THE BEHAVIORAL EFFECTS OF CHOLINE SUPPLEMENTATION ON
A RODENT MODEL OF FETAL ALCOHOL SPECTRUM DISORDERS

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Thesis of Elizabeth Jalal Abou:

The Behavioral Effects of Choline Supplementation on a Rodent Model of Fetal
Alcohol Spectrum Disorders

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Despite knowledge that alcohol consumption during pregnancy is one of the leading causes of mental retardation and may lead to a range of fetal alcohol spectrum disorders (FASD); some women continue to drink alcohol during pregnancy, producing a need to seek effective therapeutic interventions. One treatment that has gained interest as a potential treatment for FASD is choline, an essential nutrient that plays a critical role in brain development and function. The current study used a rodent model to examine the beneficial effects of choline supplementation initiated mid-gestation on mitigating alcohol related teratology caused by heavy alcohol consumption through a time period equivalent to all three trimesters of human pregnancy. Ethanol was administered to pregnant Sprague-Dawley rats at a dose of 6.0 g/kg/day from gestational days (GD) 5-20, a time period equivalent to the first two trimesters of human pregnancy and to pups at 5.25 g/kg/day from postnatal days (PD) 2-9, a time period equivalent to the third trimester of human pregnancy. Ethanol treated pups received choline supplementation at the following dose (0, 15, 50, 100, or 150 mg/kg/day) via subcutaneous injections from PD 1-21. Pair-fed (PF) and lab chow (LC) control groups received injections of saline and sham intubations. On PD 45, subjects were tested on a Morris water maze spatial learning task and on PD 70, a working memory version of that task. Developmental alcohol reduced body and brain weight and impaired spatial learning. Spatial learning deficits were only observed in males. Choline reduced the severity of alcohol-related deficits in a dose-dependent manner. Ethanol exposure did not significantly impair working memory performance. These results indicate that choline supplementation may reduce some alcohol-related learning and memory impairments when given around the third trimester of pregnancy even if heavy alcohol consumption occurred throughout pregnancy. These findings have important implications for children of women who drank alcohol during pregnancy and their caregivers.
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CHAPTER 1

INTRODUCTION

It has been well established that maternal alcohol consumption during gestation can result in physical and central nervous system (CNS) abnormalities. Given the CNS damage, children prenatally exposed to alcohol may suffer from behavioral abnormalities including attention deficits, hyperactivity, learning impairments, and motor dysfunction (Mattson & Riley, 1998; Thomas, Melcer, Weinert, & Riley, 1998). Lemoine, Harousseau, Borteyru, and Menuet (1968) were the first to discover a pattern of common characteristics among 127 children born to alcoholic mothers. These characteristics included: (1) pre and/or postnatal growth retardation, specifically noted in height and head circumference; (2) facial dysmorphologies such as short palpebral fissures of the eyes, short nose bridge, smooth philtrum, and thin upper-lip; and (3) cognitive deficits (Lemoine et al., 1968). Jones and Smith (1973) later confirmed these findings. The presence of the triad of characteristics along with confirmation of maternal alcohol exposure constitutes a diagnosis of what is now termed Fetal Alcohol Syndrome (FAS). FAS is one of the most common preventable causes of mental retardation, however some women continue to drink alcohol during pregnancy. Since not all children prenatally exposed to alcohol meet the full criteria for FAS, Clarren, Alvord, Sumi, Streissguth, and Smith (1978) described these children as suffering from fetal alcohol effects (FAE). More recently, the term ‘fetal alcohol spectrum disorders’ (FASD) has been coined to refer to the range of alcohol’s teratogenic effects, which includes FAS.

RANGE AND MAGNITUDE OF PRENATAL ALCOHOL EFFECTS

The sizeable variability in the array and degree of prenatal alcohol effects has made diagnoses of FAS and FASD challenging and therefore has affected the accuracy of prevalence rates reported. It is most likely that FAS and FASD prevalence rates from epidemiological studies are underestimated due to the diagnostic difficulties. The estimated prevalence rate of FAS in the United States is two per 1,000 live births (May & Gossage, 2001). The combined prevalence of FASD was reported to be as high as 9.1 per 1000 in the
general population (Sampson et al., 1997).

CNS dysfunction is one of the most serious consequences of prenatal alcohol exposure. Early reports of brain regions affected by prenatal alcohol exposure lacked consensus (Clarren, Alvord, Sumi, & Streissguth, 1977; Clarren et al., 1978; Jones & Smith, 1975), leading Peiffer, Majewski, Fischback, Bierich, and Volk (1979) to believe that no specific patterns of brain malformations result from prenatal alcohol exposure. Clarren et al. made a similar erroneous conclusion in 1978. These erroneous conclusions were based on early autopsy reports, which likely utilized non-generalizable FAS population samples, since mortality was a prerequisite for analysis. Both Peiffer and Clarren did state in their reports that the amount and timing of alcohol exposure likely played a role in the variability of the neuropathology seen in the autopsy reports. More recent imaging technology has allowed FASD populations to be studied, revealing specific patterns of structural abnormalities, such as overall reduction in brain size, with volumetric reductions in the parietal lobe, cerebellar vermis, corpus callosum, and the caudate nucleus (Spadoni, McGee, Fryer, & Riley, 2007). Since the time of the early autopsy reports on FASD, much has been learned about multiple mechanisms of prenatal alcohol related damage that make it more apparent as to why there is variability in the severity and pattern of alcohol-related damage.

Variability and severity of prenatal alcohol related damage may be determined by maternal, environmental, and/or genetic risk factors. Maternal risk factors include maternal age, a history of alcoholism, patterns of maternal alcohol abuse such as timing, duration, and quantity of maternal alcohol consumption (Goodlett, Horn, & Zhou, 2005). Environmental risk factors that may worsen the effects of prenatal alcohol exposure and put the child at greater risk for developing FAS include low socioeconomic status, the usage of additional drugs during pregnancy, and a lack of prenatal care. Finally, it has been shown that genetic factors such as the presence of ADH2*3 allele has protective properties against FAS by producing a more efficient alcohol dehydrogenase enzyme and therefore removing alcohol from one’s system faster (McCarver, Johnsrud, & Koukouritaki, 2003).

Aside from risk factors, it should also be noted that alcohol consumed during pregnancy acts on a variety of cells and molecular targets. The alteration of neurochemicals, cell signaling, or a delay of the developmental process may lead to a pathological cascade of events. Categories of mechanisms that play a role in the array of FASD phenotypes
suggested by Goodlett, Horn, and Zhou (2005) include disturbance in cellular energetics, cell acquisition/impaired regulation of developmental timing, dysregulation of gene expression, abnormal cell to cell interactions, disrupted cell signaling, and cell damage/death.

**USE OF ANIMAL MODELS**

The tools used to advance FASD knowledge range from cellular studies to those with animals and humans. Animal models have a distinct advantage over human studies because experimenters are able to control their subjects’ environment, both prenatal and postnatal, therefore the experimenter can conclude that any change observed in the dependent variable(s) is a direct result of manipulations made to the independent variable(s). Experimental animals generally reproduce and age quicker than humans, which helps decrease significantly the duration and cost of the experiments. Lastly, it would be unethical to answer certain experimental questions using humans. The utilization of both animal and human research is essential to broaden our knowledge and aid in the search for FASD treatments.

The rat model is the most common animal model used in developmental and biomedical research. The extensively studied model has led to well-mapped neuroanatomy and behavioral assessments (Clancy, Finlay, Darlington, & Arland, 2007). A drawback to using an animal model can be put simply by stating animals are not humans. The effectiveness of an animal model results from the ability to confidently extrapolate research findings from the non-human to human species. The timing and sequence of early brain developmental events are conserved across mammalian species (Finlay & Darlington, 1995). When equating the developmental milestones in rats and humans it should be noted that the length of gestation in rats (22 days) is equivalent to the first two trimesters in humans. The rat’s brain growth spurt, a transient period of time when the brain is growing most rapidly, takes place during the first ten days following birth, whereas in humans this occurs during the third trimester. Thus, to model alcohol exposure across all three trimesters, alcohol exposure must occur both prenatally and postnatally in the rat.

**TREATING PRENATAL ALCOHOL RELATED DAMAGE**

Animal studies can be used to examine ways that effectively block some of ethanol’s acute teratogenic effects. Some effective agents include growth factors (Bonthius, Karacay,
Dai, & Pantazis, 2003; Endres et al., 2005), neuroactive peptides (Chen, Charness, Wilkemeyer, & Sulik, 2005; Wilkemeyer et al., 2003; Wilkemeyer, Menkari, Spong, & Charness, 2002), and N-methyl-D-Aspartate receptor antagonists (Idrus, Thomas, & Riley, 2009; Thomas, Fleming, & Riley, 2001). Some environmental/behavioral treatments that attenuate damage caused by prenatal alcohol exposure in both human and animal studies include enriched environment, which ameliorated some behavioral aspects of fetal alcohol effects (Hannigan & Berman, 2000; Hannigan, Berman, & Zajac, 1993), exercise (Christie et al., 2005), and acrobatic motor training (Klintsova, Goodlett, & Greenough, 2000; Klintsova et al., 2002). Human studies have shown that with specific training, improvements can be made to alcohol related deficits in areas such as social skills (O'Connor et al., 2006), behavioral problems, and math skills (Coles, Kable, & Taddeo, 2009).

**CHOLINE: A PROMISING TREATMENT FOR FASD**

Another promising therapeutic treatment for FASD is the essential nutrient choline. Choline is critical for brain development and function (Zeisel, 2006). It is a precursor to phosphatidylcholine and sphingomyelin, which are major components of all cells, including glia and neurons. During nervous system development having an adequate supply of choline is necessary for cell division, axonal/dendritic growth, synapse formation, and myelination. Choline is also a precursor to the neurotransmitter acetylcholine (ACh), which modulates hippocampal based learning and memory. Finally, choline acts as a methyl donor and can, therefore, influence gene expression.

Perinatal choline supplementation has been studied in typically developing rats and induces lasting memory enhancements (McCann, Hudes, & Ames, 2006; Meck, Smith, & Williams, 1988; Meck & Williams, 1997, 2003). These enhancements can be attributed to morphological (Li et al., 2004; Loy, Heyer, Williams, & Meck, 1991; Williams, Meck, Heyer, & Loy, 1998), electrophysiological (Jones, Meck, Williams, Wilson, & Swartzwelder, 1999; Pyapali, Turner, Williams, Meck, & Swartzwelder, 1998), and neurochemical (Alkondon & Albuquerque, 2006; Cermak, Holler, Jackson, & Blusztajn, 1998; Cermak et al., 1999; Coutcher, Cawley, & Wecker, 1992; Meck, Smith, & Williams, 1989; Montoya et al., 2000) changes found in the brain. Choline supplementation during pre and/or postnatal development in rats results in long-lasting improvements in spatial memory, as determined
by performance on a radial-arm maze (Meck et al., 1988; Meck et al., 1989; Meck & Williams, 1999) or Morris water maze (Brandner, 2002; Plyusnina, Oskina, Shchepina, Prasolova, & Trut, 2006), and of timing and temporal memory (Cheng, MacDonald, Williams, & Meck, 2008; Meck & Williams, 1997).

Choline’s beneficial effects are not limited to typically developing rats. In fact, a growing body of literature has shown that choline may benefit those that have suffered from the damaging effects of prenatal alcohol exposure. In rodent studies, choline was able to ameliorate prenatal alcohol-related discrimination learning deficits (Thomas, La Fiette, Quinn, & Riley, 2000), reversal learning errors (Thomas, Garrison, & O’Neill, 2004), over-activity, spatial learning impairments (Thomas, Biane, O’Bryan, O’Neill, & Dominguez, 2007), and trace fear conditioning deficits (Wagner & Hunt, 2006). A commonality between all the above tasks mentioned is that they rely on the functional integrity of the hippocampal and cortical cholinergic systems and choline has demonstrated its ability to improve cholinergic neurotransmission (Blusztajn, Cermak, Holler, & Jackson, 1998).

Choline supplementation was also found to mitigate some of the behavioral alterations associated with alcohol exposure during the brain growth spurt when choline was administered from postnatal day (PD) 4-30 (Thomas et al., 2004). In a follow up study by Ryan, Williams, and Thomas (2008), it was reported that choline supplementation from either PD 11-20 or PD 21-30 improved Morris water maze spatial learning performance in rats exposed to ethanol during the brain growth spurt. It was also found that when choline was administered from PD 11-30 no added benefits over and above the PD 11-20 or PD 21-30 ranges were found. This study indicates that the therapeutic window for choline is likely to be large; suggesting that choline supplementation even when administered during late childhood may still benefit those who suffered from prenatal alcohol exposure.

**Purpose**

The purpose of this study is to inform a longitudinal clinical study that is to take place in the Ukraine. The clinical study intends to identify alcohol consuming pregnant women mid-gestation and request that they abstain from alcohol. Following the request for alcohol abstinence during pregnancy, the pregnant women will be treated with micronutrients, one of
which includes choline. It is possible that some of these women may fail to abstain from alcohol consumption during pregnancy even after the request for abstinence has been made.

Using a rodent model, the current study examined whether choline supplementation is effective if initiation occurs mid-gestation during heavy alcohol consumption through all three trimesters of development. In rats, the third trimester equivalent occurs postnatally, so alcohol was administered at a dose of 6.0 g/kg/day from gestational days (GD) 5-20 and at 5.25 g/kg/day from PD 2-9. Choline was administered via subcutaneous injections at varying doses (0, 15, 50, 100, or 150 mg/kg/day) during the third trimester equivalent and beyond (PD 1-21). The choice to extend choline supplementation beyond the third trimester equivalence stems from previous literature that showed the developmental window for choline’s effectiveness extended beyond the third trimester equivalence (Ryan et al., 2008). Subjects were tested on a standard spatial learning Morris water maze task and a working memory version of the Morris water maze, as these tasks depend on hippocampal cholinergic integrity (Everitt & Robbins, 1997; McNamara & Skelton, 1993). We hypothesized that ethanol would impair performance in spatial learning and working memory and that choline supplementation would attenuate ethanol-related impairments in a dose-dependent manner.
CHAPTER 2

METHODS

The methods section has been divided into the following subsections; subjects, prenatal treatment, postnatal treatment, blood collection, behavioral testing, and statistical analyses.

SUBJECTS

Nulliparous female Sprague-Dawley rats between (200-225 grams) were ordered from Charles River Laboratories, Inc. Hollister, CA. Following habituation to a humidity, temperature, and light (12hr light/dark cycle) controlled vivarium at the Center for Behavioral Teratology, San Diego State University; a female and male rat were housed together overnight for mating purposes. The presence of a seminal plug following overnight housing indicated conception. The conception date was designated as GD 0. The pregnant females were singly housed and randomly assigned to one of three prenatal treatment groups; ethanol, pair-fed, or lab chow. The offspring from the resulting timed pregnancies were randomly culled to 8-10 per litter and assigned to one of seven postnatal treatment groups; Ethanol + Saline, Ethanol + 15 mg/kg Choline , Ethanol + 50 mg/kg Choline, Ethanol + 100 mg/kg Choline, Ethanol + 150 mg/kg Choline, Pair-fed + Saline, and Lab chow + Saline. To control for litter effects, no more than one rat per sex and treatment group was used. This experiment was conducted in accordance to the guidelines set by the University Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health (1996) Guide for the Care and Use of Laboratory Animals.

PRENATAL TREATMENT

All dams were randomly assigned to one of three groups: ethanol, pair-fed, or lab chow and were fed AIN-93G diet (containing 2.5 g/kg Choline Bitartrate) with ad lib access to water. Ethanol-exposed dams received a measured amount of AIN-93G powder diet (100 g each day) throughout pregnancy while pair-fed dams were yoked to an ethanol dam (receiving the amount of food consumed by the ethanol dam on each equivalent day of
gestation accounting for body weight differences) to control for nutritional factors. The amount of diet remaining in the cage was measured every day between 10:00 a.m. and noon to determine daily food intake. Lab chow dams received AIN-93G food pellets ad lib throughout pregnancy.

The prenatal treatments were accomplished via a single daily oral gavage, using a 16 gauge intubation needle, from GD 5-20. Ethanol-exposed dams were exposed to 6.0 g/kg/day ethanol, pair-fed controls were treated with iso-caloric maltose solution, and lab chow controls were treated with physiological saline solution (0.89% sodium chloride). One hour prior to gavage, food was removed from the ethanol and pair-fed dam cages in order to standardize the rate of absorption of ethanol and to standardize the stress of food deprivation. To determine any possible restricted feeding effect, the lab chow group had ad lib access to food. All rats had ad lib access to water. Body weights of the dams were taken daily through GD 21. Body weights were not taken on GD 22 so that the dam was not stressed on the expected delivery day.

**POSTNATAL TREATMENT**

The pups’ date of birth was considered PD 0. On PD 1, stratified sampling by sex was used to cull pair-fed and lab chow control litters to 10 (5 males and 5 females per litter whenever possible) and ethanol litters to 8 (4 males and 4 females per litter whenever possible). Ethanol litters were culled to a smaller number than control litters to normalize body growth rate since ethanol-exposed rat pups suckle less adequately from the dam during intoxication.

Subjects received ethanol from PD 2-9 at a dose of 5.25 g/kg/day, delivered in two feedings, two hours apart, using 11.9% v/v ethanol in milk solution. Ethanol pups were also given two additional milk-only feedings (two hours apart) after the ethanol exposure on the days of treatment. These added milk-only feedings ensured pups were being nourished since ethanol-exposed pups tend to suckle less than controls during intoxication. Pair-fed and lab chow controls were sham-intubated, receiving intubations in the same manner as the ethanol-exposed group, but with no milk solution being delivered. The purpose for the sham intubation was to control for any stress associated with the intragastric intubation.
Postnatal ethanol exposure was accomplished via intubation of neonatal rats, using a length of thin flexible tubing (cannula) attached to a 25 gauge needle on a 1 mL syringe. The external distance from the mouth to the stomach was first measured and marked on the cannula to ensure the correct length of tubing is inserted and the tip correctly placed within the stomach during intubation. The tubing was lubricated with corn oil prior to intubation procedure. The cannula was then passed over the tongue into the esophagus and down into the stomach. Solution was delivered over a period of at least ten seconds, reducing any excessive force or pressure, and decreasing the risk of damage to the pups. The entire procedure (from cannula marking to intubation) was completed in less than two mins per pup, restricting the time the litter is away from the dam.

Ethanol-exposed pups were randomly assigned to one of five subcutaneous choline injection treatment doses (0, 15, 50, 100, or 150 mg/kg/day) following culling. For the dose of 0 mg/kg subjects were given injections of a saline vehicle. Pair-fed and lab chow controls were treated with saline injections only. Pups received choline injections daily from PD 1-21, a time period equivalent to the third trimester and beyond in humans.

Body weights of pups were taken from PD 1-21 and on the first day of testing for each behavioral test. On PD 6, paws of all experimental pups were tattooed for individual identification, with non-toxic ink. Pups were weaned on PD 21 and separated by sex on PD 28.

**Blood Collection**

To determine peak blood alcohol concentrations (BACs), 40 μl of blood was collected from ethanol and pair-fed dams via tail clip three hours after the alcohol intubation (GD5 and GD 20) and 20 μl of blood was collected from all pups (ethanol, pair-fed, and lab chow) via a tail clip 90 minutes after the second ethanol treatment on PD 6. Blood samples were centrifuged and supernatant was collected for only ethanol-treated rats. Samples were frozen before analysis. BACs were analyzed with the Analox Alcohol Analyzer.

**Behavioral Testing**

The behavioral outcomes of rats with FASD are important for evaluating the extent of ethanol related damage and treatment efficacy. For this reason two behavioral testing
procedures were used: Morris water maze spatial learning task, and a working memory version.

**Morris Water Maze**

The use of water to motivate learning in animals has been around since the early 1900s (Glaser, 1910; Waller, Waller, & Brewster, 1960; Wever, 1932). Rats are natural swimmers; however, when they are placed in water they display a desire to escape. The benefit of using water based tasks to motivate learning includes a lack of need to food deprive or extensively pre-train animals. Richard Morris developed the open-field Morris water maze in 1984 (Morris, 1984). In this task, the main purpose is for the animal to escape by finding a hidden platform submerged about an inch under opaque water. The animals are allotted a specific amount of time after they find the escape platform to study stationary distal cues. Finding the platform and the subsequent removal of the animal from water reinforces its desire to locate the platform on subsequent trials. Learning is inferred from the rats’ ability to find the escape platform. The task, depending on variation, can be used to measure either long-term spatial memory or working memory.

**Apparatus**

A white metal pool (175 cm diameter) centered in a room with distal visual cues (i.e. large black shapes with brightly colored boarders, posters, and signs) located on the walls was utilized as the test location. The room contained curtains around the pool that were used to conceal the distal visual cues when appropriate and were used to conceal the tester during testing. The pool was filled with water, one inch above a hidden platform (10 cm diameter). The water was made opaque by adding four cups of pro-nurse specialty milk powder and the temperature was maintained at 26 (± 1)°C.

The rats’ behavior was tracked and recorded (e.g. latency, path length, heading angle, and speed) using an HVS Image tracking system, Water 2020 program, and a pressure operated start/stop ball. Each subject’s head was marked with black non-toxic permanent marker each day prior to testing so that the tracking system could detect the contrast between the subject and the white pool water.

The pool was divided into 4 equal quadrants with two platform locations in each quadrant; arbitrarily numbered 1-8 (i.e. Quadrant I contained platform location 1 and 2,
Quadrant II contained platform location 3 and 4 etc.). The outer perimeter of the pool was numbered with 12 equally spaced numbers (1 through 12 in an analog clock like fashion) that indicated the starting positions for the task.

**Standard Reference Morris Water Maze Spatial Learning Procedure**

The standard reference Morris water maze places demand on hippocampal-based spatial memory and was conducted in three phases; acquisition (PD 45-50), probe (PD 51), and visible platform. During the acquisition phase, rats acquire spatial information that they use to find a hidden platform. The probe trial tests subjects’ spatial memory and, lastly, the visible platform establish if factors outside of memory influence water task performance.

Subject cages were placed in the testing room for 30 minutes each day prior to testing, a period known as habituation. During the habituation period, the rats had free access to food and water. Subjects were trained to 1 of 4 pseudo-randomly chosen platform locations in one of the four quadrants for 4 trials per day for 6 consecutive days. The location of the platform remained constant for each subject throughout testing. Each trial had a maximum search time of 60 seconds. During the first six days of testing, an inter-trial interval of three to five minutes was used. Once the platform was located, the subject remained on the platform for ten seconds to have an opportunity to process the distal visual cues in relation to the platform position. If the platform was not located in the allotted time, the subject was led to the platform by the experimenter and allowed to remain on the platform for ten seconds. To avoid bias, the rats were placed in the pool facing the outer perimeter, at varying start positions. On the seventh day of testing, during the probe trial, the platform was removed from the pool, and subjects were given one 60-second trial with no escape opportunity. The purpose of the probe trial was to measure the degree and accuracy of spatial bias that resulted from the spatial learning acquisition phase. After each trial, subjects were removed from the water and placed into a holding cage that was warmed by a heat lamp. When testing was completed for the day, subjects were dried off, placed in their home cage, and returned to the vivarium.

Visible platform was tested approximately a week following the probe trial. Subjects were given four trials for two days. The four trials consisted of the visible platform moving to one of four quadrants, not using the same quadrant twice in one day. All external visual
cues were hidden so that the subjects would attend only to the platform that sat slightly above water level. The visible platform task does not depend on memory, thus impairment on this task suggests that memory may not be the only factor influencing performance on the spatial acquisition task or working memory task, rather there are likely other performance factors involved confounding the interpretations of memory test results.

The outcome measures for the acquisition phase and visible platform included the following: path length, latency, speed and heading angle. For the probe trial outcome measures included percent time spent in target quadrant, number of times the platform location was passed through and percent time spent near the platform perimeter.

**Working Memory Morris Water Maze Procedure**

The working memory Morris maze task places a greater demand on the memory processes of subjects, compared to the standard reference Morris maze procedure. Since all of the subjects in the present study have already undergone Morris maze training, no pre-training will be necessary. The Morris maze was set to accommodate eight platform positions.

For this task, each session consisted of two trials, an acquisition and a test trial. The platform location did not vary within a session; however the rats’ starting position did. The platform location did change between sessions; thus, the acquisition trial of each session consisted of trial and error, and the test trial indicated the subject’s memory of the platform location during that session. Because the location changed between sessions, this task required flexibility in memory, or working memory. The maximum search time for a trial was 60 seconds. Subjects were tested on two sessions each day, one in the morning (around 9 a.m.) and one (six hours later) in the afternoon (around 3 p.m.).

On testing days 1-3, subjects were tested with a 0 second inter-trial-interval (ITI). Following successful location of the platform during the acquisition trial, the subject remained on the platform for a period of twenty seconds, allowing the subject the opportunity to process the distal cues in relation to the platform position. Directly after this post-trial interval period, the subject was removed from the platform and placed immediately back into the Morris maze tank at a pre-determined start position. On testing days 4-6, subjects were tested with a 60 second ITI. Once the subject found the platform and 20 seconds had elapsed,
the subject was removed from the tank and put into a drying cage. Following the 60 second ITI, the subject was placed back into the tank at a pre-determined start position that was different from the acquisition start position.

The outcome measures for the working memory task included path length, latency, speed and heading angle. These measures were also analyzed as savings measures (the difference in performance on the test trial compared to the training trial).

**STATISTICAL ANALYSES**

All data were analyzed using SPSS 17.0. Analyses of variance (ANOVAs) were used with treatment and/or sex as between-subject factors and days, trials, platforms, and/or time as repeated measures. In the presence of an interaction follow-up analysis of simple effects were conducted. Fisher’s Least Significant Difference post-hoc comparisons were used to determine the nature of group differences where significant independent variables had three or more levels. Differences were considered statistically significant at $p < 0.05$. 
CHAPTER 3

RESULTS

Analytical results are presented over the following subsections: dams’ body weights, sex ratio, food intake, pups’ body weights, dams’ blood alcohol concentrations, pups’ blood alcohol concentrations, Morris water maze, working memory, and brain weights.

DAMS’ BODY WEIGHTS

Dams’ body weights were recorded throughout gestation (GD 0-21) with the exception of GD 22, the expected date of delivery. From GD 0-5, a period prior to treatment implementation, all groups (e.g. lab chow, pair-fed, and ethanol) steadily gained weight with a significant effect of Day \( F(5,245) = 291.5, p < 0.001 \) (see Figure 1A). Analysis from GD 6-21 resulted in a significant Treatment x Day interaction \( F(30,735) = 4.1, p < 0.001 \). The ethanol-exposed dams had significantly lower body weights compared to the lab chow group on the majority of gestational days during prenatal alcohol treatment (GD 7 and GD 9-21) and a significantly lower body weights compared to the pair-fed dams on GD 13, GD 16, and GD 18-21 (see Figure 1B). The pair-fed dams grew faster than the ethanol-exposed dams despite being yoked, potentially due to a difference in nutrient absorption.

SEX RATIO

The number of pups born per litter and their sexes were recorded on postnatal day (PD) 1. There was no significant difference found in the number of pups born per litter nor was there a significant difference in the number of male or female pups born per litter based on treatment (see Table 1).

FOOD INTAKE

Food intake was recorded daily from GD 5-20 for ethanol and pair-fed groups. There was a significant effect of Day \( F(15,525) = 30.2, p < 0.001 \); dams from both group ate more as days of gestation increased. There was no significant treatment effect between the ethanol
Figure 1. Dam body weights over gestational days. (A) There were no significant body weight differences between groups during the non-treatment period. (B) During the ethanol treatment period ethanol-exposed subjects lagged in growth compared to the lab chow (LC) group on GD 7 and GD 9-21 and lagged in growth compared to the pair-fed (PF) dams on GD 13, GD 16, and GD 18-21. The pair-fed dams grew faster than the ethanol-exposed dams despite being yoked, potentially due to a difference in nutrient absorption. *=Ethanol group different from LC control group, +=Ethanol group different from both LC and PF control groups.

Table 1. Birth Statistics by Treatment Group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Litters</th>
<th>Mean (+SEM) Number of Pups</th>
<th>Mean (+SEM) Number of Males</th>
<th>Mean (+SEM) Number of Females</th>
<th>Ratio of Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>n = 24</td>
<td>11.9 ± 0.68</td>
<td>5.5 ± 0.49</td>
<td>6.3 ± 0.53</td>
<td>0.47</td>
</tr>
<tr>
<td>PF</td>
<td>n = 13</td>
<td>13.6 ± 0.83</td>
<td>6.2 ± 0.77</td>
<td>7.5 ± 0.67</td>
<td>0.45</td>
</tr>
<tr>
<td>LC</td>
<td>n = 15</td>
<td>13.5 ± 0.76</td>
<td>6.9 ± 0.59</td>
<td>6.7 ± 0.59</td>
<td>0.51</td>
</tr>
</tbody>
</table>

and pair-fed groups pointing out the pair-fed group was adequately yoked to the ethanol group.

**PUPS’ BODY WEIGHTS**

Pups’ body weight data were analyzed in three segments (PD 1, PD 2-10, and PD 11-21) because PD 1 represented a postnatal period prior to choline treatment administration, PD 2-10 represented a third-trimester equivalent where alcohol treatment continued and choline treatment began, and PD 11-21 represented a period where alcohol treatment ceased, but choline treatment continued. Overall, ethanol reduced body growth, an effect that choline was unable to reverse.

The PD 1 analysis confirmed a significant effect of Sex \(F(1,139) = 11.3, p < 0.05\], as males weighed more than females and a significant effect of Treatment \(F(6,139) = 3.5,\)
All ethanol-exposed groups weighed less than the lab chow controls. Though pups were randomly assigned to treatment groups and there were no significant difference among birth weights between ethanol-treated groups, it is notable that post hoc analyses indicated that ethanol-exposed subjects treated with 100 or 150 mg/kg choline weighed significantly less than the pair-fed control group. The pair-fed group did not differ in body weight compared to the lab chow controls (see Table 2 for body weights on PD 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean (±SEM) Body Weight (g) PD1</th>
<th>Mean (±SEM) BAC (mg/dl) PD6</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH - Saline</td>
<td>6.6 ± 0.18</td>
<td>301.0 ± 15.7</td>
</tr>
<tr>
<td>EtOH - 15 mg/kg Choline</td>
<td>6.6 ± 0.19</td>
<td>318.8 ± 9.4</td>
</tr>
<tr>
<td>EtOH - 50 mg/kg Choline</td>
<td>6.7 ± 0.19</td>
<td>316.1 ± 12.6</td>
</tr>
<tr>
<td>EtOH - 100 mg/kg Choline</td>
<td>6.3 ± 0.16</td>
<td>312.6 ± 11.1</td>
</tr>
<tr>
<td>EtOH - 150 mg/kg Choline</td>
<td>6.5 ± 0.17</td>
<td>320.2 ± 7.0</td>
</tr>
<tr>
<td>PF - Saline</td>
<td>7.0 ± 0.13</td>
<td>N/A</td>
</tr>
<tr>
<td>LC - Saline</td>
<td>7.1 ± 0.10</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Body weight analyses from PD 2-10 resulted in a significant effect of Day \( F(8,1112) = 2979.8, p < 0.001 \) and Day x Treatment interaction \( F(48,1112) = 15.9, p < 0.001 \). On PD 2 ethanol-exposed subjects treated with 15 mg/kg choline weighted significantly less than the pair-fed group and ethanol-exposed subjects treated with 100 or 150 mg/kg choline weighed significantly less than both control groups (see Figure 2A). From PD 3-10 all ethanol groups weighted significantly less than both control groups (see Figure 2B), however on PD 3 the 100 mg/kg choline subjects also weighed significantly less than the ethanol-exposed 0 mg/kg choline group.

Body weight analyses from PD 11-21 revealed a significant effect of Day \( F(10,1390) = 5644.9, p < 0.001 \), Day x Treatment interaction \( F(60,1390) = 1.8, p < 0.001 \), and Day x Sex interaction \( F(10,1390) = 3.9, p < 0.001 \). Ethanol-exposed subjects continued to lag in growth compared to controls throughout the choline treatment period (see Figure 3, p. 18). By PD 15, the Ethanol + Saline group no longer weighed significantly less than lab chow controls and by PD 18, no longer weighed significantly less than either control group. Importantly, there were no body weight differences among the ethanol-exposed subjects, illustrating that choline did not significantly affect body growth. Males gained weight at a faster rate than female rats.
Figure 2. Pup body weights during the early postnatal period. (A) On PD2, ethanol-exposed subjects treated with 15, 100 or 150 mg/kg choline had significantly lower body weights compared to at least one control group. (B) Ethanol-treated subjects weighed significantly less than both control groups from postnatal days 3-10. *= Ethanol group different from PF group, **=Ethanol group different from both LC and PF control groups.

**DAMs’ BLOOD ALCOHOL CONCENTRATIONS**

Blood alcohol concentrations (BACs) were collected from dams at two time points during gestation (GD5 & GD20) and analyzed with a paired t-test. The mean ± SEM BAC
### Table 1: Pups' Body Weight Across Postnatal Days

<table>
<thead>
<tr>
<th>Postnatal Days</th>
<th>EtOH - Saline</th>
<th>EtOH - 15 mg/kg Choline</th>
<th>EtOH - 50 mg/kg Choline</th>
<th>EtOH - 100 mg/kg Choline</th>
<th>EtOH - 150 mg/kg Choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>15</td>
<td>25</td>
<td>35</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>26</td>
<td>36</td>
<td>46</td>
<td>56</td>
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<td>13</td>
<td>17</td>
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<td>21</td>
<td>25</td>
<td>35</td>
<td>45</td>
<td>55</td>
<td>65</td>
</tr>
</tbody>
</table>

### Figure 3. Pups' body weights following the end of ethanol treatment. Ethanol-exposed subjects continued to lag in growth compared to controls throughout the choline treatment period, but by PD 18 Ethanol + Saline group no longer weighed significantly less than either control group.

For the ethanol-exposed group on GD 5 (135 ± 14 mg/dl) was significantly lower compared to the sample collected on GD 20 (222 ± 15 mg/dl), \( F(1,23) = 8.4, p < 0.001 \). This suggests a change in alcohol metabolism during pregnancy.

### Pups’ Blood Alcohol Concentrations

Pups’ BAC data were collected on PD 6. One blood sample data point was substituted with the group mean since it was aberrant (over 3 SD from the mean). No statistically significant effects were found, suggesting that neither choline treatment nor gender affected peak BACs (see Table 2 for BAC values on PD6).

### Morris Water Maze (MWM)

Morris water maze spatial memory data was analyzed in three segments that included acquisition, probe, and visible platform.
Acquisition

During acquisition, rats were able to find a more direct path to reach the hidden platform over testing days and trials, producing a significant main effect of Day $[F(5,690) = 167.4, p < 0.001]$ and Trial $[F(3,414) = 58.6, p < 0.001]$. There was also a main effect of Treatment $[F(6,130) = 4.0, p < 0.05]$ and a Sex x Treatment interaction $[F(5,690) = 167.4, p < 0.001]$. For a follow-up of the interaction, data were analyzed separately for each sex.

Males had a significant Treatment $[F(6,67) = 4.0, p < 0.05]$ effect. With the exception of the ethanol-exposed treated with 15 mg/kg or 100 mg/kg choline, ethanol-exposed groups were impaired, producing significantly longer path lengths compared to both controls (see Figure 4). In contrast, ethanol-exposed subjects treated with 15 or 100 mg/kg choline performed significantly better than the ethanol-exposed group that did not receive choline and not significantly different from controls. Females also had a significant effect of Treatment $[F(6,71) = 2.3, p < 0.05]$; however ethanol-exposed subjects that did not receive choline were not impaired, rather the Ethanol + 100 mg/kg Choline group performed significantly worse (longer path length) compared to all other groups including the controls with the exception of Ethanol + 150 mg/kg Choline with whom they did not differ. Thus, the Ethanol + 100 mg/kg Choline group showed impairment even in the absence of an ethanol effect (see Figure 5, p. 21).

A similar pattern was observed on the latency to find the platform. Analyses of the latency revealed a significant effect of Day $[F(5,690) = 181.5, p < 0.001]$ and Trial $[F(3,414) = 62.7, p < 0.001]$, as subjects were able to find the hidden platform faster over days and trials. A significant effect of Treatment $[F(6,138) = 5.1, p < 0.001]$ and a Sex x Treatment interaction $[F(6,138) = 2.4, p < 0.05]$ were also revealed. The Sex x Treatment interaction was followed-up by analyzing each sex separately.

There was a significant main effect of Treatment $[F(6,67) = 4.8, p < 0.001]$ among males, as the Ethanol groups treated with saline, 50 or 150 mg/kg choline took longer to locate the hidden platform compared to both control groups. The Ethanol groups treated with 15 or 100 mg/kg choline did not differ significantly from the lab chow group or the Ethanol + Saline group, but did take longer to find the platform compared to pair-fed control (see Figure 6A, p. 22). There was also a significant effect of Treatment $[F(6,71) = 2.7, p < 0.05]$ among females, where post hoc analysis indicated that the Ethanol + 100 mg/kg Choline
Figure 4. Morris water maze path length for males. (A) Path length by days of testing and (B) path length averaged over 6 days of testing demonstrated male ethanol-exposed subjects were impaired compared to controls with the exception of the Ethanol + 15 mg/kg Choline and Ethanol + 100 mg/kg Choline groups (graphs A and B). ** = Ethanol group different from both LC and PF control groups.
Figure 5. Morris water maze path length for females. (A) Path length by days of testing and (B) path lengths averaged over 6 days of testing demonstrate no apparent ethanol effect; however the Ethanol + 100 mg/kg Choline group had significantly longer path length than all groups except the Ethanol + 150 mg/kg Choline group. ***=Ethanol group different from all other groups, except Ethanol + 150 mg/kg Choline.
Figure 6. Morris water maze latency to find platform by sex. (A) Ethanol-exposed males treated with 0, 50 or 150 mg/kg choline had significantly higher latencies compared to both control groups. (B) Ethanol exposure by itself did not disrupt performance among females yet the Ethanol + 100 mg/kg Choline group took significantly longer to locate the hidden platform compared to all other groups except for Ethanol +150 mg/kg Choline. ***=Significantly different from all groups except the Ethanol + 150 mg/kg Choline group, **= significantly different from both LC and PF control groups, *=significantly different from PF control group.
group took significantly longer to locate the hidden platform compared to all groups except for Ethanol +150 mg/kg Choline group (see Figure 6B).

Unlike the path length, latency to find the platform can be affected by swimming speed. Analyses of the speed measure revealed significant interactions of Trials x Treatment \([F(18,414) = 2.4, p < 0.05]\), Day x Treatment \([F(30,690) = 1.5, p < 0.05]\), and Day x Trial \([F(15,2070) = 4.0, p < 0.001]\). To understand the Trial x Treatment interaction, each trial (Trials 1-4) was analyzed separately. There was no significant effect of Treatment for any of the trials. To analyze the Day x Treatment interaction, each test day was analyzed separately. A significant main effect of Treatment \([F(6,130) = 2.8, p < 0.05]\) was only found for the first day of testing. The post hoc analysis indicated that the Ethanol +15 mg/kg Choline and Ethanol + 100 mg/kg Choline groups swam significantly slower than the Ethanol + Saline group and compared to both control groups (see Figure 7). This treatment effect was driven by the males. Even though there was no significant effect of sex, there were no group differences in swim speed among the females. Thus, the less robust effects of choline treatment seen in latency compared to path length (males) are likely due to differences in swimming speed.

![Figure 7. Morris water maze speed for day 1 of testing. The speed of the ethanol-exposed subjects treated with 15 and 100 mg/kg choline was significantly slower compared to the Ethanol + 0 mg/kg Choline group, LC and PF groups on day 1 of testing. ***= Significantly different from LC, PF and Ethanol + 0 mg/kg Choline groups.](image-url)
Analyses of the heading angle parameter resulted in a significant three-way interaction of Sex x Day x Treatment \( F(30,690) = 1.7, p < 0.05 \). However, a follow-up analysis conducted with each sex analyzed separately, which yielded no treatment effects for either males or females (see Figure 8).

![Figure 8. Morris water maze heading angle by sex. No significant treatment effects on heading angle exist for (A) males or (B) females.](image)

**Probe**

During the probe trial, which serves as an indicator of spatial memory, there were no significant effects of treatment, only a significant effect of sex \( F(1,138) = 4.3, p < 0.05 \), as males spent less time in target quadrant compared to females (see Figures 9 and 10).
Figure 9. Morris water maze probe target quadrant by sex. Males spent significantly less percent time in their target quadrant compared to females during the probe trial. * = Male rats significantly different from female rats.

Figure 10. Morris water maze probe target quadrant by treatment group. There was no significant difference between groups for both (A) males and (B) females on the target quadrant measure of the Morris water maze probe.
Visible Platform

A Day X Platform interaction was found for the following parameters: latency $F(3,414) = 15.0, p < 0.05$, path length $F(3,414) = 14.2, p < 0.001$, and heading angle $F(3,414) = 3.4, p < 0.001$. The interactions were due to a preference for platforms located near the curtain where the experimenter hid behind was stronger on the first day of testing over the second day of testing.

Although there were no significant effects of treatment on the first day of testing, there were on the second day of testing for latency $F(6,138) = 3.9, p < 0.05$ and path length $F(6,138) = 3.1, p < 0.05$. The ethanol-subjects treated with 0 mg/kg choline did not differ in latency compared to any control group whereas ethanol 50, 100 and 150 mg/kg choline groups did significantly worse than both control groups. The ethanol-subjects treated with 100 mg/kg choline also performed significantly worse than even the 0 mg/kg choline group. The Ethanol + 15 mg/kg Choline took longer than the pair-fed group, but not longer than the lab chow control to find the visible platforms (see Figure 11). Findings for the path length parameter were similar to the latency parameter in that all ethanol groups except ethanol-exposed subjects treated with 0 or 15 mg/kg choline had longer path lengths compared to the pair-fed controls (data not show).

There was a significant effect of Day $F(1,138) = 73.8, p < 0.001$ and Platform $F(3,414) = 56.5, p < 0.001$ for the speed parameter, but no treatment effect. Subjects swam significantly faster on the second day of testing since they were likely to be more familiar with the requirements of the task. Subjects also swam significantly faster to specific platform locations, the locations near where the experimenter hid following the release of the subjects into the pool. This behavior was a likely result of subjects making an association between the experimenter and escaping.

These results indicate that performance on water tasks may have been influenced by factors other than memory (e.g. sensory, motivational, or motor). For this reason, caution should be used when interpreting results from the spatial acquisition phase. However, it is also possible that performance during the visible platform stage was affected by carryover effects from the spatial acquisition period.
Figure 11. Morris water maze visible platform latency on day 2 of testing. The visible platform latency for Ethanol + 100 mg/kg Choline group was significantly higher compared to both controls and Ethanol + Saline group, while ethanol-exposed subjects treated with 50 and 150 mg/kg choline were only impaired compared to both control groups. Ethanol +15 mg/kg Choline had a significantly higher latency compared to the PF group alone (graph A). There was no sex differences in this specific task on day 2 (graphs B and C). *=Ethanol group different from PF control group, **=Ethanol group different from both LC and PF control groups, ***=Ethanol group different from LC, PF and the Ethanol + 0 mg/kg Choline groups.

WORKING MEMORY

Overall there were no robust ethanol effects on the working memory task, although the Ethanol + 100 mg/kg Choline females exhibited impaired performance. When analyzing savings in path length, there was a significant effect of Day \( F(5,630) = 3.6, p < 0.05 \), Sex \( F(1,126) = 5.3, p < 0.05 \) and a Sex x Treatment interaction \( F(6,126) = 4.3, p < 0.05 \). Only females showed a significant Treatment effect \( F(6,64) = 2.8, p < 0.05 \). The female Ethanol + 100 mg/kg Choline group showed significantly less path length savings compared to all other groups, except the Ethanol + 15 mg/kg Choline group (see Figure 12). Similar effects were observed for savings in latency.

BRAIN WEIGHTS

On the day of perfusions, PD 85, rats were weighed and the weights of the forebrain and cerebellum were recorded. There were main effects of sex \( F(1,139) = 679.7, p < 0.001 \)
and treatment \( F(6,139) = 2.6, p < 0.05 \) on body weight. Males weighed significantly more than females. All ethanol-treated subjects, except those in the Ethanol + 150 mg/kg Choline group, weighed significantly less than the pair-fed group, but only Ethanol + 100 mg/kg Choline weighed significantly less than the lab chow group (see Table 3).

**Table 3. Body Weight on Postnatal Day 85 by Treatment Group**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean (±SEM) Body Weight (g) PD 85</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH - Saline</td>
<td>376.0 ± 21.8</td>
</tr>
<tr>
<td>EtOH - 15 mg/kg Choline</td>
<td>370.8 ± 20.6</td>
</tr>
<tr>
<td>EtOH - 50 mg/kg Choline</td>
<td>377.0 ± 21.6</td>
</tr>
<tr>
<td>EtOH - 100 mg/kg Choline</td>
<td>357.7 ± 22.1</td>
</tr>
<tr>
<td>EtOH - 150 mg/kg Choline</td>
<td>379.7 ± 20.1</td>
</tr>
<tr>
<td>PF - Saline</td>
<td>403.7 ± 22.0</td>
</tr>
<tr>
<td>LC - Saline</td>
<td>391.7 ± 19.3</td>
</tr>
</tbody>
</table>

The brain weight analyses conducted on the forebrain and cerebellum regions shared very similar results. There were significant effects of Sex and Treatment for both brain regions (forebrain: Sex \( F(1,139) = 50.7, p < 0.001 \), Treatment \( F(6,139) = 28.0, p < 0.001 \); cerebellum: Sex \( F(1,139) = 28.7, p < 0.001 \), Treatment \( F(6,139) = 8.7, p < 0.001 \)) (see Figures 13A and 13B). Males had higher brain weights compared to females. In addition,
Figure 13. Brain weights and weight ratios by region. There were no group differences when considering forebrain (A) and cerebellum weight (B) alone. This was not the case when the ratio of brain weight to body weight was considered. The forebrain brain weight to body weight ratio of the Ethanol + 0 mg/kg Choline, Ethanol + 50 mg/kg Choline and Ethanol + 150 mg/kg Choline groups were significantly smaller compared to both control groups (C). The cerebellum ratio of all ethanol treated groups was significantly smaller compared to both control groups (D). **=Ethanol group different from both LC and PF control groups.
the post-hoc analyses revealed that the lab chow and pair-fed control groups had significantly heavier brain weights for both brain regions compared to all other ethanol treated groups.

Since there were differences in body weight, a ratio of brain weight to body weight was also analyzed. There was a Sex and Treatment effect for both brain regions (forebrain: Sex $[F(1,139) = 332.7, p < 0.001]$, Treatment $[F(6,139) = 3.5, p < 0.05]$; cerebellum: Sex $[F(1,139) = 179.4, p < 0.001]$, Treatment $[F(6,139) = 22.5, p < 0.001]$). For all the measures males, had a smaller brain/body weight ratio compared to females. Ethanol-exposed subjects treated with 0, 50, or 150 mg/kg choline had significantly smaller forebrain/body weight ratios compared to both control groups (see Figure 13C). In contrast, all ethanol-treated subjects had significantly smaller cerebellum/body weight ratios compared to controls (see Figure 13D).

Overall no Ethanol + Choline groups showed improvements in comparison to the Ethanol + Saline group, however when it came to the forebrain ratio Ethanol + 15 mg/kg Choline and Ethanol + 100 mg/kg Choline did not differ from controls.
CHAPTER 4

DISCUSSION

The present study demonstrated that alcohol delivered during what is equivalent to all three trimesters of the human pregnancy resulted in developmental and behavioral alterations in rats. These alterations included body weight deficits, impaired spatial learning and aberrant brain/body weight ratios. All of these alterations are consistent with previous reports in both clinical (Coles, Brown, Smith, & Platzman, 1991; Hamilton, Kodituwakku, Sutherland, & Savage, 2003) and animal models (Berman & Hannigan, 2000; Cronise, Marino, Tran, & Kelly, 2001; Detering, Reed, Ozand, & Karahasan, 1979; Kelly, Goodlett, Hulsether, & West, 1988; Marino, Aksenov, & Kelly, 2004; Tomlinson, Wilce, & Bedi, 1998).

Birth weights for all ethanol-treated rats were deficient compared to controls and body weights from the completion of ethanol treatment until weaning (PD11-21) were also deficient demonstrating that ethanol-treated rats continued to have adverse affects on body weight long after ethanol treatment ceased. Choline supplementation’s ability to mitigate fetal alcohol body weight deficiencies have been demonstrated when choline was administered prenatally (Thomas, Abou, & Dominquez, 2009). However, similar to other studies that administered choline during postnatal development (Meck & Williams, 1999; Ryan et al., 2008; Thomas et al., 2004), this study did not find choline supplementation to be beneficial in mitigating fetal alcohol body weight deficiencies at any of the doses administered (e.g. 15, 50, 100, 150 mg/kg choline). The divergence in choline supplementation’s benefits based on the time period choline is administered suggests that there is a small therapeutic window where choline may specifically influence alcohol-related body weight deficits.

Postnatal choline supplementation did mitigate some alcohol-related deficits, however the benefits were sex and dose dependent but not linear, therefore deviating from our hypothesis. We had originally hypothesized that choline would have beneficial effects on alcohol-related damage in a linear dose-dependent manner. For example we believed that
Ethanol + Saline group would show the most severe impairment and Ethanol + 150 mg/kg Choline would have the least impairment compared to controls. Rather, we found that ethanol exposure only impaired acquisition among males. Moreover, postnatal choline supplementation mitigates spatial learning impairments during the acquisition phase for male rats receiving 15 or 100 mg/kg/day choline, but not at the 50 or 150 mg/kg/day doses. In fact, based on path length, males from the following groups (Ethanol + 15 mg/kg or 100 mg/kg Choline) performed at control levels and significantly better than the Ethanol + Saline group, indicating improvement in spatial memory. Similar effects were seen for latency; however, it should be noted that both Ethanol + Choline groups swam significantly slower than the Ethanol + Saline group on the first day of testing. Results from the path length parameter should be considered more indicative of spatial learning, since differences in swim speed can affect latency. In contrast to acquisition, there was no treatment differences found during the probe trial. This suggests that after substantial training, all groups performed equally with regard to spatial memory and that impairments were evident only during initial acquisition of spatial memory. In support of our findings, studies have reported that perinatal choline effects are generally more robust in males (Tees, 1999; Williams et al., 1998). However, alcohol exposure in females did not significantly impair performance on the Morris water maze.

Female rats exposed to ethanol in this particular study did not show significant impairment on either memory tasks. However, the female rats exposed to ethanol and treated with 100 mg/kg/day choline were impaired compared to all other groups, even in the absence of an ethanol effect. Given that this dose of choline has been effective in reducing the adverse effects of alcohol exposure in females, either following prenatal or 3rd trimester equivalent alcohol exposure, these results are difficult to interpret.

It is important to mention that based on the visible platform control task findings all ethanol-treated groups treated with the higher choline doses took significantly longer to find the visible platforms compared to both control groups. In fact, the Ethanol + 100 mg/kg Choline group showed the most impairment during the visible platform task compared to controls and even compared to the Ethanol + Saline group. Impairment on the visible platform control task could suggest that factors other than memory (e.g. motivation, visual impairment, motor impairment) may have affected the rat’s ability to locate the platform
during the memory tasks, however considering our study design it is more likely that there was a carryover effect from the earlier spatial learning task where rats learned to look for distal spatial cues that are no longer important during this phase of testing.

In line with our behavioral findings, the Ethanol +15 mg/kg Choline and Ethanol +100 mg/kg Choline groups showed no difference in brain/body weight ratios compared to any other group including controls, though all other ethanol groups had smaller ratios compared to controls. These findings suggest that choline treatment can protect against cell loss in brain locations that are imperative for learning and memory and that behavioral performance was consistent with the neuropathology. Interestingly, all ethanol-treated rats had significantly smaller cerebellum/brain ratios compared to both controls.

Based on the literature, choline supplementation has the ability to mitigate alcohol-related spatial memory impairment (Ryan et al., 2008; Thomas et al., 2004). The exact neuronal targets choline supplementation influence in ethanol-exposed subjects has not been well established; however spatial learning depends on the functional integrity of the hippocampus. The hippocampus is particularly vulnerable to alcohol exposure (Berman et al., 2000). As it pertains to the hippocampus, choline supplementation from mid-late gestation increases cell division in the neuroepithelial layer of the hippocampus, is responsible for changes in the timing of migration, and increases differentiation of hippocampal neurons (Albright, Fredrich, Brown, Mar, & Zeisel, 1999; Albright, Mar, Craciunescu, Song, & Zeisel, 2005; Albright, Tsai, Mar, & Zeisel, 1998; Craciunescu, Albright, Mar, Song & Zeisel, 2003). These events lead to long-lasting increases in cell size and basal dendritic branching in CA1 pyramidal neurons (Li et al., 2004). Perinatal choline supplementation enhances N-methyl-d-aspartate (NMDA) receptor neurotransmission (Montoya, & Swartzwelder, 2000), excitability of CA1 pyramidal cells (Li et al., 2004), and long-term potentiation (LTP) (Jones et al., 1999) Thus, it is clear that choline supplementation during development can lead to long-lasting changes in hippocampal and cortical function and this is reflected as changes in cognitive performance that are evident even in old age (McCann et al., 2006).

There are several limitations to the present study. First, compared to other studies that administered alcohol only during the third trimester equivalence (Thomas et al., 2007, 2004; Ryan et al., 2008), this study found less severe alcohol impairment on memory tasks, even
though alcohol was administered throughout all three trimesters of development. There is a potential that these rats built a tolerance for alcohol or that mechanisms responsible for alcohol metabolism may have been altered due to prolonged exposure. It is also possible that repeated animal handling that was necessary during treatment could have reduced the severity of fetal alcohol effects. This may also serve as an alternative explanation for the reduced alcohol effects considering alcohol was administered during the equivalent of all three trimesters of a human pregnancy, as neonatal handling can attenuate fetal alcohol effects (Weinberg, Kim & Wayne, 1995). It is, however, surprising, given that body weights were reduced in the alcohol-exposed subjects compared to pair-fed controls.

In light of the limitations, although some levels of choline supplementation reduced the severity of spatial learning deficits, the overall pattern in terms of sex and dose is not clear. These findings suggest that choline supplementation may effectively protect against some of ethanol's teratogenic effects, but that choline may be less effective following more prolonged ethanol exposure. Additional investigation into the effects of alcohol exposure on all three trimesters and the potential for choline as treatment for fetal alcohol spectrum disorders is needed. Particularly since this is an important area of research to reduce the adverse effects of prenatal alcohol exposure.
REFERENCES


