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Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats

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Abstract

BACKGROUND—Prenatal alcohol exposure can alter physical and behavioral development, leading to a range of fetal alcohol spectrum disorders (FASD). Despite warning labels, pregnant women continue to drink alcohol, creating a need to identify effective interventions to reduce the severity of alcohol's teratogenic effects. Choline is an essential nutrient that influences brain and behavioral development. Recent studies indicate that choline supplementation can reduce the teratogenic effects of developmental alcohol exposure. The present study examined whether choline supplementation during prenatal ethanol treatment could mitigate the adverse effects of ethanol on behavioral development.

METHODS—Pregnant Sprague-Dawley rats were intubated with 6 g/kg/day ethanol in a binge-like manner from gestational days 5–20; pair-fed and ad lib chow controls were included. During treatment, subjects from each group were intubated with either 250 mg/kg/day choline chloride or vehicle. Spontaneous alternation, parallel bar motor coordination, Morris water maze, and spatial working memory were assessed in male and female offspring.

RESULTS—Subjects prenatally exposed to alcohol exhibited delayed development of spontaneous alternation behavior and deficits on the working memory version of the Morris water maze during adulthood, effects that were mitigated with prenatal choline supplementation. Neither alcohol nor choline influenced performance on the motor coordination task.

CONCLUSIONS—These data indicate that choline supplementation during prenatal alcohol exposure may reduce the severity of fetal alcohol effects, particularly on alterations in tasks that require behavioral flexibility. These findings have important implications for children of women who drink alcohol during pregnancy.

Keywords

fetal alcohol spectrum disorders; treatment; nutrition; ethanol; fetal alcohol syndrome

Introduction

Alcohol consumption during pregnancy can disrupt development, leading to physical alterations, such as facial dysmorphology, pre- and post-natal growth deficiencies, and central nervous system (CNS) dysfunction in the offspring (Hoyme et al., 2005). Individuals that exhibit each of these features are diagnosed with fetal alcohol syndrome (FAS), which is estimated to affect 0.5–3.1 per 1000 births (Clarren et al., 2001; May and Gossage, 2001). However, the features of FAS can vary in severity and many individuals exposed to high

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levels of alcohol prenatally may not meet all of the criteria for a diagnosis of FAS. Thus, we now refer to the range of adverse prenatal alcohol effects as fetal alcohol spectrum disorders (FASD), which is estimated to affect 1 in 100 live births (Sampson et al., 1997). FASD is a serious global concern, particularly as women continue to drink alcohol during pregnancy despite prevention efforts (Warren et al., 2001).

Identification of effective interventions and treatments for FASD is therefore critical. Ideally, one would intervene at the time of alcohol exposure, thereby directly preventing or reducing the amount of alcohol-related damage. Based on putative mechanisms of alcohol-induced teratogenesis, a number of potential experimental therapeutics have been identified that may reduce the severity of fetal alcohol effects, including neurotrophic agents (Barclay et al., 2005; Bonthius et al., 2003; Endres et al., 2005; Heaton et al., 2000b; McGough et al., 2009), neuroactive peptides (Sari, 2009; Vink et al., 2005; Wilkemeyer et al., 2003; Wilkemeyer et al., 2002; Zhou et al., 2004), antioxidants (Antonio and Druse, 2008; Chen et al., 2004; Cohen-Kerem and Koren, 2003; Heaton et al., 2000a; Marino et al., 2004; Mitchell et al., 1999) and NMDA receptor antagonists (Lewis et al., 2007; Stepanyan et al., 2008; Thomas et al., 2001; Thomas et al., 2004a). Nutritional variables may also impact alcohol's teratogenic effects. Nutritional supplements may compensate for changes in the bioavailability of nutrients due to alcohol metabolism (Lieber, 2000), as well as alcohol-related reductions in nutritional intake and absorption (Dreosti, 1993). However, nutritional supplements may also alter development, even if they are not targeting the same sites as alcohol-related actions (see (Summers et al., 2008; Summers et al., 2009).

Choline is recognized as an essential nutrient (Food and Nutrition Board, 1998), and is well understood to be necessary for fetal development (Zeisel, 2006a; b). A growing literature indicates that prenatal choline supplementation in typically developing (non alcohol-exposed) rats leads to long-lasting changes in CNS structure and enhancements in cognitive functioning (McCann et al., 2006; Meck and Williams, 2003). In particular, pre- and/or early postnatal choline supplementation enhances hippocampal and prefrontal cortical functioning and performance on behaviors that rely on the functional integrity of these CNS regions, including attention and spatial learning (Meck et al., 1988; 1989; Meck and Williams, 1997; 1999; 2003). In fact, prenatal choline supplementation can advance development of spatial cue learning (Meck and Williams, 2003), improve spatial learning performance (Brandner, 2002; Schenk and Brandner, 1995; Tees, 1999; Tees and Mohammadi, 1999), increase memory capacity (Meck et al., 1988; 1989; Meck and Williams, 1997) and reduce proactive interference (Meck and Williams, 1999). Moreover, these beneficial effects are evident even when rats are over 2 years old, illustrating that a prenatal nutrient can reduce cognitive decline associated with aging (Glenn et al., 2008; Meck and Williams, 2003).

Our laboratory has reported that choline supplementation during early postnatal development can likewise reduce the severity of some ethanol-induced neurobehavioral alterations. For example, choline supplementation from postnatal days (PD) 2–21, following prenatal alcohol exposure, reduced the severity of working memory deficits in rats (Thomas et al., 2000). Similarly, choline supplementation from PD 4–30 significantly reduced the severity of spatial reversal learning deficits (Thomas et al., 2004b), hyperactivity (Thomas et al., 2004b), and trace fear conditioning deficits (Wagner and Hunt, 2006), but not motor coordination deficits (Thomas et al., 2004c) associated with alcohol exposure during the 3rd trimester-equivalent brain growth spurt. The severity of learning deficits and hyperactivity associated with 3rd trimester alcohol exposure was also reduced by choline administered during the period equivalent to postnatal development in humans (PD10–30) (Ryan et al., 2008; Thomas et al., 2007). Collectively, these data suggest that choline supplementation during the early postnatal period is particularly effective in attenuating ethanol's adverse effects on behavioral development. More recently, we have shown that choline

supplementation can reduce some of ethanol's teratogenic effects when administered during prenatal alcohol exposure. Specifically, we have shown that choline supplementation reduces the severity of alcohol-related birth weight reductions, physical anomalies, and alterations in early reflex development (Thomas et al., 2009).

The present study further examines whether choline supplementation is effective in reducing fetal alcohol effects when administered during prenatal alcohol exposure. Of particular concern is mitigation of behavioral alterations associated with alcohol-induced neuropathology. Prenatal alcohol exposure affects behavioral development in a number of domains, leading to hyperactivity, motor incoordination, alterations in social processing, and deficits in cognitive functioning (Mattson et al., 1998; Riley and McGee, 2005). Thus, the present study examines the effects of choline supplementation during prenatal alcohol exposure on a number of behavioral domains, including motor coordination, spatial learning, and working memory.

Materials and Methods

All procedures used in this study were approved by the SDSU IACUC and are in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Treatment

Timed pregnant Sprague-Dawley dams were obtained from the Center for Behavioral Teratology, San Diego State University Animal Care facilities. Gestational day (GD) 0 was designated as the day when a seminal plug was detected. Dams were housed individually in plastic cages, exposed to a 12:12 hour cycle of light and dark in a temperature- and humidity-controlled room, and received food (LabDiet® 5001, Richmond, IN, containing 2.25 g choline chloride/kg diet) and water *ad libitum*.

Pregnant dams were randomly assigned to one of three treatment groups: ethanol-exposed (EtOH), yoked pair-fed (PF), or *ad libitum* control (LC). Ethanol-exposed dams received 6.0 g/kg/day (28.5% v/v) ethanol, PF dams received an isocaloric maltose dextrin solution to control for the calories from alcohol, and LC dams received vehicle (saline), via daily oral gavage from GD 5–20. Daily food intake was measured for the EtOH dams; each PF dam was matched to an EtOH dam of similar weight and food intake was correspondingly yoked. Within each of the 3 treatment groups, dams were randomly assigned to receive either a choline supplementation (choline chloride, Blachem, New Hampton, NY; 250 mg/kg/day) or a vehicle control (saline, Sigma, Milwaukee, WI), added to the daily intubation formula. This administration increases daily choline intake by 2–3 times that of controls.

Animals were monitored until the expected day of delivery GD 22 (PD 0) and the day of birth was recorded. On PD 1, litters were culled to 10 pups (5 males and 5 females when possible). Data on litter characteristics and birth weights have been previously reported (Thomas et al., 2009). Blood alcohol levels over a 24-hour period were obtained from a separate group of pregnant rats on gestational days 5 and 20. Importantly, choline supplementation did not influence blood alcohol concentrations, which peaked at 345 mg/dL (Thomas et al., 2009), indicating that choline does not alter the amount of fetal alcohol exposure.

Behavioral Testing

Different sex pairs within each litter were tested on each behavioral task, reducing the possibility of carryover and excessive handling effects. To control for litter effects, no more than one sex pair per litter was tested on a particular task (one sex pair for spontaneous alternation, a different sex pair for parallel bar testing, and a third sex pair for spatial and

working memory). For each behavioral task, the n of litters is as follows spontaneous alternation (EtOH + Saline n=11 litters; EtOH + Choline n=12; PF + Saline n=14; PF + Choline n=10; LC + Saline n=11; LC + Choline n=13); parallel bar (EtOH + Saline n=9 litters; EtOH + Choline n=12; PF+ Saline n=11; PF + Choline n=10; LC + Saline n=8; LC + Choline n=10); morris water maze spatial and working memory (EtOH + Saline n=11 litters; EtOH + Choline n=12; PF + Saline n=14; PF + Choline n=10; LC + Saline n=11; LC + Choline n=13). All testing was conducted by experimenters blind to treatment group. Body weights for each subject were taken prior to testing and are described in Table 1.

Spontaneous Alternation—Subjects were tested during the light cycle on PD 15–17, PD 28–30, and PD 39–41 for spontaneous alternation – a measure of natural exploratory and foraging behavior that depends on hippocampal cholinergic functioning (Lalonde, 2002; Messier et al., 1990). The subject was initially placed at the bottom of the stem (start box) of a T-maze made of black Plexiglas that had a start box and two goal boxes, found at the end of the two maze arms (stem length = 60 cm, width = 13 cm; arm length = 35 cm, width = 13 cm). After 10 seconds, a guillotine door to the start box was opened and the subject was free to explore the maze until the subject had placed four paws in one of the goal boxes or 5 mins had elapsed. After 30 seconds in the goal box, the subject was removed and placed back in the starting box. After ten seconds, the start box door was opened again and the procedure repeated. Arm choices and response latencies were recorded. The percent of subjects that alternated on 2 of the 3 consecutive days at each age served as the performance measure.

Parallel Bar Motor Coordination—On PD 30–32, motor coordination was measured on a parallel bar task. Subjects were tested for 3 consecutive days during the light cycle. The parallel bar apparatus consisted of two parallel steel rods (0.5 cm diameter, 91 cm long) held between end platforms (15.5 × 17.8 cm), all of which stood 63 cm above wood chip bedding.

Subjects were initially acclimated on each platform and then situated on the rods halfway between the platforms, so that the subject had its left paws placed on one rod and right paws placed on the other. Four successive alternating steps with the hind paws on the rods constituted a successful traversal. The rods were initially spaced 3.5 cm apart; the space widened at 0.5-cm increments following each successful traversal. The trial was considered unsuccessful if the subject placed both hind paws on one rod, fell, dragged a paw, or swung under the bars. Subjects were allowed up to five trials at a given width, being tested up to a maximum of 15 trials per day. The maximum width between rods successfully traversed and the ratio of successful traversal to total trials was recorded.

Morris Water Maze—Beginning on PD 45, subjects were tested for 7 consecutive days on the Morris maze – a spatial learning task (D'Hooge and De Deyn, 2001). The task used a circular tank (175 cm diameter) filled with water (26°C) made opaque with the addition of powdered milk and a platform (10 cm diameter) submerged 1 to 1.5 cm beneath the water surface. The tank was placed in a room rich with extramaze spatial cues.

Subjects first underwent 4 days of acquisition. During acquisition, the subject was placed in the tank and allowed to swim for up to 60 seconds, until the platform was found. Subjects were tested for 6 trials/day with an ITI of 4 mins. When the platform was located, the subject was allowed 10 seconds to observe the various visual cues of the room (e.g. posters, curtains, lights) and was then removed from the platform and placed into a drying cage during its inter-trial interval. The platform's position was randomly selected from one of four possible positions, and remained stationary for each subject during acquisition trials. Subjects were placed in the maze, facing the outer rim; the starting position was different during each trial to prevent the learning of motor strategies. The swimming activity (e.g.

path latency and distance to the platform) of each subject was monitored via a video camera mounted overhead (HVS Image, 2020 Software). The acquisition phase was followed by a 60-second probe trial on the 5th day to test for spatial memory. During the probe trial, the platform was removed and the subject allowed to swim freely. Measures such as the percent of time spent near the target position and the number of passes through the previous target's position were recorded.

Finally, to determine if there were group differences in performance measures such as swimming ability or motivation, subjects were tested with a visible platform for 2 days, 4 trials/day. The platform sat above the water's surface and was marked for visibility, changing position from quadrant to quadrant between trials. All previous spatial cues were eliminated by a white curtain surrounding the tank.

Working Memory—On PD 65–66, subjects were tested on a working memory version of the Morris water maze. Each day comprised two sessions (beginning at 9:00 and 15:00), each of which consisted of 2 trials (training and test). During each session, the platform was placed in one of 12 randomly chosen spatial locations; the platform's position remaining static during the training and test trials within a session. Subjects were allowed up to 60 seconds to find the escape platform hidden below the water's surface. Once the subject found the platform during the training trial, they remained on the platform for 10 seconds and then were immediately placed at a new starting position for the test trial. Thus, the subject had to remember the platform's position from the training trial to the test trial using spatial cues. The platform's spatial position changed between the sessions, requiring working memory as the subject had to disregard previous sessions and remember the location of the escape platform during that session only. Path lengths and latencies to find the hidden platform were measured using the HVS Image system. Savings was determined by subtracting the path length to find the platform during the test trial from the path length during each training trial.

Data Analyses—Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Science, Chicago, IL). All data were analyzed with analyses of variance (ANOVAs) with a 3 (Ethanol, PF and LC) x 2 (Choline, Control) x 2 (Female, Male) design. Repeated within-subject factors of time were included in appropriate tasks. Newman-Keuls post-hoc comparisons were conducted when a significant P-value of <0.05 was found. Non-parametric Fisher's exact probability analyses were conducted for spontaneous alternation data.

Results

Body Weights

As reported in Thomas et al, 2009, the prenatal ethanol-exposed and pair-fed dams gained less weight during pregnancy compared to lab chow controls, but choline had no effect on maternal weight gain. There were no treatment effects on litter size, gestational length or sex ratio of pups. At birth, ethanol-exposed and PF pups weighed less than LC pups and choline transiently increased the body weights of the ethanol-exposed subjects only. Over time, PF subjects caught up in weight with LC controls, but the EtOH-exposed subjects did not.

Body weights for subjects during behavioral testing is shown in Table 1. Body weight measures for 44 subjects were not acquired on PD 28. A significant sex effect was found for all groups' body weights taken prior to spontaneous alternation, parallel bars and Morris water maze ($F(1,90)=25.3$, $p<0.0001$; $F(1,105)=50.8$, $p<0.0001$; $F(1,131)=137.8$, $p<0.0001$, respectively), as males weighed more than females. Ethanol-exposed subjects weighed less than PF and LC controls, producing significant effects of ethanol at all ages ($F(2,90)=3.1$,

p=0.05; F(2,105)=13.4, p<0.0001; and F(2,131)=5.3, p<0.01, for PD 28, 30, and 45, respectively). When collapsed across all treatment groups, choline significantly increased body weight at PD 28 and 45, but not PD 30 (F(1,90)=15.2, p<0.0001; F(1,105)=2.3, p=0.13; and F(1,131)=3.9, p=0.05, respectively), although choline supplementation did not significantly increase body weights within each treatment group. Importantly, no interaction between sex, ethanol or choline was found.

Spontaneous Alternation

The percent of subjects in each treatment group that alternated at each age was not significantly different on PD 15–17 or on PD 39–41. However, on PD 28–30, ethanol-exposed subjects alternated significantly less than controls, an effect that was mitigated with prenatal choline supplementation (see Figure 1). Fisher's exact probabilities confirmed that fewer ethanol-exposed subjects alternated compared to all groups, except the PF + Choline group, which did not differ from any other group. In contrast, the EtOH + Choline group performed at levels similar to that of controls.

Parallel Bars

Gestational exposure to alcohol did not significantly impair motor coordination of the offspring, as assessed on the parallel bar motor task. In addition, choline supplementation also did not affect the motor performance on any of the outcome measures, including success ratio and maximum width traversed (all p's >0.1). Success ratios for each treatment group were as follows: EtOH + Saline = 0.337 ± 0.011; EtOH + Choline = 0.342 ± 0.010; PF + Saline = 0.336 ± 0.011; PF + Choline = 0.317 ± 0.025; LC + Saline = 0.344 ± 0.012; LC + Choline = 0.335 ± 0.010; (values collapsed by sex as there were no significant sex interactions).

Morris Water Maze

There were no significant effects of prenatal ethanol exposure on Morris maze acquisition; however, choline supplementation improved performance among LC controls. Path length to find the hidden platform is shown in Figure 2. Analyses of path length revealed significant main effects of Prenatal Exposure [F(2,129)=4.3; p<0.05] and Choline [F(1,129)=4.4; p<0.05]. The Prenatal Exposure effect was produced by subtle impairments in performance of the PF groups, which took significantly longer paths to reach the platform compared to both the EtOH and LC groups. Although the interaction of Choline with prenatal exposure did not reach statistical significance, the choline effect was the result of improved performance of the LC + Choline group, which traveled significantly shorter distances to the hidden platform (Figure 2B). Learning across days and within session resulted in significant Day [F(3,387)=240.7; p<0.001], Trial [F(5,645)=92.5; p<0.001] and Day x Trial [F(15,1935)=4.7; p<0.001] interactions (Figure 2A). A similar effect was seen with latency to find the platform (data not shown). Interestingly, the LC + Choline subjects swam slower than other groups, producing a significant effect of Prenatal Exposure x Choline [F(2,129)=4.8, p<0.05] (data not shown).

Heading angle is indicative of the relationship of the subject's initial chosen path compared to the path that would lead directly to the hidden target platform. Performance improved over the course of the testing days, producing significant effects of Day [F(3,387)=53.5; p<0.001] and Trial [F(5,645)=13.0; p<0.001]. There was no effect of prenatal ethanol exposure. Although the Prenatal Exposure x Choline interaction failed to reach statistical significance (p<0.07), choline supplementation significantly improved accuracy among LC controls, but not EtOH or PF controls, producing a main effect of choline [F(1,129)=9.0; p<0.01].

Significant improvements in spatial memory induced by choline supplementation were also evident during the probe trial. Analysis of the number of passes through the platform position (target) revealed significant main effects of Prenatal Exposure [$F(2,131)=3.7$; $p<0.05$] and Choline [$F(1,131)=4.6$; $p<0.05$]. Similar to acquisition, the PF groups were significantly impaired compared to the EtOH and LC groups, exhibiting fewer passes through the platform area. Overall, choline-treated animals performed better than their vehicle counterparts on this measure.

No significant effects were observed when analyzing the percentage of time swimming in the target quadrant, the heading angle, or the swimming speed during the probe trial. Similarly, there were no significant effects of ethanol or choline on the visible platform version of the Morris water maze, suggesting that factors other than spatial memory did not influence performance.

Working Memory

In contrast to simple Morris maze acquisition, prenatal ethanol did impair spatial working memory, an effect that was significantly mitigated with prenatal choline supplementation. First, no significant differences in path length to find the platform were observed among the groups during the training trial (p's >0.05). However, during the test trial, ethanol-exposed subjects that were not choline supplemented took longer path lengths to find the platform, compared to all other groups except the PF + Choline group, producing a significant interaction of Prenatal EtOH Exposure x Choline [$F(2,129)=4.1$; $p<0.05$] (see Figure 3A). Figure 3B depicts the savings in path length during the test trial relative to the initial training trial. The EtOH group showed no evidence of learning, as the path length to find the hidden platform during the test trial was not significantly better than the training trial. In contrast, all other groups, including the EtOH + Choline group, took shorter path lengths during the test trial than during the training trial, suggesting that the memory of the immediately previous experience facilitated finding the platform (which was located in the same position during that test trial). These effects produced a significant interaction of Prenatal EtOH Exposure x Choline [$F(2,129)=3.7$; $p<0.05$]. No significant differences were evident among the Choline-treated groups in this test (p's >0.05).

Discussion

Prenatal alcohol exposure altered development of spontaneous alternation and produced deficits in spatial working memory, effects that were significantly mitigated with prenatal choline supplementation. These data suggest that choline administration during prenatal alcohol exposure can reduce the severity of neurobehavioral alterations and, more generally, that nutritional variables may modify the long-lasting expression of fetal alcohol effects.

First, ethanol-exposed subjects alternated less frequently than their control counterparts on the spontaneous alternation task. This task measures a rodent's natural foraging and exploratory tendencies to shift between spatial locations. This behavior develops around PD 25–30 (Egger et al., 1973); adult rats normally alternate 60–80% of the time (Lalonde, 2002), as was observed in our control subjects. However, ethanol-treated subjects alternated at a rate less than chance (50%). This result is consistent with earlier studies that likewise found reduced spontaneous alternation following prenatal ethanol exposure (Abel, 1982; Stone et al., 1996) and is consistent with studies that have shown that developmental alcohol exposure leads to perseverative-type behaviors (Thomas et al., 2001; Thomas et al., 2004a). However, since alternation rates did not differ significantly among treatment groups on PD 39–41, alcohol may simply delay the expression of this behavior. Abel (1982) reported that prenatally alcohol-exposed animals exhibit less spontaneous alternation on PD16, 63 and 112 (Abel, 1982), but did not differ significantly from controls at the older ages. Other

reports also demonstrate that alcohol-induced memory retention deficits can decrease in severity with age, being more pronounced in the juvenile rat (Nagahara and Handa, 1997; 1999). Thus, it is possible that prenatal alcohol exposure either delays the maturation of certain neurological systems, or that neuroadaptive changes in the CNS may eventually compensate for alcohol-related deficits.

Ethanol also impaired spatial working memory, consistent with previous clinical (Green et al., 2009; Spadoni et al., 2009) and animal model (Popovic et al., 2006) studies. In fact, ethanol-exposed subjects not treated with choline failed to show any evidence of savings across trials. This finding further illustrates that prenatal alcohol exposure disrupts working memory and behavioral flexibility. It is important to note that since the same subjects were tested on both the spatial memory and spatial working memory versions of the Morris maze, there could be some carryover effects. However, these carryover effects would also reflect impairments in behavioral flexibility. Moreover, in contrast to working memory, prenatal ethanol exposure did not significantly impair performance on the spatial memory version of the Morris water maze, although the pair-fed controls were impaired. These results were surprising, given previous clinical (Hamilton et al., 2003) and animal model studies (Toso et al., 2006; Zimmerberg and Weston, 2002) that have shown that developmental alcohol exposure disrupts performance on this spatial learning task. However, many of the spatial learning deficits associated with developmental ethanol have been the result of exposure during, or at least including, the third trimester equivalent (Goodlett and Johnson, 1997; McAdam et al., 2008; Ryan et al., 2008), a period of rapid brain development that occurs postnatally in rats. Thus, differences in results may be related to the developmental timing of alcohol exposure.

The behavioral deficits observed in the ethanol-treated subjects were attenuated with prenatal choline supplementation. These results extend our previous report that prenatal choline loading attenuates alcohol-related birth weight reductions, reduced brain size, delayed incisor eruption, and alterations in reflex development (Thomas et al., 2009). Furthermore, ethanol-exposed subjects treated with choline performed at levels that did not significantly differ from that of control subjects on all behavioral tasks that were examined. We have previously shown that postnatal choline administration in the rodent similarly mitigates deficits in spatial, working, and reversal learning associated with developmental alcohol exposure (Thomas et al., 2007; Thomas et al., 2004b; Thomas et al., 2000); however, this is the first study to show amelioration of ethanol-induced deficits in cognitive functioning following prenatal treatment with choline.

Alcohol-related deficits in spontaneous alternation and spatial working memory are likely to reflect dysfunction of the hippocampus and/or prefrontal cortex, areas that are known to be sensitive to developmental alcohol exposure (Berman and Hannigan, 2000; Wass et al., 2001; Whitcher and Klintsova, 2008). First, damage to the hippocampus can cause significant deficits in spatial and reversal learning (Morris et al., 1982), decreases in spontaneous alternation rates (Lalonde, 2002) and deficits in spatial working memory (Jo et al., 2007). Studies have further shown that hippocampal cholinergic activity affects development of spontaneous alternation (Roland and Savage, 2009; Savage et al., 2007) and spatial working memory performance (Khakpour-Taleghani et al., 2009). However, impairments in spontaneous alternating behavior and spatial working memory have also been associated with frontal lobe damage (Avery et al., 2000; Mao et al., 1999; Sarti et al., 2002). Moreover, lesions to the basal forebrain reduces cortical cholinergic activity, which similarly leads to preservative-type behavioral errors on a reversal learning task (Loy et al., 1991), impaired spatial working memory (Khakpour-Taleghani et al., 2009) and deficits in spontaneous alternation (Lalonde, 2002; Roland and Savage, 2009). The greater sensitivity of the working memory version of the Morris water maze compared to simple spatial

learning may indicate that alcohol-related deficits are only evident when the task is more difficult, but also may suggest that prefrontal cortical areas are more sensitive to prenatal alcohol exposure.

We have yet to elucidate the CNS changes associated with choline supplementation in prenatally ethanol-exposed subjects; however, prenatal choline availability has been shown to influence morphology, neurochemical, electrophysiological, and functional activity of the hippocampus and prefrontal cortex in otherwise typically developing rats. For example, prenatal choline supplementation from GD 11–17 increases cell division within the neuroepithelial layer of the hippocampus and the septum (Albright et al., 1999a; Albright et al., 1999b), reduces apoptosis (Holmes-McNary et al., 1997), and alters differentiation (Albright et al., 1999b; Zeisel, 2006b). Changes in the structure and functioning of the hippocampus and cortex are long-lasting. For example, prenatal choline supplementation leads to increased concentrations of neurotrophic factors in the hippocampus and frontal cortex (Napoli et al., 2008; Sandstrom et al., 2002) and hippocampal neurogenesis that persists into adulthood (Glenn et al., 2007; Glenn et al., 2008). Furthermore, prenatal choline loading increases the size of cholinergic neurons in the adult basal forebrain, and alters acetylcholine turnover in the hippocampus and the forebrain (Blusztajn et al., 1998; Cermak et al., 1998; Meck and Williams, 2003), reducing acetylcholine recycling and increasing acetylcholine release in choline-supplemented animals. Cholinergic neurotransmission is therefore organized to be highly efficient in supplemented subjects, months after choline supplementation is complete. Finally, the threshold for hippocampal long-term potentiation (LTP), a putative mechanism for learning and memory, is reduced following prenatal choline supplementation (Li et al., 2004; Pyapali et al., 1998). In contrast, prenatal alcohol exposure reduces hippocampal neurogenesis (Glenn et al., 2007; Klintsova et al., 2007; Redila et al., 2006), reduces neurotrophic factors (Angelucci et al., 1999; Breese et al., 1993; Climent et al., 2002; Ghiselli et al., 2003; McAlhany et al., 1999), impairs cholinergic functioning (Janiri et al., 1994; Nagahara and Handa, 1999; VanDemark et al., 2009), and impairs LTP (Berman and Hannigan, 2000; Krahl et al., 1999; Samudio-Ruiz et al., 2009). Thus, any of these potential effects could be influencing outcome following prenatal alcohol exposure.

That being said, it is also possible that choline may have some different effects on brain development and function in the presence of an alcohol-related insult. For example, choline supplementation did not significantly alter performance of control subjects on the spontaneous alternation and spatial working memory tasks. In contrast, choline supplementation improved performance on the Morris water maze in LC controls, but not EtOH or PF controls, as seen in path length and heading angle during acquisition. Further investigation of the CNS changes would elucidate how choline may differentially affect brain development in typically developing subjects and subjects that experience adverse prenatal events.

Unlike tasks that depend on behavioral flexibility, prenatal ethanol did not impair motor coordination on the parallel bar task. Although some studies have shown that prenatal alcohol exposure can disrupt motor coordination, these results were not unexpected given that the cerebellum is most sensitive to alcohol during the third trimester equivalent brain growth spurt, a period of development that occurs postnatally in the rat (from birth to PD 10) (Dobbing and Sands, 1979). Performance on the parallel bar task is strongly correlated with alcohol-induced Purkinje cell loss within the cerebellum (Thomas et al., 1998). It is interesting to note that prenatal alcohol did delay the development of grip strength and hindlimb coordination reflexes, suggesting that it may cause some transient deficits in motor function (Thomas et al., 2009), but not long-lasting motor performance. In contrast, ethanol

exposure during the 3rd trimester equivalent does impair parallel bar performance and this is not attenuated with choline supplementation from PD 4–30 (Thomas et al., 2004c).

Combined with our previous findings, the present results suggest that choline's therapeutic window is quite large, effectively attenuating ethanol's teratogenic effects whether administered during prenatal ethanol exposure or after the alcohol insult is complete. In control rats, choline administration can improve cognitive functioning when administered either during prenatal or early postnatal development (PD 15–30) (Meck et al., 2008). Choline is also effective in protecting against other types of CNS damage, either when administered before, during, or after the insult. For example, choline supplementation reduces the severity of emotional and cognitive deficits associated with early maternal separation and undernutrition when administered at the time of separation (Tonjes et al., 1986). Similarly, choline can protect against NMDA-induced toxicity when administered during the insult (Mulholland et al., 2004). However, prenatal choline supplementation can also protect against later CNS damage. For example, prenatal choline loading protects against MK-801-induced neurotoxicity initiated during either adolescence (Guo-Ross et al., 2002) or adulthood (Guo-Ross et al., 2003). Finally, choline supplementation can reduce hippocampal neuropathology associated with seizures whether administered during prenatal development, weeks before seizure induction (Yang et al., 2000), or when administered after postnatal seizures (Holmes et al., 2002). Thus, it is clear that choline can mitigate the adverse effects of various insults at varying developmental stages, although the mechanisms of choline's protective effects may depend on the developmental timing of administration.

How choline may be inducing such a wide range of beneficial effects has yet to be fully elucidated in either control or prenatally alcohol-exposed subjects. What is known is that choline is essential for normal brain development and plays a number of important roles. First, choline acts as a precursor to the neurotransmitter, acetylcholine, and, in fact, can act directly as a nicotinic receptor agonist (Albright et al., 1998; Albuquerque et al., 1997; Mike et al., 2000). As mentioned above, metabolic imprinting may lead to enhanced cholinergic functioning that is observed months after choline supplementation. In addition, choline can act as a methyl donor, influencing the methionine-homocysteine cycle (Zeisel and Niculescu, 2006) and serving as an epigenetic factor. Recent studies show that prenatal choline availability affects both global and gene-specific DNA and histone methylation (Davison et al., 2009; Kovacheva et al., 2007; Niculescu et al., 2006). Finally, choline also acts as a precursor to phosphatidylcholine, phosphocholine, and other membrane constituents and can act as a precursor to intracellular signals (Zeisel, 2006b; Zeisel and Blusztajn, 1994). Thus, choline has several potential courses of action to attenuate ethanol's teratogenic effects and lead to long-lasting changes in CNS structure and function.

Given the importance of nutritional factors during brain development, it is somewhat surprising that more studies have not focused on interactions of prenatal alcohol exposure and nutrition. To date, most studies have examined the effects of undernutrition as a risk factor for FASD. Rates of FAS are higher in areas with poor nutrition (May et al., 2000) and animal studies have shown that compromised nutrition can exacerbate many of the teratogenic effects of alcohol including low birth weight (Wiener et al., 1981), physical anomalies (Weinberg et al., 1990), and brain dysfunction (Shankar et al., 2006; Wainwright and Fritz, 1985). It should be noted that blood alcohol levels are often more elevated among malnourished subjects. Conversely, nutritional supplements, such as vitamins E, C and beta carotene (a form of vitamin A) (Cohen-Kerem and Koren, 2003), zinc (Summers et al., 2008; Summers et al., 2006; 2009) and folate (Wang et al., 2009) can attenuate the effects of prenatal alcohol exposure, although the effectiveness depends on many factors, including the level of alcohol exposure and the outcome measure (Weinberg, 1985). The present study indicates that choline is among these supplements.

It is not yet known if ethanol itself leads to changes in choline levels, either by reducing nutritional intake or by altering choline absorption or metabolism. Nor is it known how prenatal alcohol affects nutritional and metabolic status in the offspring. However, since choline influences CNS development among non-alcohol exposed subjects with adequate nutrition, it would suggest that choline's beneficial effects on CNS development are not dependent on choline deficiency.

Importantly, the effects of prenatal choline supplementation on ethanol-exposed subjects are long-lasting, choline having mitigated a variety of ethanol-related behavioral deficits that were evident into adulthood. These data suggest that prenatal dietary interventions may reduce the severity of fetal alcohol effects, including long-lasting cognitive deficits. These findings have important implications for individuals who are born to women who drink alcohol during pregnancy, suggesting that prenatal nutritional supplements may reduce the risk of some fetal alcohol spectrum disorders.

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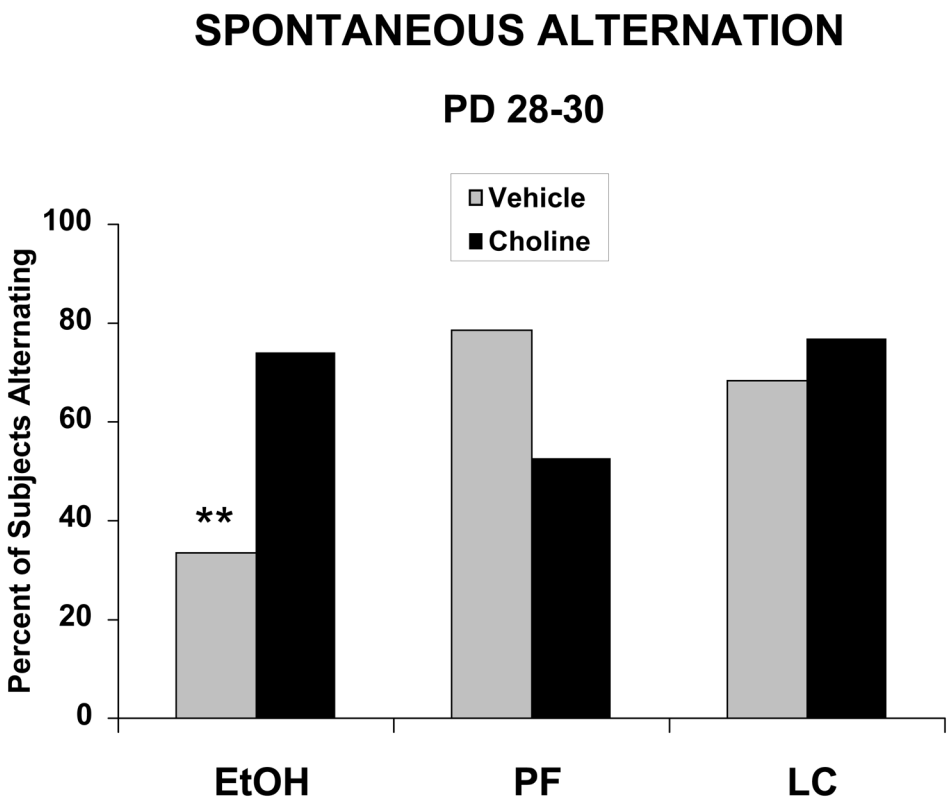

Figure 1.
Percent of subjects that spontaneously alternated on PD 28–30. Ethanol-exposed subjects alternated significantly less than controls, an effect that was significantly mitigated with prenatal choline supplementation.
EtOH = ethanol-exposed; PF = pair fed controls; LC = lab chow controls
** = significantly different from all other groups except the PF + Choline group

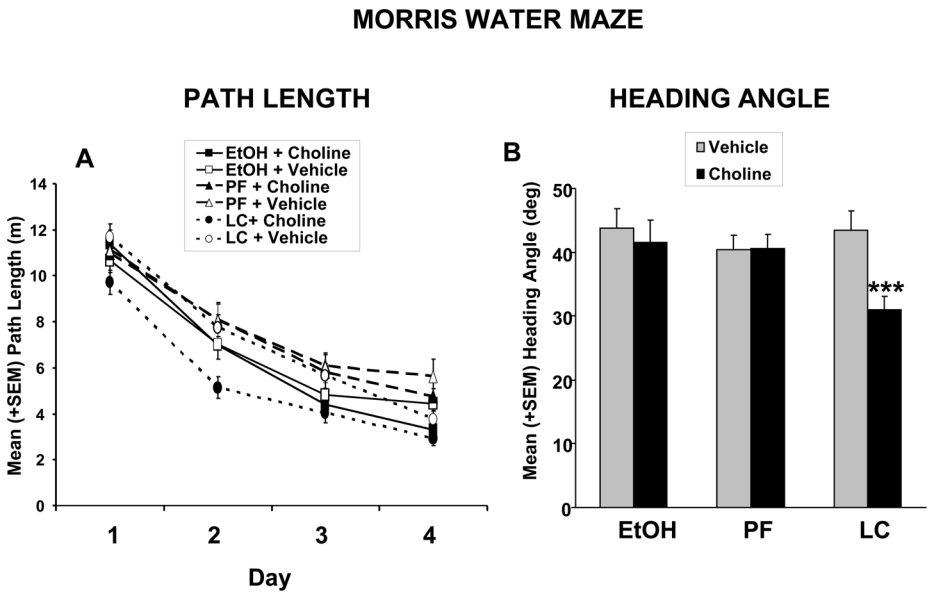

Figure 2.

(A) Mean (+ SEM) path length to find the platform in the Morris Water Maze task over testing days. Prenatal alcohol exposure did not significantly affect acquisition, although PF subjects were significantly impaired compared to both EtOH and LC subjects. Choline supplementation improved spatial learning performance among LC controls, producing a significant effect of choline. (B) Mean (+ SEM) heading angle (chosen path compared to direct path to platform) during Morris water maze acquisition. Choline supplementation significantly improved performance accuracy in LC, but not EtOH or PF subjects, producing a significant of choline. No significant effect of ethanol was found.

***= significantly different from all other groups

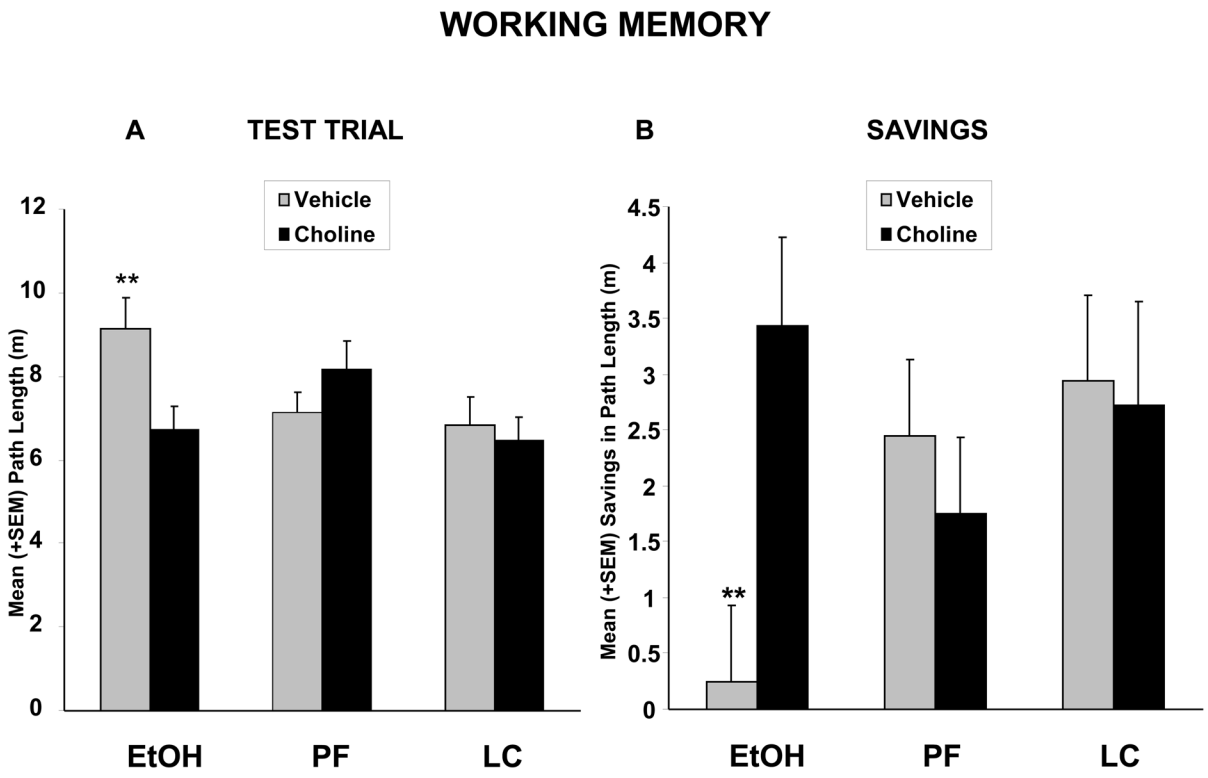

Figure 3.

Mean (+ SEM) path length during the test trials (A) of the spatial working memory task. During the test trials, ethanol-exposed subjects that were not choline supplemented took longer path lengths to find the platform, compared to all other group, except the PF + Choline group. Thus, choline supplementation mitigated ethanol-related deficits. Panel B shows the savings from training to test trial, which serves as a measure of memory retention of the platform's position. Ethanol-exposed subjects that did not receive choline supplementation showed no evidence of savings, whereas all other groups did. Indeed, the ethanol-exposed subjects treated with choline performed at levels similar to those of the control subjects.

** = significantly different from all other groups except the PF + Choline group

Table 1

Mean (± SEM) Body Weights

Prenatal Treatment							
Postnatal Day	**Sex**	**EtOH Vehicle**	**EtOH Choline**	**PF Vehicle**	**PF Choline**	**LC Vehicle**	**LC Choline**
PD 28 (Spontaneous Alternation)	Male	88.9 ± 3.2	96.1 ± 3.2	94.0 ± 3.1	100.4 ± 3.5	94.2 ± 2.9	97.4 ± 2.0
	Female	78.0 ± 4.3	88.0 ± 4.3	83.4 ± 2.3	93.1 ± 2.3	85.8 ± 2.5	90.1 ± 2.1
PD 30 (Parallel Bars)	Male	101.7 ± 1.6	106.6 ± 4.0	110.0 ± 2.3	112.3 ± 3.1	113.6 ± 2.8	112.7 ± 2.8
	Female	90.4 ± 2.9	95.1 ± 2.7	98.5 ± 2.4	99.8 ± 2.8	102.1 ± 2.0	104.1 ± 2.5
PD 45 (Morris Water Maze)	Male	216.8 ± 7.1	255.4 ± 15.0	258.4 ± 10.2	261.0 ± 13.9	268.8 ± 10.3	261.2 ± 16.4
	Female	177.5 ± 7.1	184.1 ± 4.9	180.0 ± 4.7	187.8 ± 6.4	186.8 ± 4.4	206.0 ± 8.9