induced by the consumption of the deficient diet altered, or reduced the MT and zinc responses following the ethanol challenge at GD8.

Nevertheless, these findings support the argument that the MT/zinc related aspects of ethanol teratology are primarily dependent on maternal, and not foetal, genotype. This makes sense in light of previous work, and other known functions of MT. Expression of MT in the foetus would convey beneficial effects if anything. As discussed in Chapter 1, MT has demonstrated antioxidant properties that in the foetus may help

prevent damage attributed to free radicals liberated in the metabolism of ethanol. Despite this, MT-/- foetuses are less susceptible to the deleterious of maternal ethanol exposure than MT+/+ foetuses, hence designating any antioxidant function of MT in the foetus to be of minimal importance. It should be emphasised that the relatively small number of foetuses in each genotypic group, and the low number of abnormalities observed in these studies preclude a categoric statement being made to the point of totally ruling out foetal genotype as a mediating factor.

An interesting observation arising from the MT+/- studies was the increased liver MT and zinc concentrations in MT+/+ and MT-/- foetuses from dams fed the zinc deficient diet from GD1-GD9 compared to those where dams were fed the normal diet. This up-regulated expression of MT at GD18 was independent of maternal treatment on GD8, and hence can be designated a response to decreased available zinc. The fact that liver MT concentrations were heightened well after the cessation of maternal dietary zinc deficiency implies an effect on MT gene responsiveness. Foetal liver formation begins around GD8 (the start of organogenesis), a time in these studies where there was almost certainly a pervading mild maternal-foetal zinc deficiency. With this in mind it makes sense for a compensatory up-regulation of MT gene responsiveness to have occurred. If the zinc deficient setting were maintained this would be an obvious benefit, particularly in late gestation where normal MT related accumulation of zinc in the liver is thought to be important for postnatal growth (Coyle et al, 2002). There is evidence suggesting that this purported change in MT gene responsiveness is long lasting, or perhaps permanent. A study by Vruwink et al. (1988) found that 10 week old pups originating from dams fed a 5 ppm zinc diet from GD7 onwards displayed an enhanced liver MT response following zinc injection when compared to pups from dams who were fed a normal diet during gestation. In

light of the work conducted by Barker looking at the foetal origins of adult disease (Barker et al, 1989; Barker, 1990), it would be of considerable interest to determine whether this proposed increased MT gene responsiveness has any detrimental effect in later life.

Experiments in the Chapter addressed several important issues. Firstly, the co-teratogenicity effects of maternal dietary zinc deficiency and ethanol exposure were examined in MT+/+ and MT-/- mice. The zinc deficient diet did not exert an observable effect on MT+/+ foetal development, while only slightly retarding growth in MT-/- foetuses. Evidently the dietary zinc content caused only a mild zinc deficient state in the dams. Secondly, ethanol teratogenicity was assessed in foetuses from MT+/- dams mated with MT+/- sires. In terms of teratogenicity and growth impairment, there was a definite trend for foetuses from MT+/- dams to have effects intermediate between foetuses from MT+/+ and MT-/- dams. Finally, the importance of foetal genotype was assessed in terms of mediating ethanol teratogenicity, with the findings offering support for the notion that maternal genotype is the key mediating influence.

CHAPTER 5

Effect of ethanol on placental blood flow, and glucose transfer to foetal tissues.

INTRODUCTION

It has long been suspected that the teratogenic effects of ethanol are partly caused by impaired placental blood flow, with consequent hypoxaemia and nutrient insufficiency. The foetal outcomes associated with maternal ethanol exposure are certainly consistent with nutrient deficiency. Whether or not this is due to decreased blood flow, and/or other transport effects (such as changes in maternal stores in the case of zinc), remains to be conclusively ascertained. The findings from a handful of human and animal studies addressing this issue have been conflicting, with there being evidence both for (Savoy-Moore et al, 1989; Falconer, 1990; Jones et al, 1981) and against (Erskine and Ritchie, 1986; Leichter and Lee, 1982) an effect. Both of the two studies undertaken using a mouse model examined placental blood flow following chronic maternal ethanol exposure (Jones et al, 1981; Leichter and Lee, 1982).

There are a number of techniques commonly used for measuring blood flow, including ultrasonic (typically Doppler flowmetry), electromagnetic and microsphere based methods (Tabrizchi and Pugsley, 2000). Doppler flowmetry is the most widely used of these, however the high associated costs have seen the more economically affordable microsphere techniques routinely used in animal studies. Radiolabelled,

coloured and fluorescent microspheres have all been utilised for quantifying blood flow, with radiolabelled microspheres proving the most popular. Pioneering work in producing radiolabelled microspheres was conducted by Grimm and Lindseth (1958), who incubated glass microspheres in a nuclear reactor, facilitating the conversion of ^{23}Na contained in the glass to ^{24}Na. The major problem with these microspheres was the lack of size uniformity and short half-life of ^{24}Na. The development of ceramic microspheres overcame both of these problems (Hamlin et al, 1962), however both glass and ceramic microspheres proved to be heavier than red blood cells, the constituent of blood they were designed to mimic. Production of inert plastic microspheres addressed this issue, and they have since been in use for over thirty years.

The theory behind the use of radiolabelled microspheres as indicators of blood flow is relatively simple. Microspheres are injected into the arterial systemic circulation (usually via the left ventricle or atrium), distributed, and become lodged in small capillary beds. Tissues of interest are then dissected and gamma counted to ascertain radioactive (and hence microsphere) content. This does not give a measure of blood flow in mL/min, however this can be determined by taking a reference blood sample at the time of microsphere injection. Reference blood is sampled for a set period of time, at a constant, predetermined rate downstream from the site of injection (such as the femoral artery). Counting the radioactivity in the reference sample thereby provides a measure of blood flow with respect to radioactivity, and specific tissue/organ blood flow is then calculated using the following formula (Tabrizchi and Pugsley, 2000):

Tissue blood flow = tissue radioactivity × (reference sample flow ÷ reference sample radioactivity)

Much of the work using radiolabelled microspheres has been undertaken in larger animals, most notably sheep. Although technically more challenging, experimentation in rodents has been used to good effect in addressing a number of blood flow issues (McDevitt and Nies, 1976; Wetterlin et al, 1977; Armstrong and Laughlin, 1984; Sarin et al, 1990; Dowell et al, 1992; Gompertz et al, 1996). Studies in the laboratory of Lee used ^{57}Cobalt labelled microspheres to examine the effects of chronic maternal ethanol exposure on placental blood flow in rats, with ambiguous results (Jones et al, 1981; Leichter and Lee, 1982). The effects of acute ethanol administration have not previously been assessed in this setting or in mice at all. Hence one of the aims of this Chapter was to set up and then use a radiolabelled microsphere technique to determine the effects of acute maternal ethanol injection on placental blood flow in mice.

Decreased foetal glucose transfer is often discussed as a contributing mediator of the growth related deficits commonly associated with foetal ethanol exposure. Delivery of glucose to the foetus is dependent on three primary factors; the concentration of glucose in the maternal plasma, placental blood flow, and the activity of glucose transport proteins in the placenta. Little is known about the effect of ethanol on glucose transport mechanisms in the placenta, while the issue of blood flow has been raised earlier in this Chapter. In Chapter 3 it was shown that liver and blood glucose concentrations in mice at GD8 significantly decrease following maternal ethanol injection (Chapter 3, page 58). Findings from other studies also demonstrate ethanol induced hypoglycaemia (Madison, 1967). These changes in glucose homeostasis are

related to alterations in the cellular redox state, where a reducing environment leads to inhibition of gluconeogenesis (Krebs et al, 1969). With the decrease in maternal plasma glucose concentration one could predict that foetal supply might be impaired. To this extent several studies have indeed demonstrated decreased transfer of radiolabelled glucose to foetal tissues following chronic maternal ethanol exposure in the rat (Snyder et al, 1986; Singh et al, 1989). Investigation in the second half of this Chapter examined the effect of acute ethanol treatment on maternal-foetal glucose transfer in mice using a radiotracer technique.

MATERIALS AND METHODS

Animals and mating procedure

See page 36.

Radiolabelled microspheres

^{113}Tin (Sn) microspheres were used in these investigations as Sn is a relatively unreactive element in the body, and is not known to induce MT (Yamada and Koizumi, 1991). Microspheres in saline were 15 μm in diameter, with a specific radioactivity 500 MBq/g (Perkin Elmer Life Sciences, Inc., Boston MA). The half life of ^{113}Sn is 110 days.

Radiolabelled microsphere injection

Mice were anaesthetised with halothane, and a small piece of skin was removed ventral to the ribcage just above the heart. The microsphere solution (warmed to 37°C) was vortexed, and 150 μL withdrawn using a 26 gauge needle. The needle was then inserted between the ribs into the left side of the heart (approximately 1-2 mm

deeper than the insertion point), and the solution was slowly expelled. It should be noted that several different techniques were tested with respect microsphere injection, including cutting into the thoracic cavity and directly visualising the heart, and injecting with the skin intact. The final technique was a compromise in terms of being less invasive and more reliable than the other methods respectively. Forty five sec after the completion of the injection, mice were killed via cervical luxation, and the placenti (if pregnant), maternal liver, kidneys, pancreas, gut, heart, lungs, brain, muscle and skin were dissected, weighed and radioactivity measured using a gamma counter (model 5650, Canberra-Packard, Melbourne, Australia). A skin sample of approximately 2 cm^2 was removed from the dorsal side, while a 2 cm^2 section of abdominal muscle was also dissected. Microspheres do not pass through the placenta, hence radioactivity was not assessed in the foetus itself.

The calculation for organ/tissue blood flow was performed as followed:

[(Counts in tissue / total counts in all tissues measured) x 100]/ weight of tissue

To give % total counts retained/ g tissue, as an indicator of blood flow for each tissue.

There are two key issues with respect to successful injection of microspheres into the circulation, these are; placing of the needle into the left side of the heart and hence the systemic arterial circulation, and having adequate mixing of the microspheres such that distribution in the blood is uniform. Injection into the right side of the heart results in almost complete entrapment of microspheres in the lung, while a major difference in the counts retained between the left and right kidney is indicative of inadequate mixing (Heymann et al, 1977).

Hence data was excluded if:

1. There was more than 12% total counts/g tissue in the lung.
2. There was a major difference between the counts retained in the left and right kidney, defined as being equal to or greater than 10% total counts/g tissue.

Preliminary blood flow studies

The reproducibility and validity of the microsphere technique was investigated in non-pregnant MT+/+ mice. The effect of ethanol on blood flow was also examined by treating non-pregnant mice ip with 25% ethanol 45 min prior to microsphere injection. Blood ethanol concentrations following this dose are known to peak after approximately 45 min (Carey et al, 2000a).

Effect of ethanol on placental blood flow

GD14 MT+/+ and MT-/- mice were injected with ethanol or saline, and then 45 min later with ^{113}Sn microspheres as previously described. Maternal tissues as well as placenti were examined for changes in blood flow.

Effect of ethanol on foetal glucose transfer

The method of Rofe et al. (1988) was modified for these experiments.

Pregnant MT+/+ and MT-/- mice were injected with 25% ethanol or saline on GD14, and then 45 min later with a radiolabelled glucose solution. 2-deoxy[^{14}C]glucose (specific radioactivity = 10.8 GBq/mmol; Amersham Life Science, Piscataway, NJ) in 0.85% saline containing 1μmol/mL of D-glucose was administered sc into the nape of the neck. The 300 μL injection contained approximately 3,000,000 counts per minute. Mice were killed (see page 36) 45 min after the injection of glucose and the placenti,

foetuses, liver, kidneys, spleen, heart, brain and approximately 100 μL of blood were placed in 1 mL of ice cold 5% $HClO_4$ and homogenised using an Ultra-Turrax homogeniser (Janke and Kunkel, Staufen, Germany). Homogenates were centrifuged (14 000 × g for 4 min), and 100 μL of supernatant was transferred to a glass vial containing 10 mL of a liquid scintillation cocktail (Beckman Instruments Inc., Fullerton, CA). Sample radioactivity was quantified using a liquid scintillation counter (Minaxiβ Tri-Carb 4000 series, Canberra-Packard, Melbourne, Australia).

Statistical analysis

Data were compared Student's t-test and two-way ANOVA, using Tukey's post-hoc test to determine significance.

See page 39 for additional details regarding statistical analysis.

RESULTS

Preliminary blood flow studies

Injection of radiolabelled microspheres in non-pregnant MT+/+ mice resulted in a distribution of radioactivity (giving an indication of blood flow) as can be seen in Figure 1. The majority of the counts retained accumulated in the kidneys, heart and brain (>70%). Injection of 25% ethanol 45 min prior to the injection of microspheres did not significantly alter blood distribution except in the colon, where ethanol treatment was associated with increased levels of radioactivity. There was however, a trend towards increased gut blood flow after ethanol injection.

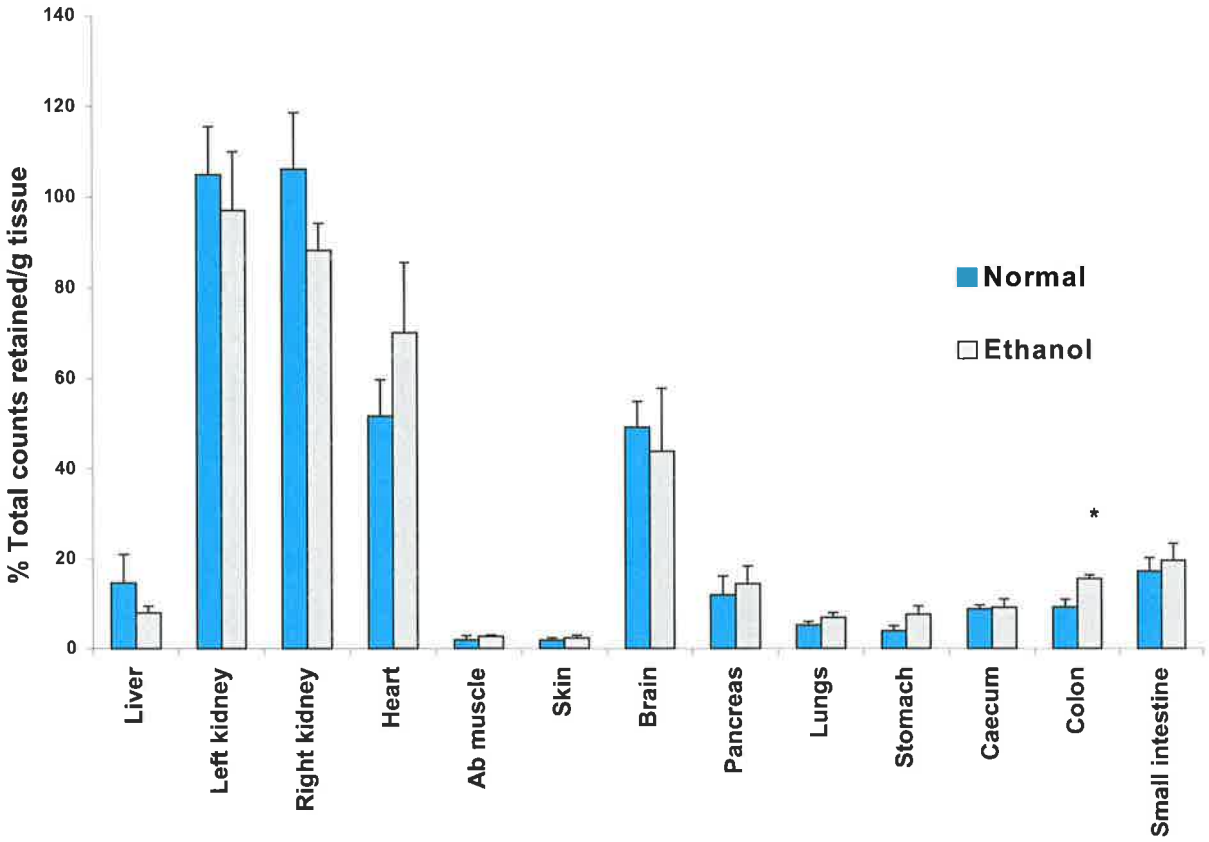


Figure 1. Blood flow was assessed in non-pregnant MT+/+ mice 45 min after ip injection of 25% ethanol. Microsphere injection was via the left ventricle, and mice were killed 45 sec after complete expulsion of the microsphere solution.

Points represent the mean ± SEM, n = 4.

* Significant difference between normal and ethanol treated mice ($p < 0.05$).

Percent successful injections

In total, 37 GD14 MT+/+ and MT-/- mice were used in experiments. Data was excluded from 10 of these mice as a result of either right ventricular/atrial injection, or poor mixing/uneven distribution between the left and right kidneys. Hence 73% of the injections were classified as successful.

Effect of ethanol on placental blood flow

In MT+/+ GD14 mice, ethanol treatment did not significantly alter placental blood flow, although the trend was for a slight decrease (Figure 2). Conversely, maternal ethanol injection in MT-/- mice significantly increased the amount of radioactivity retained in placental tissue by approximately 100 and 200% compared to placenti from saline injected MT-/- controls, and MT+/+ ethanol injected dams respectively.

Several maternal organs displayed differential blood flow. Both kidneys in MT+/+ saline exposed dams exhibited higher levels of radioactivity when compared to corresponding ethanol treated mice (Figure 3). MT-/- saline injected dams had increased radioactivity in the brain compared MT-/- ethanol and MT+/+ saline treated dams. While entrapment of microspheres in the caecum and colon of MT-/- ethanol injected dams was significantly greater than that in MT+/+ ethanol injected dams (Figure 4).

Effect of ethanol on foetal glucose transfer

Maternal ethanol injection did not effect the transfer of radiolabelled glucose to MT+/+ or MT-/- foetuses and placenti on GD14 (Figure 5). These measures were likewise unaffected by differences in genotype.

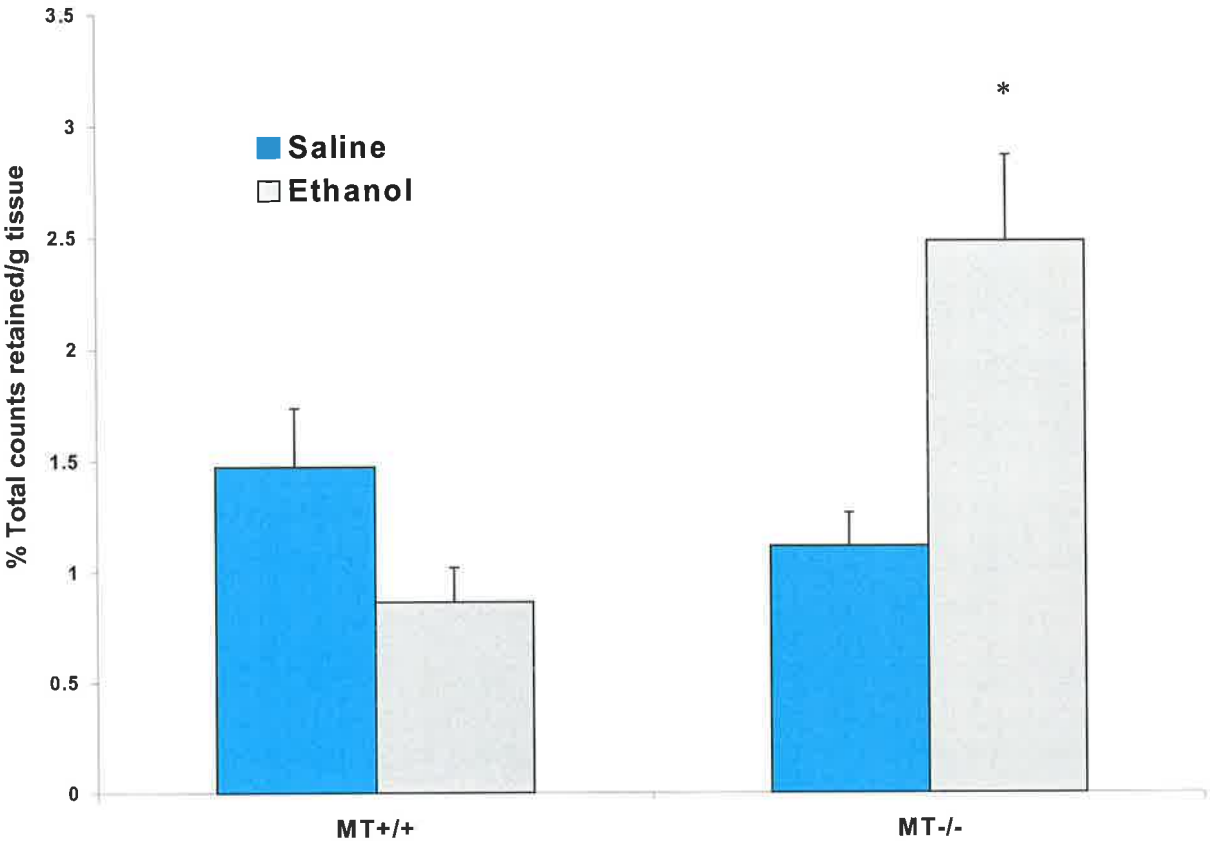


Figure 2. Blood flow to Gd14 MT+/+ and MT-/- placenti as assessed by radiolabelled ^{113}Sn microsphere distribution 45 minutes after maternal saline or ethanol injection.

Points represent the mean ± SEM, n = 36-58.

* Significantly different from MT+/+ ethanol and MT-/- saline group ($p < 0.05$).

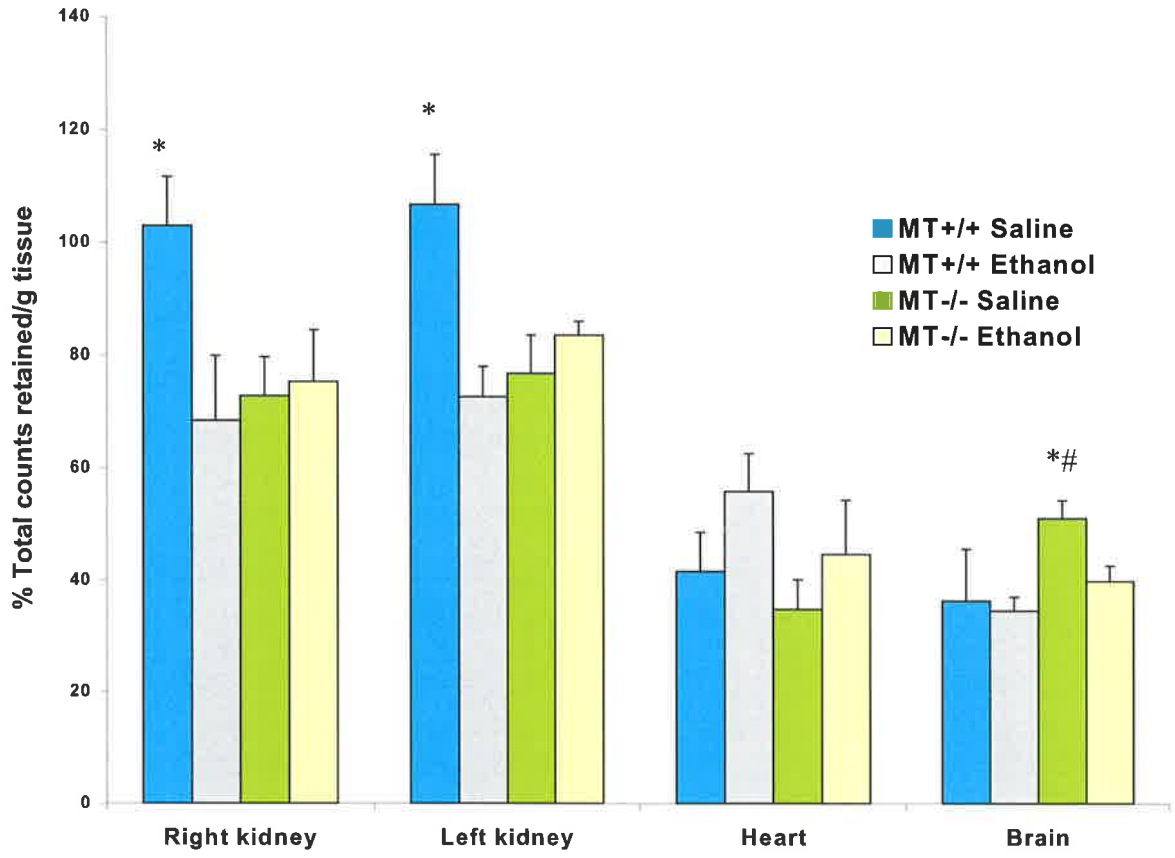


Figure 3. Blood flow to GD14 MT+/+ and MT-/- maternal organs as assessed by radiolabelled ^{113}Sn microsphere distribution 45 min after saline or ethanol injection. Points represent the mean ± SEM, n = 4-6.

* Significantly different from ethanol treated dams of the same genotype ($p < 0.05$).

Significantly different from MT+/+ dams of the same treatment ($p < 0.05$).

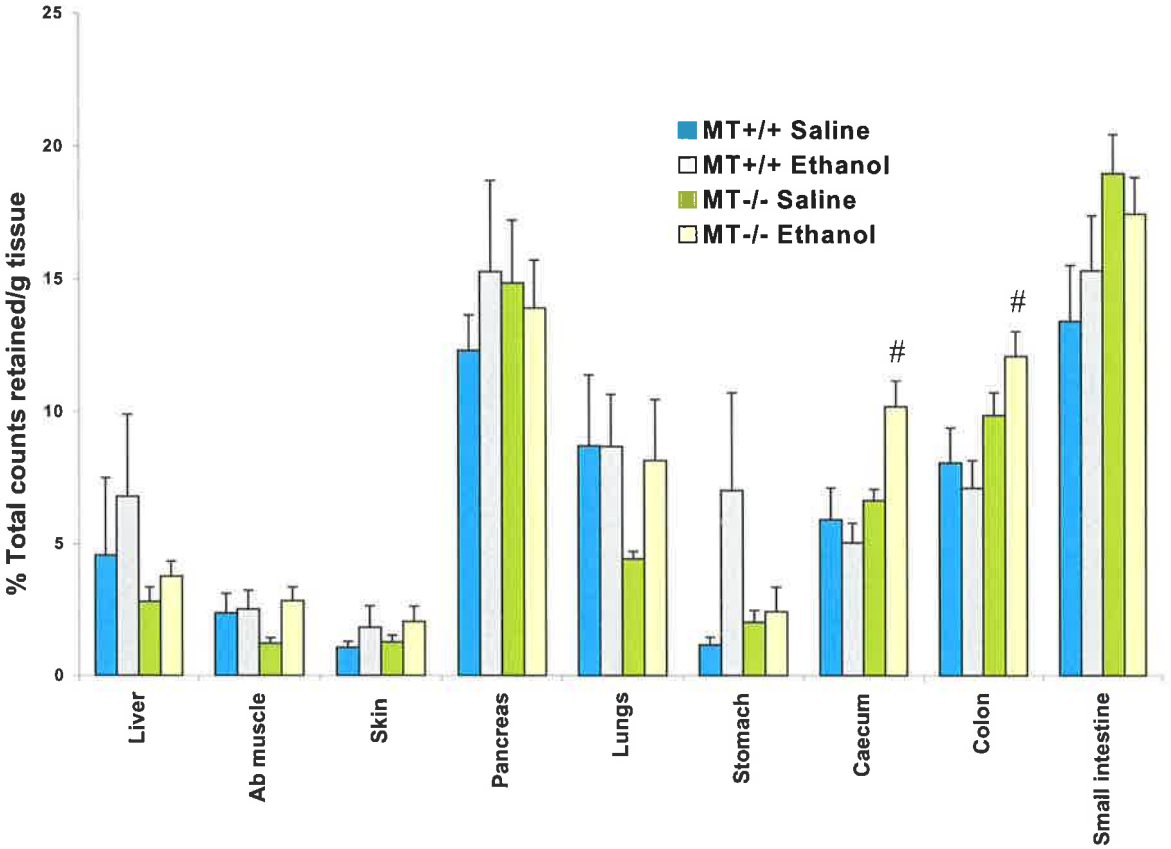


Figure 4. Blood flow to GD14 MT+/+ and MT-/- maternal organs/tissues as assessed by radiolabelled ^{113}Sn microsphere distribution 45 min after saline or ethanol injection. Points represent the mean ± SEM, n = 4-6.

Significantly different from MT+/+ dams of the same treatment ($p < 0.05$).

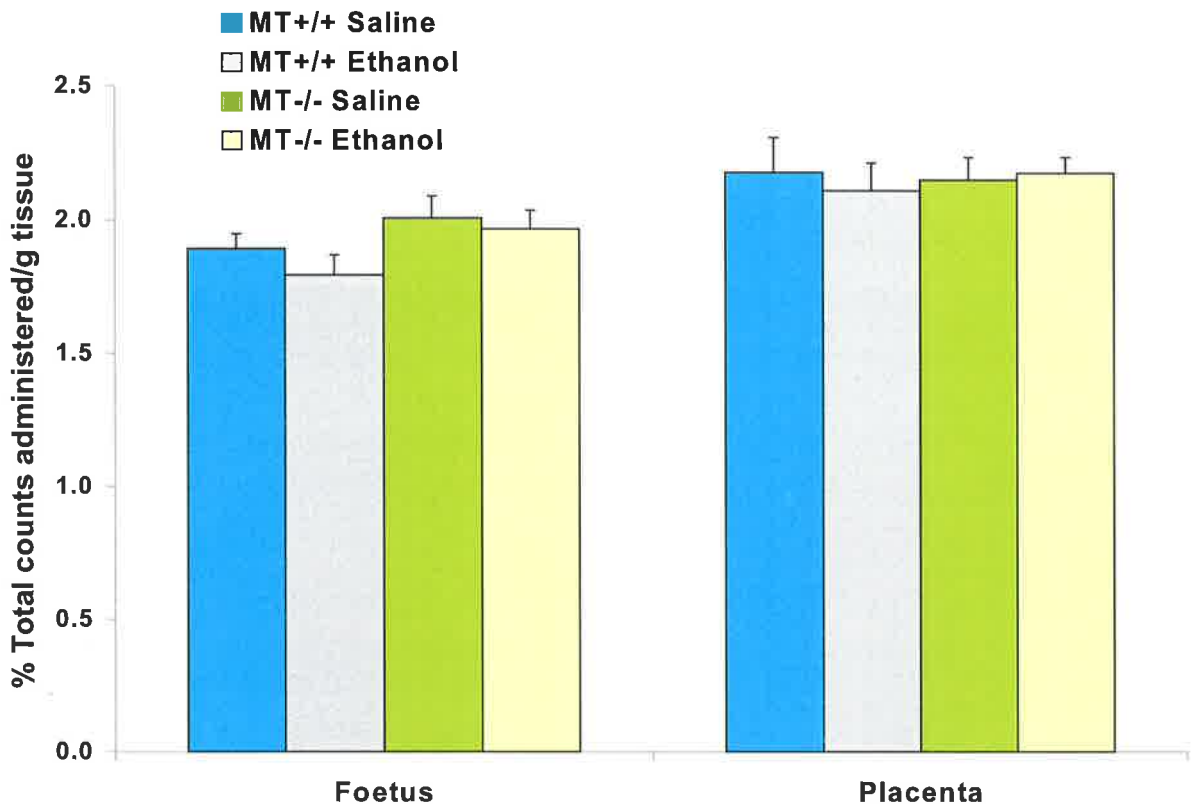


Figure 5. Effect of ethanol on foetal and placental glucose transfer in GD14 MT+/+ and MT-/- dams. Mice were injected with 25% ethanol or saline, and 45 min later sc with a radiolabelled glucose solution (2-deoxy[^{14}C]glucose, before being killed 90 min after the initial injection.

Points represent the mean ± SEM, n = 41-45 foetuses/placenti.

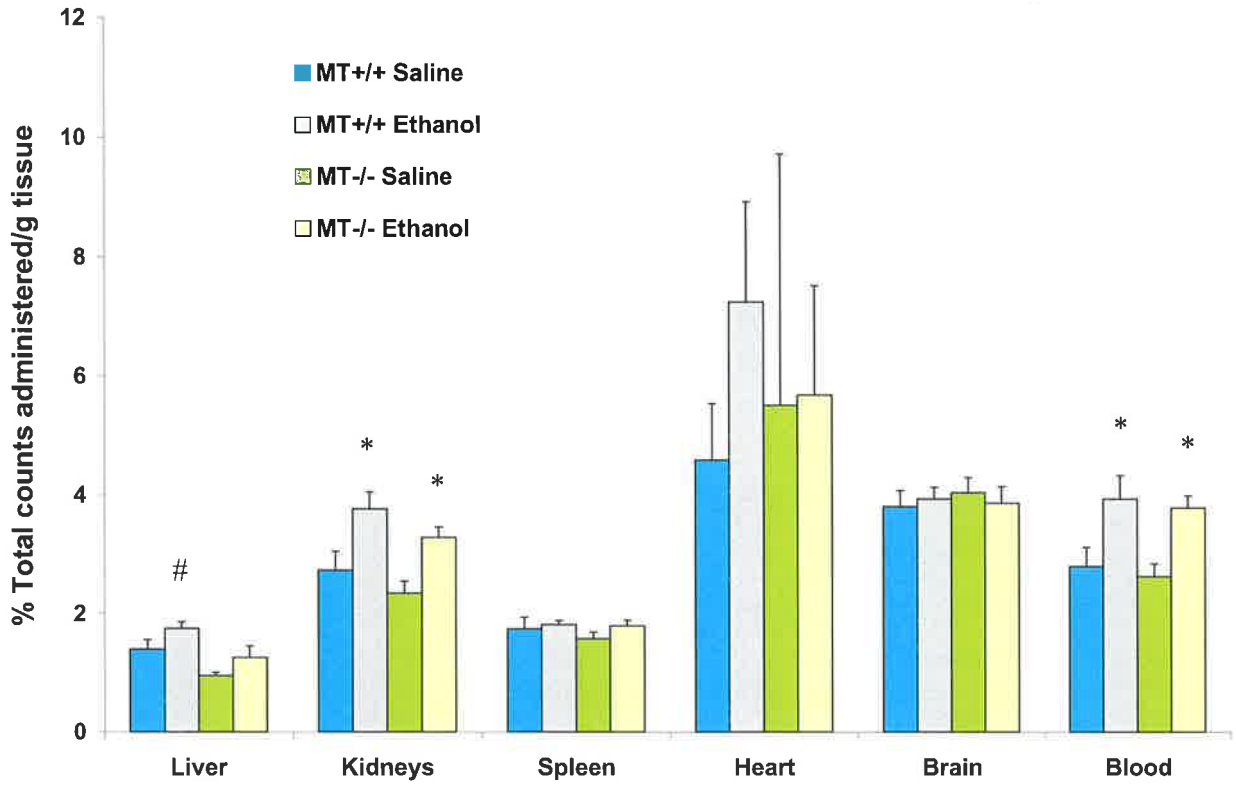


Figure 6. Effect of ethanol on organ glucose transfer in GD14 MT+/+ and MT-/- dams. Mice were injected with 25% ethanol or saline, and 45 min later sc with a radiolabelled glucose solution (2-deoxy[^{14}C]glucose, before being killed 90 min after the initial injection. Points represent the mean ± SEM, n = 6-7.

* Significantly different from saline treated mice of the same genotype ($p < 0.05$).

Significantly different from MT-/- mice of the same treatment ($p < 0.05$).

Ethanol treatment in dams was associated with increased accumulation of radiolabelled glucose in the kidneys and blood of both MT+/+ and MT-/- dams when compared to saline treated controls (Figure 6). Uptake of radiolabelled glucose to the liver of MT+/+ ethanol injected dams was greater than that observed in corresponding MT-/- dams.

DISCUSSION

Alterations in placental blood flow and foetal glucose transfer have long been proposed as mechanisms by which ethanol exerts a teratogenic effect. MT+/+ and MT-/- mice at GD14 were used to investigate these effects. These studies were conducted in later gestation due to the larger conceptus size, thereby allowing for a readily quantifiable transfer of radioactivity to the tissues of interest.

A method for assessing the impact of ethanol on placental blood flow was developed using radiolabelled ^{113}Sn microspheres. The technique of injecting a solution of ^{113}Sn microspheres into the left atrium/ventricle of the heart proved to give a reliable and reproducible indication of tissue blood flow following entrapment of the microspheres. The relatively non-invasive nature of the procedure used here is advantageous in circumventing artifactual changes in blood flow distribution associated with more invasive procedures, such as when the heart is visualised directly.

In accordance with what has long been known regarding blood flow distribution to different organs, the greatest accumulation of radioactivity (indicating blood flow) was in the kidneys, heart and brain. In non-pregnant MT+/+ female mice ethanol

injection 45 min prior to assessment did not significantly alter the pattern of blood flow distribution, except for to the colon (although there was some suggestion of an effect on other gut tissues, perhaps a direct effect of ethanol in the peritoneal cavity).

The observation that blood flow to GD14 MT-/- placenti was significantly increased in response to maternal ethanol treatment is puzzling. This effect was not observed in MT+/+ mice, where there was a trend for decreased placental blood flow following maternal ethanol injection. Blood flow to the major maternal organs in MT-/- dams after ethanol injection was no different from that observed in dams from the other treatment groups, indicating that the increased placental flow was specific for this tissue, rather than a consequence of shifts in maternal distribution. Currently, however, there is no reason to believe that the vascular structure and underlying regulatory mechanisms in MT-/- mice are any different to those in MT+/+ mice, hence the possibility of a type-1 statistical error must be considered. An alternative explanation is that differences in localised expression of nitric oxide (NO), a known vasodilator, may have led to the variable placental blood flow observed. Ethanol is known to stimulate the production of NO. It has been demonstrated that plasma NO concentrations in rats increase by over 60% following ethanol treatment, resulting in significant vasodilatation in the mesenteric vessels (Baraona et al, 2002). With this finding in mind, placental blood vessels (which originate from the mesentery) could well dilate following maternal ethanol injection, and increase blood flow. Obviously this did not occur in the present MT+/+ setting. To this extent enhanced MT expression, as occurs in MT overexpressing mice and following zinc and cadmium treatment, is associated with decreased production of NO (Penkowa et al, 2002; Rana and Kumar, 2001). It seems probable that MT induction associated with ethanol exposure would also exert a similar effect. The mechanism underlying such an

outcome may relate to competition between MT and nitric oxide synthases (NOS) for zinc binding. The catalytic activity of all forms of NOS (neuronal, inducible and endothelial) are dependent on the formation of a zinc-thiolate cluster (Zou et al, 2002). Hence a decrease in available zinc could conceivably result in diminished NOS activity and consequently less production of NO. In the absence of MT, the lack of competition for zinc between MT and NOS may lead to increased production of NO, thus possibly explaining why blood flow to MT-/- placenti was increased following maternal ethanol injection.

Although in general, blood flow distribution in the various groups was similar, there were some effects, most notably where MT+/+ dams treated with saline displayed increased renal blood flow. Other differences were; higher blood flow to the large intestine in MT-/- dams injected with ethanol, and higher brain blood flow in MT-/- saline injected dams. Part of the variability in these pregnancy studies may be explained to some extent by slight differences in maternal size, litter size and extent of development, which in turn may have influenced blood volume and distribution. Preliminary experiments were undertaken using mice of similar weights and age in order to reduce variability. It is difficult achieve this degree of uniformity in pregnancy-related studies.

Maternal ethanol injection on GD14 did not effect the transfer of radiolabelled glucose to foetuses or placenti. While other studies have examined the effects of chronic maternal ethanol exposure on foetal glucose supply in rats (Snyder et al, 1986; Singh et al, 1989), this is the first to address the issue with respect to acute exposure and indeed the first in which mice have been used as the experimental animal. The chronic administration experiments, conducted in rats, involved dams

being fed ethanol as part of a liquid diet throughout gestation, before foetal glucose transfer was assessed in late gestation (Singh et al, 1989; Snyder et al, 1986). Considering the way ethanol interferes with glucose metabolism it is hardly surprising that long term treatment impairs foetal delivery. As mentioned in the introduction, ethanol inhibits gluconeogenesis and leads to hypoglycaemia, which of course has negative implications for the foetus. In Chapter 3 it was shown that acute ethanol treatment also leads to maternal hypoglycaemia, hence the reasoning that this may in turn restrict foetal glucose supply. There are several plausible reasons why an effect was not observed in these experiments.

The first of these relates to possibly confounding differences in specific radioactivity in the maternal blood. In both MT+/+ and MT-/- dams treated with ethanol prior to glucose administration the specific activity in the blood was significantly higher than in saline injected controls. This is most likely related to decreased maternal blood glucose levels, resulting in a relatively more concentrated pool of radiolabelled glucose in the blood. Hence although there may have been an effect of ethanol on placental blood flow (as indeed there was in MT-/- mice), the higher specific radioactivity in maternal blood resulted in a seemingly normal transfer of glucose to foetal tissues, when in fact there could well have been a reduction.

The second explanation concerns glucose transport. Perhaps the nature of the acute insult is such that, despite a maternal blood glucose decrease, there is a compensatory upregulation of transport mechanisms in the placenta which serve to maintain supply. Future investigation could examine the effect of ethanol on the expression and activity of glucose transporters in the placenta.

There was a consistent effect of ethanol on GD14 dams, independent of genotype, leading to increased transfer of radiolabelled glucose to the kidneys and accumulation in the blood. The probable reasons underlying this effect on the level of blood radioactivity have already been discussed. A similar argument could also be put forth with regard to the kidneys, in that ethanol is known to inhibit gluconeogenesis (Krebs et al, 1969). In this respect, the lower glucose concentrations in the kidneys would favour the transfer of radiolabelled glucose from the blood to the kidneys.

A technique for assessing blood flow was developed in this Chapter, and utilised to assess the impact of ethanol on placental blood flow. Surprisingly, maternal ethanol treatment led to increased MT-/- placental blood flow, while there was no such effect in the MT+/+ setting. This unexpected finding may be a consequence of increased NO expression in the placental region, which in the absence of MT would result in vasodilatation. Further experimentation is required to confirm the findings here, and if replicated to examine the possible mediating influence of NO.

The second component of this Chapter examined foetal and placental glucose uptake/transport in response to maternal ethanol injection. Ethanol was found to have no effect to this extent, however between treatment differences in specific radioactivity in the maternal blood may have confounded the results to some extent.

CHAPTER 6

Teratogenic effects of lipopolysaccharide

INTRODUCTION

Bacterial lipopolysaccharide (LPS, or endotoxin) is a component of the gram-negative bacterial wall, and a potent inflammogen. During pregnancy in mice, exposure to LPS can lead to abortion/intra-uterine foetal death (IUFD) or preterm delivery, depending on the timing of the event (Kohmura et al, 2000; Silver et al, 1995; Kaga et al, 1996). In humans, elevated levels of LPS are seen with bacterial vaginosis (BV), a condition in which the normal vaginal flora of hydrogen peroxide producing lactobacilli are overwhelmed by aerobic and anaerobic bacteria (Platz-Christensen et al, 1993). BV is commonly associated with preterm birth and low birth weight (Romero et al, 1988; Gravett et al, 1986; Holst et al, 1994). There is some debate as to how LPS mediates these effects on pregnancy, but there is little doubt that LPS itself is an intermediate and not directly responsible. To this extent, it has been suggested that LPS-induced increases in TNF-α, eicosanoids, and NO may contribute to the resultant pathology (Silver et al, 1994; Platz-Christensen et al, 1992; Silver et al, 1995; Athanassakis et al, 1999).

Relatively few animal studies have examined the teratogenic effects of low dose LPS treatment, instead research in this area has tended to focus on the mechanisms underlying the abortigenic nature of LPS. This is somewhat surprising considering the prevalence of BV is quite high in pregnant women with estimates of incidence ranging from 9 to 23% of pregnancies (Guise et al, 2001). Several investigators have

examined teratogenicity in golden hamsters, and found that while higher intravenous doses of LPS administered on GD8 resulted in significant IUFD, lower dosing regimens led to the development of malformed foetuses as assessed in late gestation (Lanning et al, 1983; Collins et al, 1994). The types of abnormalities observed included limb, eye, neural tube defects, and cleft palate. In another study, mice given sc LPS on GD13 had significant foetal resorptions (IUFD), while the surviving foetuses were phenotypically normal (Coid, 1976). This is perhaps not unexpected considering that the time of exposure was outside the critical organogenic period (GD7-12 in mice).

None of these studies examined what the mechanism(s) underlying LPS teratogenicity may be.

It has previously been demonstrated that ethanol teratogenicity is linked to the expression of MT. LPS is a very potent inducer of liver metallothionein (De et al, 1990; Philcox et al, 1995), hence it is predicted that, as with ethanol, LPS mediated changes in maternal-foetal zinc distribution may contribute to resultant foetal dysmorphology. The following presents evidence to this effect.

MATERIALS AND METHODS

Animals and Mating

See page 36.

LPS treatment

Mice were injected sc into the nape of the neck with 0.5 μg/g body weight of LPS in 0.85% saline. Control mice were injected with saline only. *E. Coli* LPS (serotype O111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). To control for any nutritional related confounding effects, food was removed for 4 hr following treatment.

Previous studies in this laboratory found this dose and route of administration to initiate highly reproducible induction of hepatic MT and associated hypozincaemia. There is also the further advantage of avoiding potential complications associated with ip injection in pregnant mice.

Effects of LPS on metallothionein and zinc homeostasis

Non-pregnant MT+/+ and MT-/- female mice were injected with LPS, and the timecourse of changes in liver MT, and liver and plasma zinc were assessed. Measurements were made at 0, 6, 16, 24 and 48 hr. Following treatment, dams did not exhibit any behavioural abnormalities, or overt signs of toxicity (such as piloerection or diarrhoea). For each time point 4-6 mice were anaesthetised using halothane, bled by cardiac puncture, and killed by cervical luxation. Livers were immediately dissected and processed for MT and zinc analysis, while blood plasma was frozen at –20°C for later zinc measurement.

LPS and foetal dysmorphology

MT+/+ and MT-/- mice were injected with LPS or saline on GD8, and subsequently killed (as previously described) on GD18. Uteri were then excised and the number of foetal resorption sites ascertained. Individual foetuses were separated and examined

for dysmorphology (see page 37). Foetuses from dams exposed to LPS were further processed to ascertain any differences in bone development (see page 75).

LPS, zinc treatment and foetal dysmorphology

A further subset of MT+/+ dams were injected with LPS and $ZnSO_4$ on GD8, as described in Chapter 2 (page 36), and killed on GD18 to examine the effect of zinc treatment on teratogenicity. Findings were compared with those where MT+/+ dams were injected with saline, or LPS.

Metallothionein and zinc analysis

See pages 37, 39.

Statistical analysis

MT concentrations were compared by one-way ANOVA, as were the variables pertaining to zinc treatment, where the MT+/+ groups were saline, LPS and LPS + zinc injected. All other data was compared by two-way ANOVA. Further statistical details are described on page 39.

RESULTS

Liver metallothionein, and plasma and liver zinc

LPS administration caused a rapid increase in liver MT levels in non-pregnant MT+/+ mice (Figure 1). Concentrations peaked at 155 nmol Cd bound/g wet weight 24 hr after the initial injection, and declined thereafter, although at the last time point assessed (48 hr), levels were still above basal. Plasma zinc concentrations in these mice fell from 13.7 μmol/L to 1.6 μmol/L at 6 hr, and remained significantly below

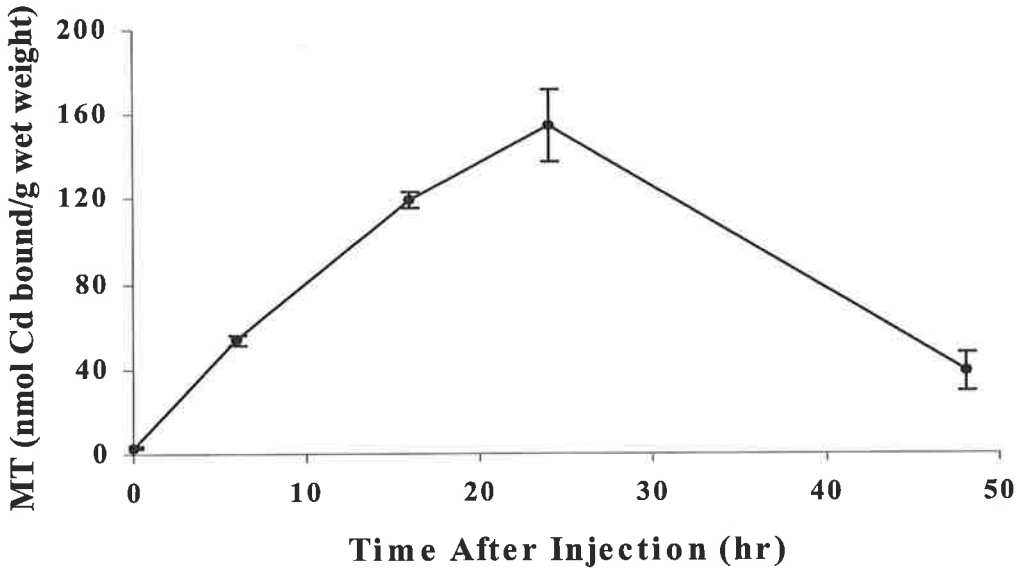


Figure 1. Effect of LPS on liver MT concentrations in nonpregnant MT+/+ mice over 48 hr. Mice were injected subcutaneously with LPS in saline (0.5 μg/g body weight). Each point represents the mean ± SEM, where n = 4-6. MT concentrations were significantly raised from baseline levels from 6 hr onwards ($p < 0.05$).

basal levels for 24 hr, before recovering over the ensuing 24 hr (Figure 2). Conversely in MT-/- mice, plasma zinc concentrations were significantly increased between 6 and 16 hr, before returning to normal by 24 hr. In keeping with the high liver MT levels, liver zinc concentrations in MT+/+ mice were also significantly elevated from 6 hr onward, with peak values of 666 nmol/g wet weight attained 16 hr after LPS injection (Figure 3). Liver zinc levels in MT-/- mice did not differ from baseline over the 48 hr, but were significantly lower than MT+/+ levels from 6 hr onwards.

Pregnancy success, and GD18 foetal dysmorphology

MT+/+ mice treated with LPS on GD8 completed pregnancy 7 out of the 21 times a plug was detected (33% success), while for MT-/- mice it was 7 out of 17 (41%). MT+/+ dams that were injected with LPS and $ZnSO_4$ completed pregnancy on 6 out of 9 occasions (67%). The normal % success rate as assessed over a number of years in this laboratory is between 80 and 90%. Both MT+/+ and MT-/- saline injected control groups fell within this range.

MT+/+ foetuses from dams treated with LPS on GD8 had by far the greatest incidence of external abnormalities, whereas MT-/- foetuses from the same treatment cohort exhibited abnormalities at a frequency that was no different from saline treated controls (Table 1). Of the abnormal MT+/+ foetuses, 45% had eye deformities (either microphthalmia or anophthalmia), while 36% were exencephalic. Other defects observed included micromelia, micrognathia and syndactyly. MT+/+ foetuses exposed to LPS also were also significantly lighter and shorter than control foetuses of the same genotype, while MT-/- LPS foetuses remained unaffected in terms of weight, but were also shorter than MT-/- controls.

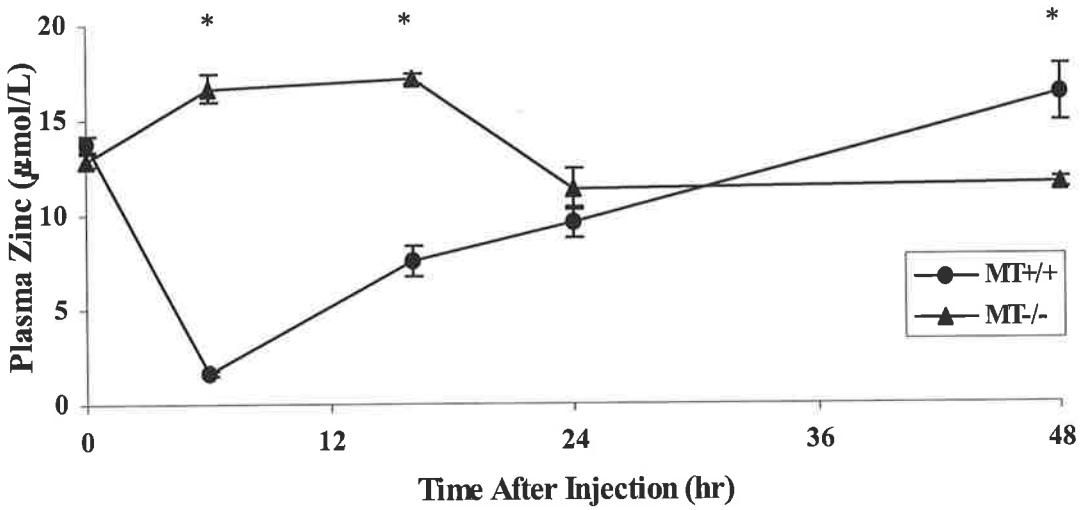


Figure 2. Effect of LPS on plasma zinc concentrations in nonpregnant MT+/+ and MT-/- mice over 48 hr. Mice were injected subcutaneously with LPS in saline (0.5 µg/g body weight). Each point represents the mean ± SEM, where n = 4-6. Plasma zinc concentrations in MT+/+ mice were significantly lower than basal levels between 6 and 24 hr, and higher at 48 hr. Plasma zinc concentrations in MT-/- mice were significantly higher than basal levels between 6 and 16 hr.

*Significant difference between MT+/+ and MT-/- ($p < 0.05$ for all comparisons).

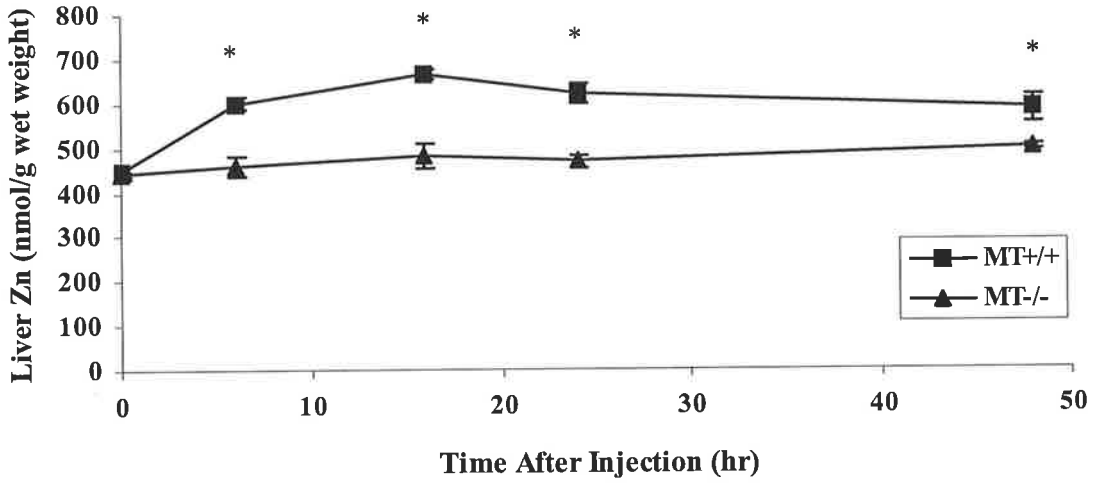


Figure 3. Effect of LPS on liver zinc concentrations in nonpregnant MT+/+ and MT-/- mice over 48 hr. Mice were injected subcutaneously with LPS in saline (0.5 μg/g body weight). Each point represents the mean ± SEM, where n = 4-6. Liver zinc concentrations in MT+/+ mice were significantly higher than basal levels from 6 hr onwards, while in MT-/- mice there was no change.

*Significant difference between MT+/+ and MT-/- ($p < 0.05$ for all comparisons).

Table 1. GD18 MT+/+ and MT-/- foetal parameters, following GD8 maternal LPS or saline treatment.

	Genotype and treatment				
	-/- Saline	-/- LPS	+/+ Saline	+/+ LPS	+/+ LPS + Zn
No. litters	7	7	8	7	6
No. fetuses	54	38	69	36	49
Litter size	7.7 ± 0.5	5.4 ± 1.0	8.6 ± 0.5	5.1 ± 1.0[ac]	8.2 ± 0.7
No. resorptions	7	10	4	22	
Resorption sites per litter	1.0 ± 0.4	1.4 ± 0.6	0.5 ± 0.3	3.1 ± 1.3[ac]	0.5 ± 0.3
No. abnormal fetuses	4	1	3	11	2
No. litters with abnormal fetuses	3	1	1	5	2
% Abnormal fetuses per litter	6.7 ± 3.4	4.8 ± 4.8	4.2 ± 4.2	32.4 ± 9.2[abc]	5.4 ± 3.6
Weight (mg)	872 ± 20	838 ± 13	846 ± 13	798 ± 19[a]	825 ± 14
Crown-rump length (mm)	19.24 ± 0.16	18.88 ± 0.09[a]	19.12 ± 0.09	18.62 ± 0.16[ac]	19.19 ± 0.09

Values represent the means ± SEM where applicable.

Data analysed by two-way ANOVA and Tukey's post hoc test, except for the comparisons between MT+/+ dams that were treated with saline, LPS and LPS + $ZnSO_4$ respectively, in which case one-way ANOVA was used.

[a] Significantly different from saline treated foetuses of the same genotype, $p < 0.05$.

[b] Significantly different from MT-/- foetuses of the same treatment, $p < 0.05$.

[c] Significantly different from MT+/+ foetuses from dams treated with LPS and $ZnSO_4$, $p < 0.05$.

MT+/+ foetuses from dams treated with $ZnSO_4$ in addition to LPS exhibited significantly fewer birth defects, and were developmentally more advanced (although only significantly so for crown-rump length) than MT+/+ foetuses from dams treated with LPS alone. There were no significant differences between the MT+/+ saline and MT+/+ LPS + zinc groups for any of the variables compared.

Both MT+/+ and MT-/- LPS exposed dams had decreased foetal numbers per litter compared to control levels. However this decline was only significant in the MT+/+ group. There was an approximate 6-fold increase in the presence of foetal resorption sites in MT+/+ LPS dams, as compared to saline treated controls. Zinc treatment in MT+/+ dams attenuated both the decrease in litter size, and the increase in the number of resorption sites.

Bone ossification in GD18 MT+/+ and MT-/- foetuses from dams treated with LPS on GD8

Within the LPS treatment groups there was significantly less ossification in the metacarpal bones from MT+/+ compared to MT-/- foetuses, whereas ossification in the posterior phalanges was less pronounced in MT-/- foetuses (Table 2).

DISCUSSION

Prior work in this laboratory demonstrated a link between the teratogenic effects of ethanol and changes in maternal-foetal zinc homeostasis (Carey et al, 2000a). It was found that this teratology was dependent on the induction of MT by ethanol in the maternal liver, leading to movement of zinc from the maternal plasma into the liver, hence decreasing the zinc pool available for foetal transfer. MT-/- foetuses exposed to

Table 2. Extent of GD18 MT+/+ and MT-/- foetal bone ossification from dams injected with LPS on GD8.

Ossification centre	Foetal genotype	
	MT+/+	MT-/-
Metacarpus[1]	6.769 ± 0.290[a]	7.529 ± 0.194
Metatarsus[1]	8.269 ± 0.171	8.0 ± 0
Anterior phalanges[1]	1.346 ± 0.490	0.706 ± 0.418
Posterior phalanges[1]	1.577 ± 0.606[a]	0 ± 0
Sternum	2.842 ± 0.377	3.400 ± 0.335
Caudate vertebra	2.115 ± 0.202	1.705 ± 0.166

Values represent the means ± SEM.

Data was analysed by Student's T-test.

[1] Left and right ossification centres were counted independently and then added together.

[a] Significantly different from MT-/- group ($p < 0.05$).

[b] Significantly different from MT-/- saline group ($p < 0.05$).

ethanol in early gestation fared no worse than saline controls in terms of the frequency of birth defects. It was hypothesised that other substances known to be inducers of MT, may in turn exert similar effects on maternal-foetal zinc homeostasis and result in teratogenicity.

Foetal exposure to endotoxin during pregnancy is known to trigger abortion, and is also associated with the development of birth defects. BV is one such human condition in which the associated release of LPS may jeopardise the foetus. Despite the relative frequency of BV, and hence the increased risk of LPS related teratogenicity, we do not fully understand the underlying mechanisms. Experimentation in this chapter has endeavoured to address this issue, by examining how LPS alters aspects of maternal-foetal zinc homeostasis with respect to resultant teratology.

A single sc dose of 0.5 μg/g LPS was found to significantly induce hepatic MT in non-pregnant female mice, increasing levels from basal by a maximum of 30-fold 24 hr after treatment. The change in hepatic MT expression also had dramatic effects on both the liver and plasma zinc concentrations over time. The flux of zinc from the plasma into the liver (for incorporation into MT), resulted in a severe and sustained fall in plasma zinc concentration (80% by 6 hr). Other studies examining the inductive effect of LPS on MT have employed different routes of administration (mainly ip), hence it is important to note that the MT and zinc responses elicited in this investigation are typical (Abe et al, 1987; Rofe et al, 1996; Philcox et al 1995). Furthermore, the changes are also comparable to those triggered by ethanol exposure, although the MT response to LPS is more exaggerated (Carey et al, 2000a). The time disparity between maximal liver MT induction and minimum plasma zinc

concentration (i.e. liver MT was maximal at 24 hr, while plasma zinc was lowest at 6 hr) is not unusual, and most likely reflects compensatory release of zinc from other tissues to bolster the depleted plasma reservoir.

The sustained rise in plasma zinc concentration following LPS treatment in MT-/- mice has not previously been demonstrated. However, this finding has strong parallels with the ethanol effect in these mice, where a similar increase in plasma zinc concentration is observed following ethanol treatment (Carey et al, 2000a). In the case of ethanol, this is related to release of zinc from the skin and muscle into the plasma compartment (Chapter 3). Although this also occurs in MT+/+ mice, the raised hepatic MT levels direct zinc away from the plasma into the liver. LPS has demonstrated hepatotoxic properties (Aschkenasy, 1978; Hewett and Roth, 1995), therefore it is plausible that in MT-/- mice there is a release of zinc from the liver following exposure, leading to plasma accumulation. Furthermore, the inflammatory response initiated by LPS may elicit additional tissue zinc release.

In accordance with other studies, we found LPS to have a significant teratogenic impact on the MT+/+ foetus (Lannings et al 1983; Collins et al 1984). More importantly, and in agreement with the previous study on ethanol teratogenicity conducted in this laboratory (Carey et al, 2000a), the implicit involvement of MT in this teratology was evidenced by the observation that MT-/- foetuses were not effected by this treatment. Furthermore, foetal teratogenicity was reduced to basal levels when MT+/+ dams were treated with $ZnSO_4$ at the time of LPS injection. These findings lend very strong support to the suggestion that LPS related abnormalities are in part caused by the previously discussed MT induction and associated changes in maternal (and hence foetal) zinc homeostasis.

As previously seen with ethanol, LPS treatment resulted in a high prevalence of exencephaly and ophthalmic defects in MT+/+ foetuses, which is suggestive of underlying interference with optic cup invagination and neural tube closure. LPS was also associated with decreased growth in GD18 MT+/+ foetuses, as indicated by lower weight and crown-rump lengths. This finding is consistent with prior studies utilising both LPS (Ornoy and Altshuler, 1976; Lanning et al, 1983) and ethanol (Hickory et al, 1979; Sauerbier, 1987). Furthermore, the general teratogenic and growth and development impediments seen here with LPS have parallels with the foetal dysmorphology associated with maternal consumption of zinc deficient diets during pregnancy (Keen and Hurley, 1989; Dreosti et al, 1986; Hickory et al, 1979). This fact further supports the notion that LPS teratogenicity is at least in part caused by an underlying foetal zinc deficiency.

The abortigenic nature of LPS was also evident in this study. Following detection of a vaginal plug, both MT+/+ and MT-/- dams treated with LPS on GD8 were considerably less successful in completing pregnancy compared to controls (30-40% compared to normal 80-90%). This is indicative of complete IUFD and subsequent resorption of foetal tissue. Furthermore, in successful MT+/+ pregnancies, not only were there significant foetal anomalies, there was also an increased number of resorption sites. These findings are interesting in that on one hand MT-/- and MT+/+ foetuses appear to be equally vulnerable to complete foetal resorption, while on the other (when pregnancy is successful) there is a greater degree of IUFD in MT+/+ foetuses. Perhaps the LPS exposure can be viewed as a double insult, with direct effects on the foetus causing IUFD, and indirect effects associated with the changes in maternal-foetal zinc homeostasis causing abnormal development, and/or IUFD.

Assuming the foetus survives the direct effect of LPS, the MT-/- foetus is then at an advantage, while its MT+/+ counterpart is further compromised as a consequence of the inadequate maternal plasma zinc concentration.

The most important findings from experiments conducted in this Chapter are those indicating that zinc treatment at the time of LPS injection in MT+/+ dams ameliorates, almost completely, the deleterious effects otherwise associated with LPS exposure. The dose of zinc injected is identical to that used in Chapter 2, where zinc treatment was also found exert beneficial effects in reducing ethanol teratogenicity. The injection itself increases concentrations of zinc in the maternal plasma, thereby preventing the normal decline in plasma zinc levels associated with MT induction. This undoubtedly leads to adequate foetal transfer, improved zinc nutrition and unhindered growth and development.

Experiments in this Chapter have demonstrated that MT-/- foetuses are afforded a significant degree of protection from the teratogenic effects of LPS compared to MT+/+ foetuses. The changes in maternal-foetal zinc homeostasis leading to a foetal zinc insufficiency in MT+/+ foetuses appears to be the key factor, particularly in light of the observation that zinc treatment at the time of LPS injection ameliorates resultant teratology. In short, findings in this Chapter lend support for a new aetiological model of LPS teratogenicity, whereby MT induced changes in zinc homeostasis lead to impaired foetal zinc supply.

CHAPTER 7

SUMMARY

Prenatal ethanol exposure is associated with a myriad of costly negative outcomes including spontaneous abortion, low birth weight, physical defects and cognitive abnormalities. Most dramatically, maternal consumption of ethanol during gestation had been quoted as being the leading cause of mental retardation in the US (Abel and Sokol, 1987). Traditionally research in this area has focussed on how chronic maternal ethanol exposure effects the foetus, however acute, or binge type episodes of consumption can also have equally severe consequences depending on the timing of the insult. The foetus is most vulnerable to teratogenic insult during the period of organogenesis, which encompasses weeks 3 to 9 in humans. Hence it is possible that a woman, not knowing she is pregnant, may inadvertently compromise the developing foetus in a single drinking session.

It is probable that there are a number of causative factors underlying ethanol teratogenicity. Recent work in this laboratory has focussed on the changes ethanol causes in maternal-foetal zinc homeostasis in mice, with the rationale being that a transient foetal zinc deficiency may eventuate. Zinc is essential for a multitude of processes that underlie growth and development, and a deficiency per se (as occurs with maternal zinc deficiency) is associated with the development of birth defects, similar to those that occur following ethanol exposure.

Alterations in zinc homeostasis after ethanol treatment are mediated by heightened expression of MT in the liver. This leads to a net movement of zinc out of the plasma, into the liver where it is bound by MT, with the result being a significant decrease in the plasma zinc concentration. Hence the following hypothesis was proposed: the deleterious effects of ethanol on the foetus are in part due to a MT mediated decrease in maternal plasma zinc which compromises foetal zinc supply. Earlier work in this laboratory demonstrated that foetuses from MT-/- dams are significantly less susceptible to the teratogenic effects of ethanol than are foetuses from MT+/+ dams, thereby lending strong support to the hypothesis (Carey et al, 2000a). This thesis focussed on a number of important additional issues pertaining to the hypothesis.

During gestation, MT expression in both dam and foetus is known to change quite dramatically. In the foetus, liver MT concentrations are particularly high in late gestation and early postnatal life. Changes in dam MT levels have been well characterised in terms of mRNA (Liang et al, 1996; Andrews et al, 1984), however information is lacking as to exactly when protein concentrations begin to rise, peak and fall. With respect to the working hypothesis, it was therefore important to assess the ontogeny of MT protein expression in the dam. If liver MT concentrations at GD8 were maximally induced as a consequence of pregnancy itself, then the argument that 'MT induction by ethanol is a major mediator of resultant teratogenicity' would be invalid. Findings in Chapter 2 demonstrated that although liver MT levels in MT+/+ dams were indeed naturally elevated at GD9, the increase was only 3-4 fold basal, and hence would not have significantly impacted on the MT inductive capacity of ethanol on GD8.

It was interesting to note that administration of ethanol to GD12 dams resulted in the development of lighter and shorter GD18 foetuses regardless of genotype (Chapter 3). Observable teratogenicity was minimal. It would appear that the impact and interference of ethanol with foetal development at this time is independent of MT. When one considers that maternal hepatic MT concentrations at GD12 are naturally higher (Chapter 2) than those attained following ethanol treatment, this is hardly surprising. On GD12 ethanol does not have the capacity to further induce liver MT in the dam, and therefore the alterations in maternal zinc homeostasis seen at GD8 are not apparent.

The key argument for the involvement of zinc, and foetal zinc deficiency per se, in mediating the teratogenicity of ethanol is that ethanol causes the maternal plasma zinc concentration to decrease dramatically. Critically, in MT-/- dams, ethanol has the opposite effect, leading to large increases in plasma zinc levels (Carey et al, 2000a). In Chapter 3 this increase was shown to be due to release of zinc from the major stores in the muscle and skin, presumably as a result of damage in these tissues. Although ethanol exerted a similar effect in MT+/+ mice, the concurrent high liver MT concentrations prevented accumulation of zinc in the plasma. So while in the MT+/+ setting the decreased maternal plasma zinc levels would lead to decreased foetal transfer, in the MT-/- scenario the raised plasma zinc concentrations would maintain, or perhaps even increase delivery of zinc to the foetus.

The high plasma zinc levels in MT-/- dams following ethanol injection appears to be the key factor in protecting foetuses. Logically then, and in keeping with the original hypothesis, averting the drop in plasma zinc in MT+/+ dams should also prevent/or

reduce the teratogenic effect of ethanol by increasing the amount of zinc transferred to the foetus. Experiments in Chapter 2 provided critical evidence to this effect. MT+/+ dams were treated with $ZnSO_4$ such that 8 hr after the first injection of ethanol, plasma zinc concentrations were around 20 μmol/L, a level which is comparable to what is seen in MT-/- mice at that time following ethanol injection alone. Most importantly, this injection of zinc at the time of the first ethanol exposure in MT+/+ dams resulted in a significant reduction in teratogenicity such that the percentage of abnormal foetuses was not different from control levels. Furthermore foetal development, with respect to weight and crown-rump length, was comparable to that seen in foetuses from saline injected control dams. These findings represent conclusive evidence that foetal zinc deficiency is the predominant mediator of the ethanol related teratogenic outcomes assessed in this thesis.

Theoretically any substance which induces maternal liver MT and thereby disrupts zinc homeostasis should also exert a teratogenic effect in MT+/+, but not MT-/- foetuses. In Chapter 6 experiments were conducted to confirm this assertion using LPS as the agent of MT induction. LPS was chosen, not only because it is a potent inducer of hepatic MT, but also because its affects have definite parallels with the common human condition of bacterial vaginosis (BV), which is associated with preterm labor (Romero et al, 1988). As with ethanol, LPS administered to dams on GD8 was found to cause significant teratogenicity in GD18 MT+/+, but not MT-/- foetuses. In fact, the percentage of abnormal foetuses (32%) was in excess of that associated with ethanol teratology (14-24%, Chapter 2; Carey et al, 2000a). This is most likely a result of the heightened hepatic MT induction and more exaggerated decrease in plasma zinc concentration associated with LPS treatment. Despite this, zinc treatment (identical to that given to dams in the ethanol studies in Chapter 2) at

the time of LPS injection in MT+/+ dams still greatly reduced teratogenicity, foetal resorptions, and retardation of growth and development.

The evidence presented thus far clearly shows that maternal genotype is the critical factor in mediating ethanol teratogenicity. Nevertheless it was deemed important to search for any possible influence of foetal genotype. To this extent MT+/- dams and sires were mated, leading to the conception of embryos from all three genotypes within a given litter (Chapter 4). Although numbers were relatively small, and perhaps best viewed as preliminary, the treatment of dams with ethanol on GD8 did not appear to favour the development of foetuses from one particular genotype over another. This finding is consistent with the original hypothesis.

While the results from the investigations regarding the involvement of MT in ethanol teratogenicity are compelling, the possibility that ethanol may be differentially affecting placental blood flow and glucose uptake was considered (Chapter 5) . It was thought that any effect with respect to blood flow would be independent of genotype. Quite unexpectedly, however, blood flow to MT-/- placenti was found to be increased after dams were injected with ethanol. Ethanol increases the synthesis of NO (a vasodilator), while MT has been shown to inhibit the generation of NO (Baraona et al, 2002; Penkowa et al, 2002; Rana and Kumar, 2001). Therefore the heightened MT-/- placental blood flow may have been due to increased activity of NO, in turn leading to mesenteric vasodilatation in the absence of MT. Despite the increased blood flow to MT-/- placenti, there was no follow on alteration in glucose transfer to the foeto-placental unit (Chapter 5).

CLINICAL APPLICATIONS

Obviously maternal abstinence is the only way to completely prevent the occurrence of FAS and the like. Despite the simplicity of this solution, an alarming percentage of women continue to consume alcoholic beverages whilst pregnant (Ebrahim et al, 1998). This implies that there is a lack of understanding in the general community with respect to the critical nature of this issue (Kaskutas, 2000). It would seem that many women believe that problems only occur with long term consumption (i.e. alcoholism), when in reality there is no known safe level. In short, there is a need for further public education outlining the specific effects that ethanol has on the foetus, and stressing the point that all manner of drinking should be avoided during pregnancy.

Therapeutic intervention is not something that is commonly discussed with regard to preventing the occurrence of ethanol teratogenicity. This is perhaps because many of the underlying mechanisms proposed are not amenable to therapy as such. In this thesis it was demonstrated in mice that maternal zinc treatment significantly ameliorated teratogenicity, as well as the foetal growth and development impairments associated with ethanol exposure. This encouraging finding suggests that zinc therapy may also be of benefit in the human situation. In a controlled clinical setting, it would be a relatively simple process to administer aqueous zinc via the intravenous route, thereby rapidly bolstering plasma zinc levels and helping maintain foetal supply. In the studies conducted in this thesis, zinc was injected within minutes of ethanol treatment. Obviously this regimen would not be applicable to the human setting, where the timing of zinc treatment would without doubt be hard to control. Further

work using animal models is required to determine whether zinc treatment at later (and variable) times after ethanol exposure is beneficial in preventing teratology.

The observation that zinc treatment reduces LPS related teratology in mice is of perhaps more immediate clinical relevance. As previously mentioned, BV in humans is associated with increased levels of LPS, and more importantly preterm birth and low birth weight (Platz-Christensen et al, 1993; Romero et al, 1988; Gravett et al, 1986; Holst et al, 1994). It is conceivable that BV may also be associated with an increased incidence of birth defects. The high prevalence of this condition in both pregnant and non-pregnant women denote it to be a significant health concern (Guise et al, 2001), particularly in light of its problematic treatment (Ugwumadu, 2002). The negative pregnancy outcomes associated with BV may in part be due foetal zinc deficiency, secondary to maternal hepatic MT induction by LPS. If this is the case then maternal zinc therapy, perhaps in conjunction with antibiotic treatment, would be of benefit to both mother and foetus.

FUTURE DIRECTIONS

There are a number of important studies that could be conducted in light of findings in this thesis.

Perhaps the most pressing of these is to address the issue as to whether zinc treatment at later times after ethanol exposure is of benefit to the foetus. As already mentioned, the answer to this question is of relevance to human pregnancy. A mouse model could be used to determine the exact time frame within which zinc treatment exerts a positive effect. It is possible that zinc treatment, even many hours after the initial

ethanol exposure, may prove beneficial. If this were the case, intervention and testing in the human setting would be warranted.

The possibility of implementing dietary zinc supplementation to offset the deleterious effects of ethanol on the foetus was briefly discussed in Chapter 2. It is questionable whether excess zinc in the diet would be sufficiently absorbed so as to raise plasma zinc concentrations to any great extent. The handful of investigations conducted addressing this issue have for the most part failed to demonstrate an effect (Keppen et al, 1990; Tanaka et al, 1982; Tanaka et al, 1988; Tanaka, 1998). Nevertheless, because a successful form of this therapy would be particularly relevant to humans, further animal experimentation is warranted. In particular, it needs to be established which zinc compounds are absorbed most efficiently, and what other factors influence/enhance absorption. In doing this it may be possible to optimise a dietary (or oral) zinc formulation such that plasma zinc concentrations can be increased, and ethanol teratogenicity decreased. To this extent, unpublished data from this laboratory indicates that it is possible to rapidly increase plasma zinc concentrations in mice by bolus oral gavage of aqueous zinc. An analogy here can be made to the fortification of human beverages with zinc. This would be a relatively simple and cheap intervention, perhaps most applicable for use in alcoholic women. Of course, one would have to be wary in promoting a therapy such as this, in that some women may see it as an opportunity to continue drinking whilst pregnant, when otherwise they may have abstained.

Ethanol and LPS are both potent inducers of hepatic MT. As discussed in Chapter 1, there are a multitude of compounds which have the capacity to induce MT. To further validate the proposed model, it would be prudent to determine whether other agents

of induction, for instance dexamethasone, alpha-hederin, and cadmium, also exert less of a teratogenic effect on MT-/- compared to MT+/+ foetuses.

The induction of MT associated with LPS administration is thought to be mediated by cytokines, in particular IL-6 and TNF-α (De et al, 1990; Liu et al, 1991). It is possible that other infectious conditions, which increase release of these substances, may also indirectly interfere with foetal development via the induction of MT and alterations in maternal-foetal zinc homeostasis. More specifically the so-called TORCH syndrome may manifest effects in this manner. The TORCH acronym refers to a group of maternal infections that can ultimately result in birth defects, and include toxoplasmosis, rubella, cytomegalovirus and herpes. It would be of considerable interest, using animal models, to examine whether changes in MT and zinc homeostasis are apparent in these conditions, and if so, whether zinc treatment has an ameliorating effect.

This thesis has provided strong evidence that foetal zinc deficiency is a key mediator of ethanol teratogenicity. Exactly which combination of the multitude of zinc dependent enzymes, proteins and processes this deficiency is interfering with is unknown. Gene array technology has recently been used to assess how dietary zinc deficiency in rats impacts on the expression of specific genes in the intestine (Blanchard et al, 2001). There is no reason why similar methodology could not be employed with respect to ethanol teratogenicity, whereby the expression of genes in the foetus could be examined following maternal ethanol exposure, with or without concurrent zinc treatment.

It should be emphasised that only gross external abnormalities were considered in the studies undertaken in this thesis. As previously mentioned, there are a myriad of other less subtle external, internal and cognitive defects commonly associated with gestational exposure to ethanol, which were without doubt present in many of the foetuses examined. Future work might incorporate additional histological, morphological and indeed behavioural (with respect to detecting CNS impairment) examinations to detect these dysmorphologies.

CONCLUDING STATEMENT

The precise mechanisms underlying ethanol teratogenicity are unclear. This thesis presents strong evidence that ethanol-induced alterations in maternal-foetal zinc homeostasis, leading to foetal zinc deficiency, are paramount in mediating this teratogenicity. Zinc supplementation proved to be beneficial in preventing ethanol teratology in mice, hence therapeutical intervention in humans may help decrease the occurrence of foetal alcohol syndrome and the like.

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