Dietary Zinc Supplementation Throughout Pregnancy Ameliorates Spatial Memory Defects in C57Bl/6J Mice Offspring Acutely Exposed to Ethanol *In Utero*

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Abstract:

Background: Ethanol exposure *in utero* can cause spatial learning and memory problems. A suggested mechanism for ethanol's teratogenesis relates to its induction of fetal zinc deficiency. We propose that dietary zinc supplementation can limit these cognitive deficits, by replenishing maternal plasma zinc levels, thereby maintaining adequate supply of this metal for the fetus. This has been previously demonstrated for physical birth defects. **Methods:** Pregnant C57Bl/6J dams were allocated a control (35mgZn/kg) or zinc supplemented (200mgZn/kg) diet on Gestational Day (GD) zero. On GD8, each mouse received two intraperitoneal injections, separated by four hours of either; 25% ethanol, (2.9g/kg body weight (0.015ml/g)), or saline. Adult offspring were tested in a water, cross-maze escape task, designed to assess spatial learning and memory, at Post Natal Day (PND) 60-66 (learning), 78 (memory I), and 105 (memory II). **Results:** No differences in spatial learning were detected between treatment groups; however, ethanol treated mice showed impairments in spatial memory, as demonstrated by increased escape latency and decreased number of correct trials. Dietary zinc supplementation prevented memory deficits, with zinc+ethanol mice performing at the same level as saline and zinc+saline groups. **Conclusion:** These results indicate that spatial memory impairments in mice, brought about by acute ethanol exposure, can be ameliorated with dietary zinc supplementation throughout pregnancy. Further research is warranted to determine if this modality reduces the incidence and severity of ethanol-induced birth defects in humans.

Key Words: Ethanol Zinc Cognition Spatial learning Spatial memory Dietary supplementation Pregnancy Birth defects

Abbreviations: ANOVA, One-Way Analysis of Variance; CNS, Central Nervous System; EP, Escape Platform; FAS, Fetal Alcohol Syndrome; GD, Gestational Day; IMVS, Institute of Medical and Veterinary Science; LSD, Least Significant Difference; logit, Logistic Regression; MT, Metallothionein; PND, Post Natal Day; REML, Restricted Maximum Likelihood; RNA, Ribonucleic Acid; SEM, Standard Error of the Mean; SHIRPA, SmithKlein Beecham Pharmaceuticals, Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit, Imperial College School of Medicine at Saint Mary's Hospital, Royal London Hospital, Saint Batholomew's and the Royal London School of Medicine, Phenotype Assessment; Zn, Zinc.

Introduction:

Alcohol consumption during pregnancy is a public health concern, due to the detrimental consequences for the unborn child, and subsequent costs to the community. The sequelae include physical and more commonly behavioural abnormalities (e.g. decreased intelligence quotient, poor attention span, with learning and memory problems). Additionally, there is a risk of spontaneous abortion, increased perinatal mortality, and Fetal Alcohol Syndrome (FAS)[1-5]. These defects are encompassed under fetal alcohol spectrum disorder, which has an estimated prevalence of one in 100 births[1, 4, 6]. Bailey, *et al.*[1] estimates that 50% of women of child-bearing age consume alcohol, with 20% continuing to drink during pregnancy. Women tend to reduce their consumption of ethanol when they discover they are pregnant; however, they may have already placed their unborn child at risk[7-8]. A single binge of alcohol can have a detrimental impact, notwithstanding the stage of pregnancy[8-9]. Therefore, alcohol is classified as one of the most prevalent teratogenic agents consumed by humans[9-10].

Patterns of ethanol ingestion influence outcomes. Binge drinking, characterised by consumption of more than five standard drinks in one hour, (one standard drink being equivalent to ten grams of ethanol), is associated with greater abnormalities, compared with the same quantity consumed over several days[1, 5-6, 8-9, 11]. It results in a higher peak blood alcohol concentration, which remains raised for longer.

Ethanol freely crosses the placenta, producing similar blood alcohol concentrations for the mother and fetus[5, 9, 12]. The mechanisms by which it acts as a teratogenic agent are unclear, with suggestions including; direct toxic effects on fetal cells, oxidative damage related to ethanol metabolism, interruption of fetal blood supply, nutrient transport, and apoptosis[2, 12-15]. Much evidence supports a detrimental effect on zinc homeostasis[2, 13-14, 16]. Zinc is vital for growth and development, as it is essential for the function of multiple enzymes, be they regulatory, structural, or catalytic[17-23]. It modulates the activity of certain hormones and neurotransmitters, has antioxidant and anti-apoptotic capabilities, and is involved in metabolism of proteins, nucleic acids, and carbohydrates[17, 23-26]. Zinc deficiency has been found to be embryo-toxic and teratogenic[24, 27]. Birth defects resulting from zinc deficiency and ethanol exposure during pregnancy share striking similarities, suggestive of a common underlying pathway towards teratogenesis (*Table 1*)[10, 14, 28-30].

Evidence linking zinc dyshomeostasis to ethanol consumption exposed ethanol's interaction with the zinc binding protein, Metallothionein (MT). MT is involved in zinc homeostasis and is thought to function in intracellular zinc chelation and storage[17, 21]. Of its four isoforms, MT-I and –II are inducible, responding to various environmental stimuli, (e.g. free zinc ions, and inflammatory mediators)[21]. Ethanol stimulates inappropriate induction of MT-I and –II in the maternal liver[13, 20, 31]. This sequesters free zinc ions in hepatocytes, creating a concentration gradient, whereby zinc moves from the maternal plasma to the maternal liver, creating a functional zinc deficiency for the fetus. Our research group found that the plasma zinc concentrations decreased by 65% over 16 hours after administration of ethanol to mice[20]. However, in mice which lack the genes for MT-I and –II (MT-/-), these levels increased by 50%. This is related to a lack of zinc sequestration in the liver, and release of this ion from skin and muscle stores in response to the assault[13, 20]. The alcohol-induced zinc deficiency in pregnant mice possessing the MT genes (MT+/+), has detrimental

effects on the rapidly growing fetus, [increased physical birth defects in offspring, compared to saline treated controls, (27.4% versus 6.4%, respectively)][2, 11, 20]. However, in ethanol treated MT-/- mice, there is a decrease in the incidence of abnormalities compared with controls, (2.2% versus 6.4%, respectively).

Table 1: **Birth Defects from Zinc Deficiency and Alcohol Exposure – A Comparison**

Birth Defects from Zinc Deficiency	Birth Defects from Alcohol Exposure
Growth retardation including; interuterine growth retardation, failure to thrive, and small for gestational age infants.	Growth retardation including; interuterine growth retardation, failure to thrive, and small for gestational age infants.
Cleft palate and cleft lip	Cleft palate and cleft lip
Neural tube defects	Neural tube defects
Skeletal abnormalities including; short or missing mandible, abnormal spinal curvature, clubbed feet, syndactyly, fusion of ribs, missing vertebrae	Skeletal and facial abnormalities including; short or missing mandible, hypoplastic nails, shortened fifth digit, radioulnar synostosis, flexion contractures, camptodactyly, clinodactyly, ectrodactyly, polydactyly, syndactyly, scoliosis, hemivertebrae, hockey stick palmar crease, flat midface, long smooth philtrum, thin vermillion border, low nasal bridge, short, upturned nose, underdeveloped ears, epicanthal folds, short palpebral fissures
Heart and lung malformations including; atrial septal defects, ventricular septal defects and aberrant great vessels, including aorta	Heart malformations including; atrial septal defects, ventricular septal defects and aberrant great vessels, including aorta
Neurological abnormalities including; hydrocephalus and hydroanencephalus, and alterations in brain structure and function	Neurological abnormalities including; microcephaly, cerebellar hypoplasia, agenesis of the corpus callosum, and alterations in brain structure and function
Urogenital malformations	Urogenital malformations
Optic abnormalities including; microphthalmia and anophthalmia	Optic abnormalities including; microphthalmia and anophthalmia
Other abnormalities including; of the lung and herniation syndromes and behavioural/ learning deficits	Numerous other abnormalities including; behavioural/ learning deficits

Contributions from the following papers:
Andrews & Geiser, 1999; Bailey, *et al* ,2004; Beach, *et al.*, 1982; Becker, *et al.*, 1996; Berman & Hannigan, 2000; Carey, *et al.,* 2000[a]; Carey, *et al.*, 2000[b]; Coyle, *et al.,* 2002; Dalton, *et al.,1996;* Dreosti, 1993; Dunty, *et al.*, 2001; Huizink & Mulder, 2006; Mattson & Riley, 1998; Minetti, *et al.*, 1996; Nulman, *et al.*, 2004; Rogers, *et al.,* 1995; Singh, *et al.*, 1989; Streissguth, *et al.*, 1980; Sulik, *et al.*, 1981; Summers, *et al.*, 2006; Tran & Kelly, 2003; Uecker & Nadel, 1998; Wattendorf & Muenke, 2005

The correlation between zinc deficiency and ethanol-induced birth defects is further supported by detection of impaired transfer of radioactive zinc-65 to the fetus of alcohol treated MT+/+ dams[13]. This results in a reduction of total fetal zinc, and is not observed in MT-/- mice[13]. Furthermore, we have shown that subcutaneous zinc treatment, given concurrently with ethanol, prevents fetal abnormalities, with the incidence of malformations equal to those observed in controls[20]. This parenteral intervention increased the maternal plasma zinc concentrations by 500%, ameliorating the effects of the ethanol-induced functional zinc deficiency[11, 20]. These positive findings led us to examine dietary zinc supplementation, which would be a more practical mode for providing zinc. We discovered that this repletion improves postnatal survival and reduces the incidence of physical birth defects to basal levels (Summers, *In Press*).

We have also demonstrated that a subcutaneous injection of zinc given to pregnant dams at the time of ethanol exposure, prevents spatial memory defects in offspring[11]. Zinc plays a vital role in the development of the Central Nervous System (CNS), which is very susceptible to damage, due to its extended period of development, (throughout pregnancy and into postnatal life)[19, 22, 25]. Indeed, the highest concentrations of zinc in the human body are in the hippocampal mossy fibres,

synaptic vesicles, and boutons[19, 22, 25, 32]. The hippocampus in the temporal lobe of the brain is central to the formation of memory (especially spatial memory), and is susceptible to the effects of ethanol[33-35]. Zinc deprivation has been associated with behavioural problems including; learning, memory, and attention deficits, (commonly observed in offspring exposed to ethanol *in utero*)[32]. Tanaka, *et al*.[36] have contributed further evidence to support this. They provided zinc supplementation to pregnant rats at the time of ethanol exposure and measured offspring cerebral weights and Ribonucleic Acid (RNA) content, comparing the measurements to control (saline) and ethanol-treated dams, (minus zinc supplementation). They discovered that zinc repletion increased cerebral weight and RNA content, compared to rats given ethanol alone, though not to the basal levels of control rats.

Positive findings pertinent to physical birth defects and postnatal survival suggest that oral zinc repletion is efficacious (Summers, *In Press*). However, studies have found that a high dietary zinc intake is generally not associated with increases in plasma zinc concentrations[37]. Notwithstanding this, oral supplementation may be beneficial, as it generates a reservoir of this ion in the gastrointestinal tract[20-21]. Lowered plasma zinc concentrations induced by ethanol exposure may be restored, as zinc moves from the gut into the plasma, down the concentration gradient. We aimed to demonstrate the benefits of oral zinc repletion on spatial learning and memory, with this water, cross-maze study on mice acutely exposed to ethanol *in utero* (Gestational Day [GD] eight).

Methods:

The C57Bl/6J mice used for these experiments were purchased from the Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia. Studies of this strain have shown that they are susceptible to alcohol-related teratogenesis[38]. The mice were housed in the same room at the IMVS animal facility; maintained at 22 degrees Celsius, with a 12 hour light-dark cycle. They were given unrestricted access to water and commercial laboratory chow (Milling Industries, Adelaide, Australia), (except where otherwise stated). Ethical approval for this project was obtained from the IMVS Animal Ethics Committee.

Virgin dams (7-14 weeks of age) were paired with a proven male, (overnight in separate cages). The following morning, females were isolated and checked for the presence of a vaginal plug. A positive presentation was considered as confirmation of pregnancy. The date was recorded as GD0, and females were housed in individual, opaque cages. They were commenced on a casein-based diet, (ICN Biomedical Australasia, AIN-93G)[11]. Fifty percent of mice were given a control diet, (35mgZn/kg). The remaining half were fed a zinc supplemented diet, (zinc sulphate, 200mgZn/kg). Mice were provided their diet and water *ad libitum*.

On GD8, pregnant females were given two intraperitoneal injections, separated by four hours, (i.e. one injection at zero hours, and the second at four hours). This mouse gestational stage corresponds to the third or fourth week of human pregnancy, when organogenesis is occurring and women are unlikely to realise they are pregnant[39]. Half of each treatment group (35mgZn/kg, or 200mgZn/kg), were given two intraperitoneal injections of 25% ethanol in 0.85% saline (v/v). This dose is a well-established model for FAS in mice, at 2.9g/kg body weight (0.015ml/g)[40]. It has been found to give a mean blood alcohol concentration of 0.2-0.3% over eight hours, with a

maximum of 350mg/dL, thirty minutes after the intraperitoneal injection, declining by 100mg/dL per hour, over 3.5 hours[2, 11]. The remaining mice in each treatment group were given two intraperitoneal injections of saline, (0.85% NaCl v/v). The mice were returned to their cages to recover. Food and water were not restricted. Four distinct treatment groups were created; Control+saline (35mgZn/kg plus two intraperitoneal injections 0.85% NaCl) [Saline], Control+ethanol (35mgZn/kg plus two intraperitoneal injections 0.015ml/g ethanol) [Ethanol], Zinc+saline (200mgZn/kg plus two intraperitoneal injections 0.85% NaCl), and Zinc+ethanol (200mgZn/kg plus two intraperitoneal injections 0.015ml/g ethanol).

In the morning of GD18, cages were inspected for pups, (C57Bl/6J gestation period; average 19 days, range 18-21 days)[38]. The day pups were discovered was Post Natal Day (PND) zero, when the litter sizes were recorded and mothers were switched to the non-zinc supplemented, commercial laboratory diet. On PND three, the number of surviving pups and their weights were recorded. PND14 pups were weighed, and litter size documented. Those with more than seven pups were culled to a maximum of seven, to limit the effects of competition for milk, (e.g. weight decrements)[11].

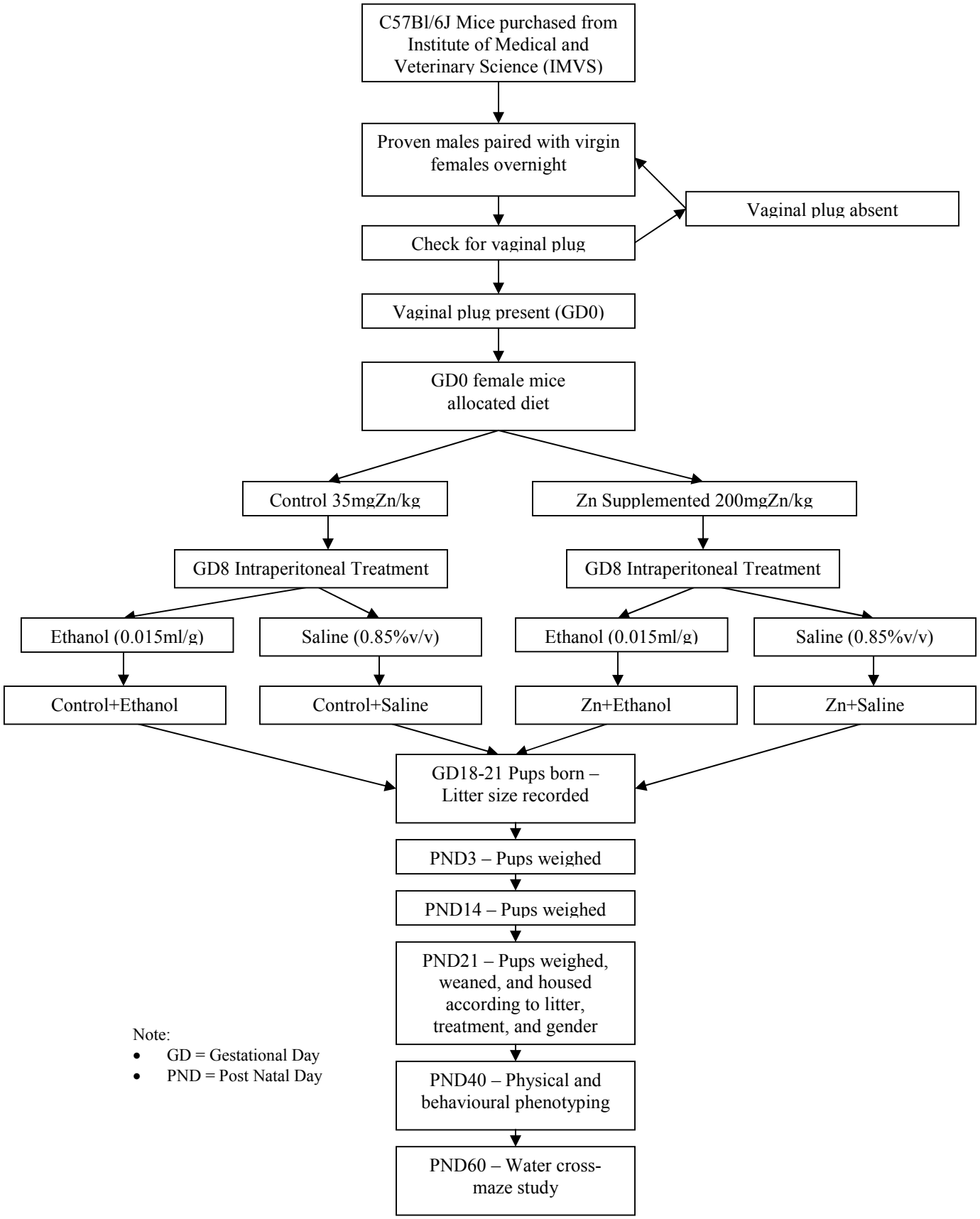
PND21 pups were weaned, weighed, and housed (in opaque cages, limiting visual stimulation, which affects cognitive ability) according to; litter, gender, and treatment. At PND35-40 (average 40 days), mice underwent a phenotyping protocol including measurements of; weight, body length, and tail length. The protocol was a condensed, modified form of the ***S**mithKlein Beecham Pharmaceuticals, **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit, **I**mperial College School of Medicine at Saint Mary's Hospital, **R**oyal London Hospital, Saint Batholomew's and the Royal London School of Medicine, **P**henotype **A**ssessment* (SHIRPA)[41] (*Table 2*). Modifications included additional tests for; swimming ability, and a screen for visual abnormalities (e.g. microphthalmia, anophthalmia, and cataracts, common birth defects for C57Bl/6J mice)[42]. This screen was necessary to identify anomalies which would impair the ability of the mouse to use spatial cues while in the maze. If any were identified, the mouse was excluded from the study (*Figure 1*).

Table 2: Modified SHIRPA pre-maze behavioural and phenotyping screen.

Area under study	Test	Brief Description/ Comments.
Physical Characteristics	Weight	(grams)
	Body length	Measured from nose to base of tail (millimetres)
	Tail length	Measured from base of tail (millimetres)
	Coat colour	-
	Skin colour	Observation of skin colour on hind paw pads
	Hair morphology	Including; straight, curly, etc
	Tail morphology	Straight, curly (impairs swimming ability)
	Tail elevation	(millimetres above or below horizontal)
	Limb morphology	Including screening for syndactyly, ectrodactyly, and polydactyly
	Pelvic elevation	(millimetres)
Neurological Reflexes	Tremor	Presence of a resting or intention tremor
	Touch escape	Response to approach by experimenter
	Postural passivity/ Irritability/ Vocalisation	Response to being held by tail (including vocalisation, struggling movements, etc)
	Toe pinch	Withdrawal of hind leg after paw pinch (rapid, slow, or no response)
	Provoked biting/ Aggression	Biting of object held close to mouth in response to being held upside down (by tail)
	Righting reflex	Ability to return to equilibrium/ upright posture after being placed on back
	Gait	Signs of normal/ abnormal gait
Neuromuscular Strength	Limb grasping/ Wire hanging test	Ability to grasp and hang upside down on a wire mesh, for maximum 60 seconds
Hearing	Acoustic startle reflex	Response to loud sound (e.g. freeze, jump, pinnae retraction)
Visual Ability	Palpebral closure	Observation of eyes and palpebral characteristics including; screening for cataracts, anophthalmia, and microphthalmia
	Visual cliff test	The testing apparatus consists of an elevated platform, ending at a cliff. This cliff is covered by a piece of clear perspex, which extends over the drop, (to prevent the animal from falling over the precipice). The animal is placed on the platform and encouraged to walk towards the cliff edge, to determine if it will walk over it. Mice lacking visual perception walk onto the perspex (over the cliff).
	Visual placement	Mouse extends towards the ground using front paws before reaching/ touching it, when lowered by tail.
	Corneal reflex	-
Smell	Olfactory smell test	Ability to detect foreign smell in cage, (e.g. cat food buried under bedding) – maximum 2 minutes to detect and dig up food
Motor Functioning	Open field test	Including; measures of spontaneous activity (number of squares crossed in 5 minutes), defecation, freezing, urination, rearing on hind limbs, digging, grooming, sitting, etc
	Swimming ability	Ability to swim for 30 seconds in a bath of warm water

Modified from: Rogers, *et al.*, (1997).

Figure 1: **Breeding and Treatment Method.**



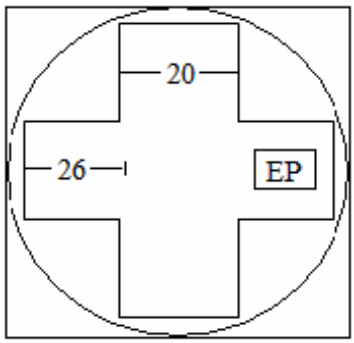
Note:

- GD = Gestational Day
- PND = Post Natal Day

At PND50, ten days before cognitive testing, mice of normal appearance (as determined by the SHIRPA) were randomly selected for a water cross-maze test, to assess their spatial learning and memory abilities. Twelve males and twelve females were selected from each treatment group. They were anaesthetised with halothane and ear-tagged. At PND60, these mice were tested in the water cross-maze, which has been successfully used in previous studies[11, 39].

The maze consisted of a clear plastic cross with arm lengths of 26cm and widths of 20cm (*Figure 2*). This was placed within a circular black pool measuring one metre diameter, filled with water maintained at 23 degrees Celsius. The pool was surrounded by 90cm high walls, covered with black plastic. Milk powder made the water opaque to conceal an Escape Platform (EP), 0.5cm below the surface in the east arm. Spatial (visual) cues were placed around the maze, including the experimenter, who always stood opposite the EP, at the west side. These cues remained constant.

Figure 2: **Cross Maze Dimensions.**

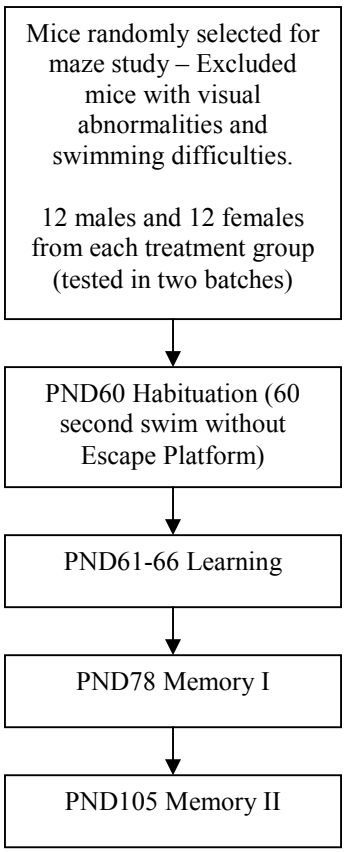


Note: EP = Escape Platform.
The EP was 5cm from the edge of the maze, and the maze boundaries were 14cm from the edge of the circular pool. All values are in centimetres (cm).

On PND60, mice were weighed. They were individually placed in the water maze for sixty seconds (minus EP), allowing for environmental habituation. During PND61-66 (six days of testing), mice were required to learn the position of the EP. The north, south, and west arms were chosen as the starting position in a semi-randomised pattern. The mice were given six trials daily (undertaken in the morning); two sets of three trials, separated by a thirty minute rest. Each mouse was placed in the distal end of the starting arm, facing the wall, and allowed sixty seconds to find the EP. Those which found the EP in the required time were left on it for ten seconds, before being removed to a holding cage for ten seconds. Subsequent trials were performed, starting from a different arm. Those failing to find the EP in the allocated time, were guided there by the experimenter, left for ten seconds, removed to the holding cage, then trials were continued. The semi-randomised pattern varied between the two daily trial sets, and between days. The learning progress of the mice was monitored by two measures; *Escape Latency,* (time taken for the mouse to find the EP), and *Number of Correct Trials,* (mouse entered the arm containing the EP and found it on the first attempt)[11].

Twelve days after the final day of learning, (PND78), mice were tested for memory of the EP position. The protocol for this was identical to that used in the learning phase of the experiment. The escape latency, and number of correct trials were recorded. A second memory test was performed one month later, (PND105), to determine long-term memory retention. The logistics (96 mice, and variations in ages of pups), necessitated testing in two batches over a two month period, (48 pups in each batch). The same experimenter collected the data for learning, memory one, and memory two, reducing variability attributable to different animal handling (*Figure 3*).

Figure 3: **Cross-Water Maze Testing Method.**



Note: PND = Post Natal Day

This study yielded observational data; two measurements per mouse, six times a day for six consecutive days for learning, and two measurements per mouse, six times a day for memory one, and memory two. These data were analysed using Minitab Statistics Software, (Minitab Incorporated, State College, Philadelphia). The litter sizes, and body weights at PND3 and PND14 were analysed using one-way analysis of variance (ANOVA). PND21, PND40, and PND60 body weights were analysed using a generalised linear model, allowing for detection of gender, treatment, and gender×treatment effects. Tukey's *post-hoc* test was used to detect significance.

Correct trial data were analysed using a generalised linear model, assuming a binomial distribution (binary data–yes/no–whether the mouse swam directly to the EP), and a logit link (logistic regression) [where $\text{logit}(p)=\log p/(1-p)$][11]. Data were presented as a percentage (number of correct trials/total number of trials×100%). Deviance tests, (with an approximate chi-squared distribution) were used to test for significance of factors and interactions.

Escape latency was analysed using ANOVA, (a general analysis of variance). F-tests were used to detect statistical significance for learning and memory one. A saline-treated male died between memory one and memory two, so the escape latency for memory two was analysed using Restricted Maximum Likelihood (REML), (different numbers of mice in each treatment group). Statistical significance for this was determined with a Wald test, (approximate chi-squared distribution). Any factor or interaction detected to be statistically significant was subjected to Least Significant Difference (LSD) tests, allowing determination of where those differences occurred. Differences were classified as significant provided $p<0.05$. Where applicable, results have been presented as Mean ± Standard Error of the Mean (SEM).

Results:

Litter Size and Body Weight Analysis (PND3, PND14, PND21, PND40, & PND60):

Analysis of the litter size and body weight data revealed no significant differences between treatment groups for either factor on PND0 (litter size), PND3 (body weight), or PND14 (body weight) (*Tables 3 and 4*). At PND21 (weaning), no differences in weights were found between treatment groups or genders (*Table 4*). When mice were weighed for SHIRPA analysis (PND40), there were no differences for treatment or treatment×gender effects. However, regardless of treatment group, males were significantly heavier than females, (17.38±0.04g versus 15.71±0.04g, respectively; $p<0.001$) (*Table 4*). Similar effects were found on PND60 for randomly-selected mice weighed prior to commencement of cognitive testing. No treatment or treatment×gender effects were observed; however, regardless of treatment, males were significantly heavier than females, (20.85±0.04g versus 17.84±0.04g, respectively; $p<0.001$) (*Table 4*).

Table 3: Litter Size Comparison (PND0)†.

Treatment*	Number of Litters	Mean Litter Size (±SEM)
Saline	20	5.5 ± 0.4
Ethanol	18	5.7 ± 0.5
Zn+Saline	18	5.67 ± 0.5
Zn+Ethanol	18	6.33 ± 0.5
F value	0.6579	
p value	0.5807 (NS)	

No significant differences were found between litter sizes of different treatment groups. Data were analysed using ANOVA, with Tukey's *post-hoc* test for significance.

* Treatment of mother on GD8. † Number of Live Pups on PND zero. NS, Not Significant. PND, Post Natal Day

Table 4: Body Weight Data Comparison (PND3, PND14, PND21, PND40, & PND60).

Treatment* or Group	PND3	PND14	PND21	PND40	PND60
Saline	1.30 ± 0.04 (n = 16) †	5.35 ± 0.23 (n = 15) †	6.61 ± 0.04 (n = 15) †	16.50 ± 0.07 (n = 15) †	19.31 ± 0.08 (n = 24) ∞
Ethanol	1.30 ± 0.04 (n=17) †	5.47 ± 0.20 (n = 16) †	6.87 ± 0.05 (n = 14) †	17.16 ± 0.09 (n = 14) †	19.33 ± 0.08 (n = 24) ∞
Zn+Saline	1.30 ± 0.05 (n=17) †	5.13 ± 0.19 (n = 16) †	6.46 ± 0.04 (n = 15) †	16.45 ± 0.08 (n = 15) †	19.68 ± 0.08 (n = 24) ∞
Zn+Ethanol	1.21 ± 0.03 (n=18) †	4.90 ± 0.24 (n = 17) †	6.20 ± 0.04 (n = 15) †	16.06 ± 0.09 (n = 15) †	19.05 ± 0.08 (n = 24) ∞
All Females	-	-	6.51 ± 0.02 (n = 55) †	15.71 ± 0.04 (n = 54) †	17.84 ± 0.04 (n = 48) ∞
All Males	-	-	6.56 ± 0.02 (n = 58) †	17.38 ± 0.04 (n = 54) †	20.85 ± 0.04 (n = 48) ∞
Main Effects Treatment	F Value = 1.2938 *p* Value = 0.2841 (NS)	F Value =1.3867 *p* Value = 0.2555 (NS)	F Value = 1.70 *p* Value = 0.171 (NS)	F Value = 1.06 *p* Value = 0.369 (NS)	F Value= 0.49 *p* Value = 0.692 (NS)
Main Effects Gender	-	-	F Value = 0.04 *p* Value = 0.844 (NS)	F Value = 15.49 *p* Value = <0.001	F Value = 65.41 *p* Value = <0.001

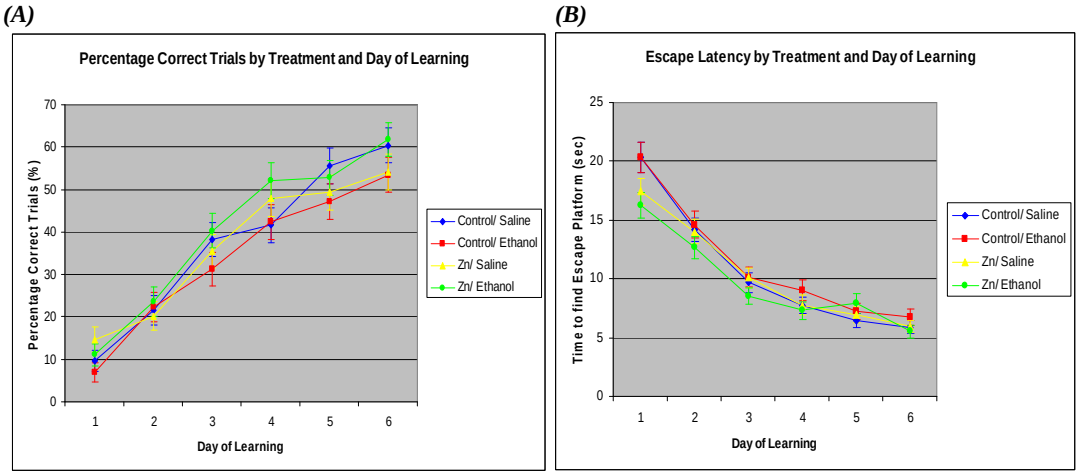
No significant differences were found between the body weights of mice in different treatment groups on PND3, PND14, PND21, PND40, or PND60. An effect of gender was found on PND40 and PND60, with males being significantly heavier than females. No treatment×gender effects were observed. Data from PND3 and PND14 were analysed using ANOVA, with Tukey's *post-hoc* test for significance. Data from PND21, PND40, and PND60 were analysed using a generalised linear model, with Tukey's *post-hoc* test for significance. Weight data are presented as mean ± SEM.

* Treatment of mother on GD8. NS, Not Significant. †, n refers to the number of litters. ∞, n refers to the number of pups. PND, Post Natal Day

Spatial Learning Analysis (PND61-66):

Figure 4 illustrates the trends for number of correct trials and escape latency for the six days of learning. Mice from all treatment groups learnt the position of the EP. This is indicated by the decreasing escape latency and increasing incidence of correct trials, over the six days. Both indicators demonstrated a day effect, and on the sixth day of learning, all mice had attained the same level of performance. The escape latency data did not show any significant differences between treatment groups on any single day. However, a gender×day effect was found, with female mice tending to reach the EP faster than males, regardless of treatment ($p<0.005$) (*Table 5*). No treatment×gender effect was observed. The correct trial data did not show any differences between treatments on any particular day. As there was no conclusive gender×day or treatment×gender effect, the data for correct trials of males and females were combined, allowing for analysis of a larger sample size.

Figure 4: Spatial Learning. (A) Correct Trial Data by Treatment and Day of Learning. (B) Escape Latency by Treatment and Day of Learning.



These graphs show an increasing number of correct trials and decreasing escape latency for all groups over the six days of training. No significant differences occurred between the treatment groups for either parameter on any single day of learning. Each data point was obtained by taking an average of the six daily trials for all mice, (24 mice per treatment group). Correct trial data are represented as percentage correct trials on each day (Number of correct trials / Total number of trials × 100% ± SEM%). Escape latency are represented as mean ± ANOVA pooled SEM (sec).

Table 5: Gender Effect on Escape Latency per Day of Learning.

Day of Learning	Female Mean Escape Latency ± SEM (sec)	Male Mean Escape Latency ± SEM (sec)
1	14.0 ± 1.2	14.4 ± 1.2
2	9.8 ± 1.2	10.1 ± 1.2
3	7.5 ± 1.2	6.3 ± 1.2
4	5.3 ± 1.2	5.6 ± 1.2
5	4.7 ± 1.2	5.0 ± 1.2
6	3.8 ± 1.2	4.9 ± 1.2
p value	< 0.005	

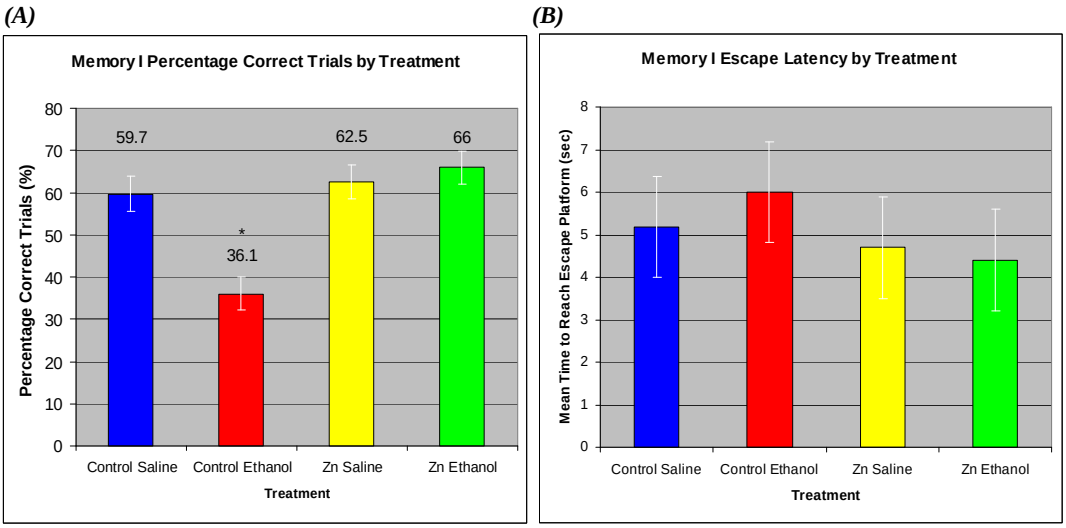
Females tended to find the EP faster than males on each day, regardless of treatment group. Each data point was obtained by averaging the escape latency for all trials on a particular day for each gender. Escape latency are represented as mean ± ANOVA pooled SEM (sec).

Spatial Memory I and II Analysis (PND78 and PND105):

Figures 5 and *6* give the outcomes for correct trials and escape latency for memory I and II, respectively. The correct trial data for memory I show a difference between treatment groups. Ethanol treated mice had significantly fewer correct trials (36.1±3.9%) compared with those of the saline (59.7±4.1%), zinc+saline (62.5±4.0%), and zinc+ethanol (66±3.9%) groups, (the latter three are not significantly different) (p<0.001). Escape latency data for memory I indicates differences between treatment groups. Ethanol treated mice took longer (6.0±1.2 sec) to find the EP, compared with zinc+saline (4.7±1.2 sec), and zinc+ethanol (4.4±1.2 sec) (p=0.047). However, no difference was found between ethanol (6.0±1.2 sec) and saline (5.2±1.2 sec), even though ethanol treated mice tended to require a longer swim time. Moreover, the escape latency for saline (5.2±1.2 sec) was not significantly different to that of zinc+saline (4.7±1.2 sec) or zinc+ethanol (4.4±1.2 sec). When the male and female data for escape latency were compared, a gender effect was observed, (regardless of treatment group), with females being faster than males

(4.7±1.1 sec versus 5.4±1.1 sec, respectively, p=0.028). A gender effect was not observed for correct trials, nor was a treatment×gender effect seen for either parameter.

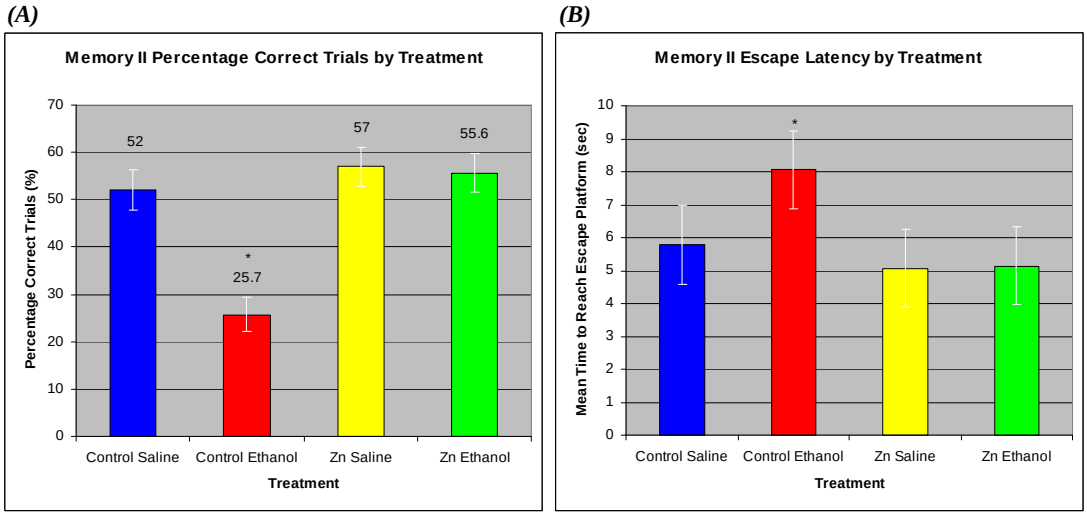
Figure 5: Spatial Memory I. (A) Percentage Correct Trials by Treatment. (B) Escape Latency by Treatment.



These graphs indicate that ethanol impairs spatial memory, as ethanol treated mice had significantly fewer correct trials compared with other treatment groups. Ethanol treated mice also had a significantly longer escape latency compared with zinc+saline, or zinc+ethanol mice. However, their escape latency was not different to that of saline treated mice, despite the tendency towards a longer swim time. Nor was the saline treated mice escape latency different to that of zinc+saline or zinc+ethanol. Correct trial data are represented as percentage correct trials (Number of correct trials / Total number of trials × 100% ± SEM%). Escape latency are represented as mean ± ANOVA pooled SEM (sec).
* Control+ethanol treated mice significantly different to other treatment groups (p<0.001).

Testing in the water cross-maze for memory II, displayed more overt differences. Correct trial data revealed that ethanol treated mice had fewer correct trials (25.7±3.6%) compared with saline (52.0±4.2%), zinc+saline (57.0±4.1%), and zinc+ethanol (55.6±4.1%) (p<0.001) (*Figure 6*). The correct trial data for saline, zinc+saline, and zinc+ethanol were not significantly different. When treatment was ignored, a gender effect was found, with females having more correct trials (52.0±2.8%) than males (43.0±2.9%) (p=0.025). A gender×treatment interaction was not observed. Escape latency showed a treatment effect, with ethanol treated mice being slower to find the EP (8.1±1.2 sec) than all other groups (saline, 5.8±1.2 sec; zinc+saline, 5.1±1.2 sec; or zinc+ethanol, 5.1±1.2 sec) (p<0.001). No significant differences were seen between saline, zinc+saline, and zinc+ethanol groups for escape latency. No gender or treatment×gender effects were found for this parameter. These data demonstrate that ethanol treated mice had impaired memory of the position of the EP, compared with other treatment groups, as demonstrated by fewer correct trials, and longer escape latency.

Figure 6: Spatial Memory II. (A) Percentage Correct Trials by Treatment. (B) Escape Latency by Treatment.



These graphs show that ethanol impairs spatial memory, as ethanol treated mice had significantly fewer correct trials, compared with other treatment groups. Ethanol treated mice also had a significantly longer escape latency, compared with saline, zinc+saline, or zinc+ethanol groups. Correct trial data are represented as percentage correct trials (Number of correct trials / Total number of trials × 100% ± SEM%). Escape latency are represented as mean ± ANOVA pooled SEM (sec).

* Control+ethanol mice significantly different to other treatment groups (p<0.001).

Discussion:

This study provides further evidence of the teratogenicity of ethanol, with the resultant physical and behavioural abnormalities manifesting in various ways[5, 38].

Litter Size and Body Weights:

Ethanol did not have an impact on the litter size (PND0), nor the body weights of mice at any age (PND3, PND14, PND21, PND40, or PND60). This contradicts the findings of other studies, which have reported decrements in these factors in response to ethanol[38-39]. However, Minetti, *et al.*[34] and our laboratory have previously found no effect of alcohol on body weight, or litter size[11]. These disparities could be related to variations in treatment protocols, timing of exposure, or the random nature of teratogenesis[38]. Not every mouse exposed to ethanol *in utero* is impaired. Those damaged, fall into a spectrum of affectedness, including prenatal mortality and growth retardation. Different cohorts of animals will show variations in numbers affected. In this cohort, we found that males were significantly heavier than females, regardless of treatment, (at PND40 and PND60). This was expected, due to hormonal differences between genders, (i.e. testosterone promoting the formation of muscle bulk in males)[11].

Pre-Maze Study Behavioural Screening:

Ethanol exposure *in utero* is associated with eye malformations, and C57Bl/6J mice are also innately prone to these, (specifically; anophthalmia, microphthalmia, and cataracts)[10, 20, 38, 42]. To alleviate this confounding factor, all mice underwent phenotyping, incorporating screening for eye abnormalities. Those with anomalies were excluded from the water cross-maze, as was the case in previous studies[11, 39]. Defects in the retinogeniculocalcarine pathway, and occipital lobe, (areas involved in vision and visual processing of spatial information) were not ascertained. However,

this appears not to have affected the results, as mice from all treatment groups achieved a similar level of performance during the learning phase of the experiment, (which required a functioning visual system)[11]. Streissguth, *et al*.[5] have noted that motor impairments can occur as part of the spectrum of defects resulting from intrauterine ethanol exposure. Screening of swimming ability for all mice was performed as a part of behavioural testing. Those with impairments were excluded from the maze.

Ethanol, Spatial Learning and Memory:

Our results demonstrate that acute prenatal exposure to ethanol does not affect spatial learning; as the ethanol treated mice performed as well as the saline group in the learning task. The ethanol treated mice did show impairments in spatial memory compared with all other groups, when tested 12 days (memory I) and one month (memory II), post-training. These observations corroborate those of previous studies[11, 33-34, 39]. Our research group has recently shown an ethanol effect on spatial memory, using a similar protocol[11]. Similarly, a water-maze study by Minetti, *et al*.[34], (using rats that had been exposed to ethanol on GD8) found that the ability to learn the EP position was not impaired, while spatial memory was affected in females. Dumas & Rabe[39] found memory impairments in C57Bl/6J mice tested in a water cross-maze. However, these were observed in aging mice (24 months), and not in young mice (at three months, and 12 months), which contrasts with this study. This may be due to the heterologous protocols used in the studies, such as dose (5.8g/kg versus 2.9g/kg), day of insult (GD9 versus GD8; when different structures are forming in the embryo), and mode of administration of ethanol (one versus two intraperitoneal injections)[11, 39]. Different doses affect the peak blood alcohol concentrations, (initially higher with one intraperitoneal injection of dose 5.8g/kg), and the duration of the insult, (longer with two intraperitoneal injections of dose 2.9g/kg, separated by four hours), thereby, impairing brain function to different extents. Detrimental effects of ethanol on memory are also widely reported in humans[7, 9, 43].

Gender, Spatial Learning and Memory:

There was a gender specific difference for spatial learning and memory. Females tended to find the EP faster than males on any particular day, during training and memory I, regardless of treatment. Memory II data display a gender effect for correct trials (but not escape latency), with females having more correct trials than males. Gender specific effects have previously been reported by our laboratory (C57Bl/6J mice), and Minetti, *et al*.[34] (Wister rats). In these studies, females proved more impaired than males[11, 34]. LaBuda, *et al*.[44] have found that CD1 male mice had a superior performance in a spatial processing task, while Goodlett & Peterson[45] found that female C57Bl/6J mice were less affected than males with spatial learning. The outcome differences may relate to random variations in cohorts, differences in timing of exposure to teratogens, different testing methods, and strain and species specific vulnerabilities. The relative vulnerability of male and female fetuses to ethanol-induced teratogenesis requires attention in future studies, to examine how variations in genetic and hormonal constitutions impinge on outcomes.

Zinc Supplementation, Ethanol Exposure, and Spatial Memory:
Mice exposed to ethanol *in utero*, whose mothers had been provided with a zinc supplemented diet, performed at the same level as saline treated mice, during memory testing. Our laboratory has previously reported that a subcutaneous injection of zinc given concurrently with acute ethanol exposure on GD8, prevents spatial memory defects in offspring[11]. These studies provide support for the assertion that zinc dyshomeostasis is associated with ethanol-induced teratogenesis[11, 20]. The ethanol is probably responsible for the disruption of zinc homeostasis, by induction of the zinc-binding protein, MT, in the maternal liver[2]. The precise mechanism for this is unknown, whether it has a direct effect on the promoter region of the gene coding for MT, or by interaction with secondary messengers. Further research is necessary.

MT induction sequesters zinc ions in hepatocytes, creating a concentration gradient resulting in movement of zinc to the mother's liver. Therefore, the fetus experiences a transient zinc deficiency, with associated negative consequences. It has been proposed that zinc supplementation at the time of alcohol exposure, prevents birth defects resulting from MT induction, and the ensuing functional zinc deficiency. This is achieved by raising the plasma zinc levels, (up to five-fold with subcutaneous zinc injection)[11]. Previous studies have found that dietary zinc supplementation does not produce inordinate changes in plasma zinc concentrations[37]. This is attributable to a MT related mechanism in the gastrointestinal tract, (restricting absorption when luminal zinc concentrations are high)[20, 46]. This implies that dietary zinc repletion would be inadequate to ameliorate a transient zinc deficiency caused by ethanol. It is theorised that a high zinc concentration in the intestines could act as a reservoir, replenishing the plasma zinc, following ethanol-related MT induction in the liver, with benefits for the fetus[20].

This study is the first to find that dietary zinc supplementation can ameliorate ethanol-induced cognitive defects in mice. We have recently shown that dietary zinc repletion has positive effects in terms of preventing physical birth defects and improving post-natal survival (Summers, *In Press*). Tanaka, *et al*.[47] have performed experiments on pregnant rats (provided with a high dietary zinc intake), which demonstrated beneficial effects for offspring. However, Keppen, *et al*.[16] have reported no effect of zinc repletion on the incidence or severity of FAS in mice fed a liquid diet containing four times the recommended daily intake of zinc. The experimental procedures may have facilitated the cited benefits in our study. Keppen, *et al*.[16] administered alcohol throughout pregnancy, whereas we provided an acute assault of ethanol, (mimicking a "binge"). Additionally, our supplementation of zinc was almost six times the recommended daily intake, compared with four times for Keppen, *et al*.'s study. We were able to prevent the detrimental effects of ethanol, possibly because the exposure was transient, and the available zinc higher. Further research is required to determine the threshold for a protective action in mice and higher species, (including humans).

It is unclear how the ethanol-induced zinc deficiency produces cognitive defects. This insufficiency may be a primary aetiology for teratogenesis, especially considering zinc is necessary for the function of many molecular processes involved in growth and development[19]. For the CNS, these include; neurogenesis, differentiation, migration, synaptogenesis, and neurotransmission[19]. We exposed mice to ethanol during early embryogenesis (GD8), equivalent to the third or fourth week of human development (Carnegie Stages 7-13)[39, 48]. These stages correspond to a period when

teratogenic insults can potentially have a greater impact, as the basic structural layout of the embryo is emerging. Specific areas of the brain are destined to form, (e.g. the hippocampus), and major structural deviations can result from damage to the stem cells responsible for formation of these areas[49].

Zinc supplementation may help to prevent abnormalities produced directly by ethanol, (e.g. apoptosis). Apoptosis is induced by ethanol in diverse cell populations, and developing neurons have been found to be particularly susceptible[15, 50]. It has been purported that zinc functions in anti-apoptotic pathways[27, 51-53]. This metal may preserve compromised neurons that would otherwise be destined for apoptosis[52]. Ethanol has been found to influence the anatomical structure of the hippocampus. This is by reduction of the overall number of neurons (through inhibition of cellular proliferation during development), decreasing connectivity between neurons (fewer dendritic spines), and repression of cellular plasticity[49]. Further research is required to determine if zinc supplementation at the time of ethanol exposure can promote normal hippocampal development.

Conclusion:

This study demonstrates that even a single binge of alcohol during early pregnancy can have detrimental effects on mice offspring, (such as spatial memory defects). Dietary zinc supplementation throughout pregnancy can ameliorate these and may be useful in the clinical setting to prevent CNS disturbances in humans. However, as with all supplementation strategies, consideration needs to be given to the impact on other elements (e.g. iron and copper). Our results have important implications for health care. Encouraging women to abstain from alcohol, particularly large quantities in a short period of time (binge drinking) during pregnancy, is vital to promote positive fetal outcomes. Additionally, promoting maternal nutrition, including zinc intake, is fundamental to ensure fetal health. The mechanisms whereby zinc acts as a protective agent are unknown. Further research is warranted, including study to determine the protective threshold of zinc, and whether supplementation following a teratogenic insult can prevent or minimise physical and neuro-developmental anomalies. Zinc repletion may have wider applications, (e.g. other teratogenic insults inappropriately inducing MT in the maternal liver). Optimising zinc intake through oral supplementation (similar to folate protocols for neural tube defect prevention), is a promising option to reduce the incidence or severity of birth defects caused by ethanol, promoting the health of future generations.

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