

Choline Supplementation Following Third-Trimester-Equivalent Alcohol Exposure Attenuates Behavioral Alterations in Rats

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Despite the known adverse consequences of prenatal alcohol exposure, some pregnant women continue to drink alcohol, making it imperative to identify treatments for children with fetal alcohol spectrum disorders. The authors recently reported that perinatal choline supplementation can reduce some fetal alcohol effects (J. D. Thomas, M. Garrison, & T. M. O'Neill, 2004), and the present study examined whether choline supplementation is effective when administered after third-trimester-equivalent ethanol treatment. Rat pups were exposed to 6.0 g/kg/day ethanol during the neonatal brain growth spurt (Postnatal Days [PD] 4–9) and treated with choline chloride (0, 10, 50, or 100 mg/kg) from PD 10–30. Behavioral testing occurred after choline treatment had ceased. Female subjects exposed to ethanol were overactive and exhibited spatial learning deficits, effects that were attenuated with all doses of choline supplementation. These data indicate that choline supplementation can alter brain development following a developmental insult. Moreover, the data suggest that early dietary interventions may reduce the severity of some fetal alcohol effects, even when administered after birth.

Keywords: fetal alcohol syndrome, overactivity, teratogenic, treatment, alcohol-related neurodevelopmental disorder

Prenatal alcohol exposure can affect the developing central nervous system (CNS), which in turn alters the course of behavioral development. Children exposed to alcohol during gestation may exhibit learning impairments, attention deficits, motor dysfunction, and altered social behavior (Kelly, Day, & Streissguth, 2000; Mattson & Riley, 1998; National Institute on Alcohol Abuse and Alcoholism, 2000; Riley & McGee, 2005). Although much is known of the adverse consequences of prenatal alcohol exposure, less is known of how to treat individuals with various alcohol-related neurodevelopmental disorders. Given that some women continue to drink alcohol during pregnancy, it is important to identify treatments that might mitigate the teratogenic effects of alcohol.

Ideally, one would prevent the CNS damage during the alcohol exposure period. Animal studies have identified a number of experimental therapeutics that might minimize the severity of alcohol-related neuronal damage, including neurotrophic agents (Bonthuis, Karacay, Dai, & Pantazis, 2003; Endres et al., 2005; Heaton, Mitchell, & Paiva, 2000b; Heaton, Paiva, Swanson, & Walker, 1993; Luo, West, & Pantazis, 1997; McAlhany, West, & Miranda, 2000; Mitchell, Paiva, Walker, & Heaton, 1999), neuroactive peptides (Chen, Charness, Wilkemeyer, & Sulik, 2005;

Vink, Auth, Abebe, Brenneman, & Spong, 2005; Wilkemeyer et al., 2003; Wilkemeyer, Menkari, Spong, & Charness, 2002; Zhou, Sari, Powrozek, & Spong, 2004), antioxidants (Chen, Dehart, & Sulik, 2004; Cohen-Kerem & Koren, 2003; Heaton, Mitchell, & Paiva, 2000a; Marino, Aksenov, & Kelly, 2004; Mitchell, Paiva, & Heaton, 1999), and *N*-methyl-D-aspartate receptor antagonists (Thomas, Fleming, & Riley, 2001; Thomas, Garcia, Dominguez, & Riley, 2004). However, it is challenging to identify interventions that would be safe to administer to a pregnant woman and that would not alter nontargeted developmental processes in adverse ways. In addition, it may not be possible to intervene during the alcohol exposure periods. Various behavioral and environmental treatments administered to alcohol-exposed offspring, such as motor training (Klintsova, Goodlett, & Greenough, 2000; Klintsova et al., 2002), enriched environments (Hannigan, Berman, & Zajac, 1993; Rema & Ebner, 1999), and even exercise (Christie et al., 2005), have been shown to reduce the severity of fetal alcohol effects, suggesting that experience can influence brain function even after prenatal alcohol exposure (see Hannigan & Berman, 2000, for discussion).

We have been investigating the possibility that choline may serve as an effective treatment for fetal alcohol spectrum disorders. Choline is recognized as an essential nutrient by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences (Food and Nutrition Board, 1998). Choline serves as a precursor to the neurotransmitter acetylcholine as well as to membrane constituents, such as the phospholipids phosphatidylcholine and sphingomyelin, signaling factors like platelet-activating factor and sphingosylphosphorylcholine (Zeisel & Blusztajn, 1994) and intracellular messengers like diacylglycerol and ceramide (Meck & Williams, 2003). Animal studies have demonstrated that pre- and perinatal choline supplementation leads to long-lasting cognitive enhancement that is evident even into old

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age, far beyond the period of choline administration (Brandner, 2002; McCann, Hudes, & Ames, 2006; Meck, Smith, & Williams, 1988, 1989; Meck & Williams, 1997c, 1999, 2003; Tees & Mohammadi, 1999). For example, choline-supplemented subjects exhibit enhanced memory and reduced proactive interference on tasks of spatial learning like the radial arm maze and Morris water maze (Brandner, 2002; Meck et al., 1988, 1989; Meck & Williams, 1997b, 1999, 2003; Tees & Mohammadi, 1999). Perinatal choline supplementation also leads to earlier maturation of relational cue processing and mitigates age-related declines in spatial memory (Meck & Williams, 2003), enhances temporal memory (Cheng, Meck, & Williams, 2006; Meck & Williams, 1997a, 1997c), and facilitates attentional processing (Meck & Williams, 1997b). Given that alcohol exposure during development leads to cognitive deficits, we hypothesized that choline might reduce the severity of some of these effects.

We first reported that choline administration during Postnatal Days (PD) 2–21 reduced the severity of learning deficits in adult rats exposed to alcohol during gestation (Thomas, La Fiette, Quinn, & Riley, 2000). More recently, we demonstrated that administration of choline during PD 4–30 reduced the severity of hyperactivity and spatial reversal learning deficits, but not motor impairments, observed in subjects exposed to alcohol during the neonatal brain growth spurt (PD 4–9; Thomas, Garrison, & O'Neill, 2004; Thomas, O'Neill, & Dominguez, 2004). Similarly, choline administration during PD 4–30 also effectively reduced the severity of trace fear conditioning deficits associated with neonatal alcohol exposure (Wagner & Hunt, 2006). In our study, we administered choline both during and after ethanol treatment to maximize choline's effectiveness. The present study examined whether choline was effective when administered after ethanol treatment was complete and during a period of brain development that would be equivalent to early infancy and childhood in humans. In this study, we exposed rats to alcohol during the third-trimester-equivalent brain growth spurt (PD 4–9) and then administered choline daily during PD 10–30. After choline treatment was completed, activity level and Morris maze spatial learning were monitored.

Method

Subjects

Subjects were 61 male and 66 female Sprague–Dawley rats (9–13 per sex per group), which were derived from timed births at the Center for Behavioral Teratology, San Diego State University Animal Care facilities. A Sprague–Dawley male and female rat were housed together overnight, and the presence of a seminal plug in the morning indicated mating and designated Gestational Day 0. Pregnant females were then singly housed in a temperature- and humidity-controlled room with ad lib access to food (LabDiet 5001; Richmond, IN; which contains 2.25 g choline chloride/kg diet) and water. On the morning following birth, litters were randomly culled to 10 pups, maintaining 5 males and 5 females when possible. To control for litter effects, no more than one pup per litter was assigned to a particular treatment group.

Treatment

To control for nutritional variables, we exposed subjects to ethanol via an artificial rearing procedure. On PD 4, subjects were randomly assigned to one of six treatment groups. Four groups were exposed to ethanol (EtOH) and then treated with one of four doses (0, 10, 50, or 100 mg/kg)

of choline chloride. The fifth group served as artificially reared gastrostomy controls (GC), and the final group served as normally reared suckle controls (SC). Control groups were injected with saline vehicle. All procedures included in this study were approved by the San Diego State University Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health (1996) *Guide for the Care and Use of Laboratory Animals*.

On the morning of PD 4, SC subjects were fostered to a lactating dam along with nonexperimental pups, maintaining a litter size of 10. Subjects in the EtOH and GC treatment groups underwent gastrostomy surgery. Briefly, subjects were anesthetized with a halothane–oxygen mix, and a gastrostomy tube was guided through the mouth into the stomach and out through the abdominal wall, where it was anchored in place with press-fit washers. For details on this procedure, see Thomas, Garrison, and O'Neill (2004).

Following surgery, pups were artificially reared using the “pup-in-a-cup” method (Diaz & Samson, 1980). Pups were placed individually in Styrofoam cups filled with wood chips and artificial fur. Wood chips from the mother dam's cage were also added, to provide familiar odor cues. Each cup floated in a water-filled tank that maintained the temperature inside the cup at 35° C. Every 2 hr, a nutritionally balanced milk diet (West, Hamre, & Pierce, 1984) was delivered into the gastrostomy tube for a 20-min delivery period via a timer-controlled infusion pump (Harvard Apparatus, Model 980566; Holliston, MA). Pups were weighed each morning, and the mean body weight (in grams) was calculated. The daily volume of milk diet (in milliliters) was calculated as 33% of the mean body weight (in grams) for pups maintained on each artificial rearing apparatus. Pups were bathed twice a day, and their anal–genital areas were stimulated to facilitate excretion. Double-distilled water was injected into the gastrostomy tubes twice each day to keep the tubes patent.

From PD 4 through PD 9, ethanol (6.8% vol/vol) was added to the diets of EtOH subjects during the first four consecutive feedings each day, for a total dose of 6.0 g/kg/day. Thus, subjects were exposed to binge-like alcohol treatment each day. Feedings began between 0900 and 1100. During ethanol feedings, isocaloric maltose dextrin was added to the diets of GC subjects. Milk diet only was delivered during the remaining eight feedings each day. Subjects were maintained in the artificial rearing environment and fed only milk on PD 10 and 11 to allow the pups to undergo any withdrawal before being fostered back to a lactating dam, replacing the nonexperimental subjects to maintain litter size at 10. On PD 11, India ink was injected in the subjects' paws for later identification, and on PD 12, subjects were fostered back to a lactating dam along with the SC pups. The pups remained with the lactating dam until PD 21, at which time they were weaned. Litters remained group housed until separated by sex on PD 28 and were housed under a 12:12 light–dark cycle in a temperature- and humidity-controlled animal facility.

From PD 10–30, EtOH subjects received daily subcutaneous injections of one of the three doses (10, 50, or 100 mg/kg, with a dosing volume of 6.66 ml/kg) of choline chloride solution (DuCoba; Verona, MO) or saline vehicle. Thus, subjects were treated with choline after ethanol exposure was complete. These doses were based on our earlier findings that choline doses as low as 10 mg/kg may be effective in reducing the adverse effects of alcohol on behavioral development (Thomas, LeGrand, & O'Neill, 2002). Because we did not find effects of choline treatment on activity level or spatial learning abilities in controls when choline treatment occurred for a longer period of time, from PD 4–30 (Thomas, Garrison, & O'Neill, 2004), only saline was administered to control groups.

Blood Alcohol Level

On PD 6, 1.5 hr after the start of the last alcohol feed, 20 μ L of blood were drawn from a tail clip from each artificially reared subject to determine blood alcohol level. Previous studies have shown that this represents the peak blood alcohol level following alcohol exposure during this period

of development (Kelly, Bonthius, & West, 1987). Blood samples were analyzed using the Analox Alcohol Analyzer (Model AM1, Analox Instruments; Lunenburg, MA).

Behavioral Testing

Open field activity. On PD 31 through PD 34, activity level was measured in an automated open field (16 in. wide \times 18 in. long \times 15 in. high [41 cm \times 46 cm \times 38 cm]). The Plexiglas open field was contained in a sound-attenuated chamber with a fan, which provided masking noise and ventilation. The open field contained a grid of infrared beams (Digiscan Model RXYCM; Omnitech Electronics, Inc.; Columbus, OH) that tracked each subject's movement.

Subjects were placed in the testing room 30 min prior to testing to allow for acclimation. Each subject was then placed in the center of the activity chamber, and activity was recorded. Chambers were cleaned prior to testing of each subject to eliminate odor cues. Activity was recorded in 5-min bins for a period of 1 hr per day for 4 consecutive days during the subjects' dark cycle. Total distance traveled and time spent in the center of the chamber served as the performance measures. Data from 6 subjects were lost due to equipment failure.

Morris water maze spatial learning. Beginning on approximately PD 145, subjects were tested on the Morris water maze, a spatial learning task. One male EtOH + 0 subject died prior to Morris water maze testing. This task utilizes a circular water tank (175-cm diameter) filled with water (26°C) made opaque with the addition of powdered milk. An escape platform (10 cm diameter) was hidden 1.5 cm below the surface and could not be seen by the subject because of the opacity of the water. The tank was housed in a room filled with spatial cues (e.g., sink, lights, posters, and the experimenter, who remained in the same location throughout testing). Each subject was marked with a nontoxic ink to allow a video tracking system to track the animal within the white pool.

Subjects were trained for 5 consecutive days. During each acquisition trial, the subject was placed in the tank, facing the periphery, and allowed to swim until the escape platform was found. If the subject failed to escape within 60 s, the subject was manually placed on the platform. The subject remained on the platform for 10 s before being removed from the tank. The location of the escape platform remained constant throughout testing; however, to eliminate the learning of motor strategies, the starting position of the subject was changed following a pseudorandom pattern with the condition that the subject must start once in each of the four quadrants of the tank each day. Between trials, subjects were kept in a heated environment (31°C) to prevent hypothermia. Subjects were tested for four trials each day with an intertrial interval of 3–5 min. Path length, latency to the platform, heading angle, and swimming speed served as performance measures.

On the days following hidden platform acquisition training, subjects were tested with a visible platform, to determine whether any group differences were related to performance measures, like vision, swimming ability, or motivation. On these days, white sheets surrounded the pool, removing spatial cues, and a brightly marked platform was set above water level to be easily visible. The location of the platform changed on each trial, and so subjects had to learn to swim to the platform, regardless of spatial location. Subjects were tested for four trials on each of 2 days, with the platform located in each of the quadrants per day.

Data Analyses

All data were analyzed with analyses of variance, using SPSS software. Treatment and sex served as between-subjects factors on all measures. Body weight data were analyzed with day as a repeated measure, activity level was analyzed with day and 5-min bin as repeated measures, and Morris maze data were analyzed with trial and day as repeated measures. Follow-up comparisons were conducted with least significant difference post hoc analyses with $p < .05$.

Results

Body Weight

Body weight is shown in Figure 1. Beginning around PD 7, artificially reared pups lagged in growth compared with SC pups. Males weighed more than females, and the difference between SC and artificially reared subjects was slightly more evident among male subjects, which led to a significant interaction of Treatment \times Sex \times Day, $F(130, 2990) = 1.5, p < .001$. There were also significant interactions of Treatment \times Day, $F(130, 2990) = 15.0, p < .001$, and Sex \times Day, $F(26, 2990) = 32.5, p < .001$, as well as main effects of treatment, $F(5, 115) = 26.0, p < .001$; sex, $F(1, 115) = 23.3, p < .001$; and day, $F(26, 2990) = 9,055, p < .001$. Follow-up analyses illustrated that SC subjects were significantly heavier than artificially reared subjects by PD 7 in males and by PD 8 in females and continued to weigh more up to the time of behavioral testing at PD 31. It is important to note that there were no significant differences in body weight among artificially reared subjects. Thus, neither alcohol nor choline supplementation had a significant effect on body weight.

There was catch-up in growth over days, and by the time of Morris water maze testing, the SC subjects differed significantly only from the GC and the EtOH + 10 mg/kg choline subjects, producing a significant effect of treatment, $F(5, 114) = 4.5, p < .001$ (see Table 1). There was also a significant effect of sex, $F(1, 114) = 406.1, p < .001$, as males weighed more than females. Although the Treatment \times Sex interaction failed to reach significance ($p = .12$), the treatment effects were due to body weight differences among males, and there were no significant effects of treatment on body weight among females (see Table 1).

Blood Alcohol Level

Mean peak blood alcohol levels for alcohol-treated groups are shown in Table 1. There were no significant differences in peak blood alcohol level among alcohol-treated subjects.

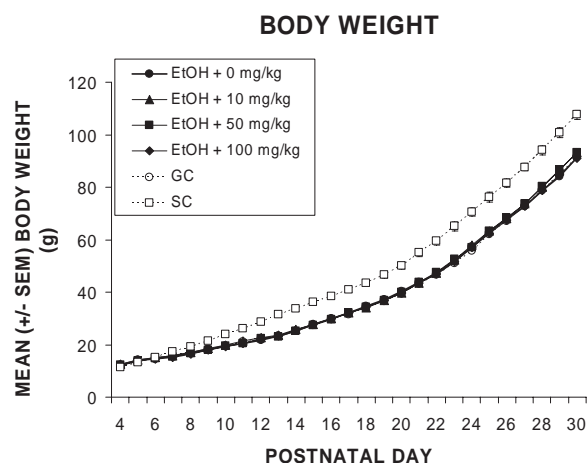


Figure 1. Body weight. Artificially reared subjects (EtOH and GC) began to lag in growth compared with SC subjects beginning on Postnatal Day (PD) 7, an effect that was still evident at the termination of choline treatment (PD 30). It is important to note that there were no significant differences in body weight among artificially reared groups at any time. EtOH = ethanol group; GC = gastrostomy controls; SC = suckle controls.

Table 1
Mean (\pm SEM) Peak Blood Alcohol Levels and Body Weights at Postnatal Day 145

Group	Blood alcohol level (mg/dl)	Body weight (g) ^a	Males	Females
EtOH + 0	331 \pm 17	434 \pm 28	529 \pm 27	315 \pm 17
EtOH + 10	324 \pm 21	408 \pm 29*	530 \pm 28	308 \pm 14
EtOH + 50	344 \pm 17	428 \pm 28	516 \pm 21	304 \pm 16
EtOH + 100	334 \pm 19	415 \pm 26	551 \pm 27	325 \pm 11
GC		391 \pm 27*	488 \pm 31	295 \pm 9
SC		461 \pm 31	618 \pm 12	328 \pm 13

Note. EtOH groups were treated with either 0, 10, 50, or 100 mg/choline chloride. EtOH = ethanol group; GC = gastrostomy controls; SC = suckle controls.

^a As measured at the time subjects performed the Morris water maze.

* $p < .001$ (for difference from SC group).

Activity Level: Total Distance

Female subjects exposed to alcohol during development were overactive in the open field chamber, an effect that was attenuated with 50 or 100 mg/kg/day choline supplementation. Total distance traveled in the activity chamber as a function of treatment group and sex is shown in Figure 2. One ethanol-treated female injected with vehicle (EtOH + 0 mg/kg) was extremely overactive, traveling almost 70,000 in. (177,800 cm) over the 4 days of testing. This value was over three times the second highest activity level achieved in that group and over three standard deviations from the group mean. With this outlying subject included in the analyses, the EtOH + 0 mg/kg group was significantly different from all treatment groups on all measures. However, to be conservative, we excluded this subject from further data analyses. After doing so, there was still a significant effect of treatment, $F(5, 109) = 4.0, p < .01$, as well as Treatment \times Bin, $F(55, 1199) = 2.3, p < .001$, and Treatment \times Sex \times Day \times Bin, $F(165, 3597) = 1.2, p < .05$, interactions. Because of the four-way interaction, data were analyzed separately for males and females.

Within the female subjects, the treatment effect was significant, $F(5, 56) = 2.9, p < .05$, as was the Treatment \times Bin interaction, $F(55, 616) = 1.5, p < .05$. There was also an effect of day, $F(3, 168) = 47.5, p < .001$, and a Day \times Bin interaction, $F(33, 1848) = 15.2, p < .001$, due to habituation both within and between sessions. Post hoc comparisons indicated that the EtOH + 0 mg/kg group was significantly more active as compared with the control groups and with the EtOH groups treated with the two higher doses of choline (50 and 100 mg/kg; see Figure 2). Indeed, 5 of the 9 female subjects treated with alcohol traveled over 10,000 in. (25,400 cm) over the 4 testing days, compared with only 1 of 10 ethanol-exposed subjects treated with 100 mg/kg/day choline, 1 of 9 GC subjects, and 1 of 13 SC subjects.

The effects of ethanol and the beneficial effects of choline supplementation were most evident during the first half of each testing session among females, producing the Treatment \times Bin interaction. There were significant effects of treatment during the first five bins of testing (see Figure 3A). When data are collapsed across the first 25 min of testing, the EtOH + 0 mg/kg subjects were significantly more active compared with all groups except the EtOH + 10 mg/kg group. The EtOH + 10 mg/kg group was also

significantly more active compared to both control groups; however, the EtOH + 50 and EtOH + 100 mg/kg groups were not significantly different from controls.

In males, the main effect of treatment failed to reach statistical significance, $F(5, 53) = 1.8, p = .12$; however, there was a significant interaction of Treatment \times Bin, $F(55, 594) = 1.7, p < .001$. As seen in Figure 3B, the SC group was less active during the early part of the session (Bins 3 and 5), and choline effects were more obvious during the later periods of training (Bins 9–11). During Bins 9–11, EtOH + 10 mg/kg subjects were significantly more active compared to the EtOH + 50 mg/kg, EtOH + 100 mg/kg, and SC subjects. In addition, administration of the highest dose of choline (100 mg/kg) significantly reduced activity levels compared with the EtOH + 0 mg/kg group, although the EtOH + 0 mg/kg group was not overactive compared with controls ($p = .08$ compared with SC subjects). Thus, treatment effects among the males were less robust than those observed in females, a finding that might be influenced by a potential floor effect. Finally, there were also significant effects of day, $F(3, 159) = 38.4, p < .001$; bin, $F(11, 583) = 413.0, p < .001$; and Day \times Bin, $F(33, 1749) = 12.5, p < .001$, due to habituation within and between sessions.

Activity Level: Center Time

Alcohol exposure during development also led to increases in the amount of time spent in the center of the open field among females, an effect that was attenuated by all doses of choline supplementation. Figure 4 illustrates the amount of time spent in the center of the chamber for all groups, collapsed across day and bin. There were significant effects of treatment, $F(5, 109) = 5.7, p < .001$, as well as interactions of Treatment \times Sex, $F(5, 109) = 3.3, p < .05$; Treatment \times Day, $F(15, 327) = 1.9, p < .05$;

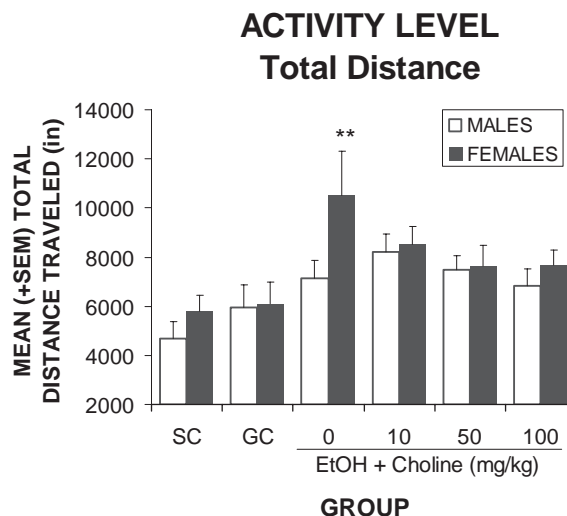


Figure 2. Activity level: Mean (+ SEM) total distance traveled in the open field collapsed across day and bin. Female subjects exposed to ethanol and treated with vehicle were significantly more active compared with controls. Choline treatment reduced the ethanol-related hyperactivity, with the 50- and 100-mg/kg/day doses significantly attenuating ethanol's effects. SC = suckle controls; GC = gastrostomy controls; EtOH = ethanol group. **Significantly different from all other female groups except EtOH + 10 mg/kg.

TOTAL DISTANCE

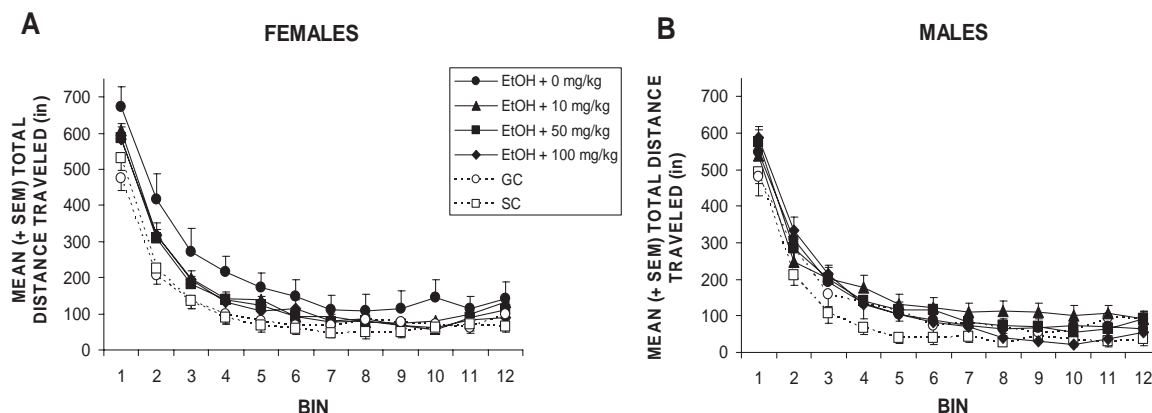


Figure 3. Average daily total distance traveled within sessions, collapsed across testing day. Ethanol effects were most evident during the first 25 min of the session in females (A), during which the ethanol group treated with vehicle was significantly more active than all groups except the EtOH + 10 mg/kg group. In males (B), there were some treatment effects during the latter portion of the session (Bins 9–11). The EtOH + 10 mg/kg group was significantly more active compared with the EtOH + 50 mg/kg, EtOH + 100 mg/kg, and SC groups. The EtOH + 100 mg/kg group was also significantly less active than the EtOH + 0 mg/kg group. Error bars represent standard error. EtOH = ethanol group; GC = gastrotomy controls; SC = suckle controls.

Treatment \times Sex \times Bin, $F(55, 1199) = 1.5, p < .05$; Treatment \times Sex \times Day \times Bin, $F(165, 3597) = 1.3, p < .01$; and Bin \times Sex, $F(11, 1199) = 1.9, p < .05$. Because of the interactions with sex, data were analyzed separately for males and females.

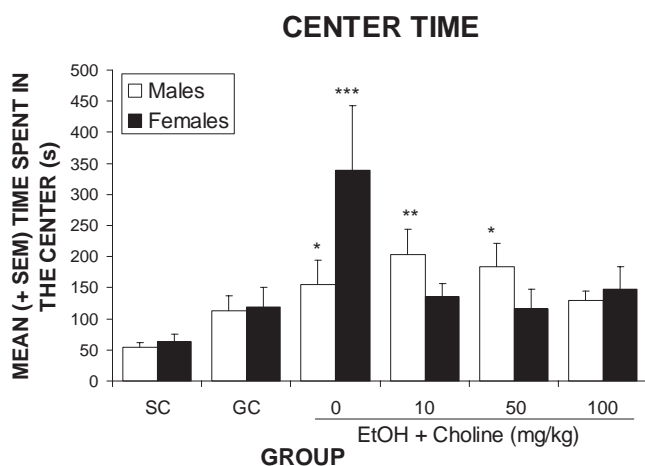


Figure 4. Mean (+ SEM) time spent in the center of the activity chamber. Females exposed to ethanol and treated with vehicle spent significantly more time in the center of the chamber compared with all other treatment groups. Thus, administration of all doses of choline significantly attenuated ethanol's effects on this measure. In males, ethanol-exposed subjects treated with vehicle or the two lowest doses of choline (10 and 50 mg/kg) spent significantly more time in the center compared with suckle controls (SC). The EtOH + 10 mg/kg group also spent more time in the center compared with gastrotomy controls (GC). EtOH = ethanol group. *Significantly different from SC. **Significantly different from both control groups. ***Significantly different from all other treatment groups.

The effects of treatment were mostly observed in the females. Follow-up analyses on data from females illustrated that ethanol-exposed subjects treated with vehicle spent significantly more time in the center of the activity chamber compared to all other groups, including all ethanol-exposed groups that were treated with choline (10, 50, or 100 mg/kg), producing a significant effect of treatment, $F(5, 55) = 4.6, p < .001$. This effect was most obvious during the beginning and end of the activity sessions, producing a Treatment \times Bin interaction, $F(55, 605) = 1.4, p < .05$ (data not shown).

In males, the treatment effect was also significant, $F(5, 54) = 3.1, p < .05$. Follow-up analyses indicated that ethanol-treated groups injected with vehicle or with 10 or 50 mg/kg choline spent significantly more time in the center compared with the SC group and that the EtOH + 10 mg/kg group spent more time in the center compared with the GC controls. There were no significant differences among ethanol-treated groups. Thus, the EtOH + 100 mg/kg group did not differ significantly from either controls or other ethanol-treated groups.

Morris Water Maze

Alcohol exposure during the third-trimester equivalent impaired spatial learning performance in female subjects, and all doses of choline supplementation attenuated ethanol-related impairments. Path length to find the platform collapsed across days is shown in Figure 5. Data are separated by sex, as there was a significant effect of sex, $F(1, 114) = 12.4, p < .001$, as well as Treatment \times Sex, $F(5, 114) = 2.4, p < .05$, and Treatment \times Sex \times Trial, $F(15, 342) = 2.0, p < .01$, interactions. There were also significant effects of day, $F(4, 456) = 88.1, p < .001$; trial, $F(3, 342) =$

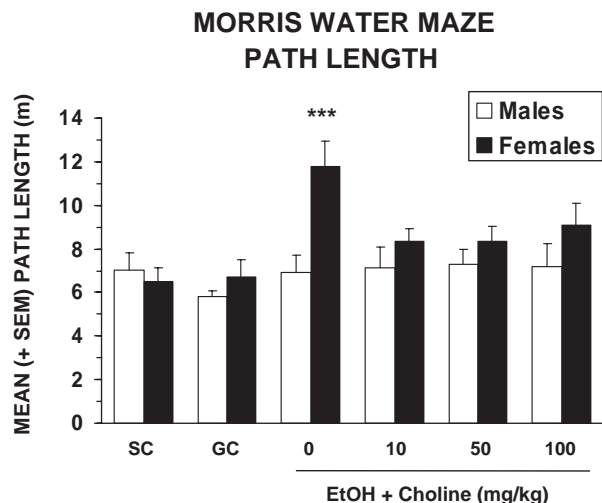


Figure 5. Mean (+SEM) path length to find the escape platform in the Morris water maze spatial learning task. Female subjects exposed to ethanol during the brain growth spurt exhibited impaired performance, taking significantly longer paths to find the hidden platform. This effect was significantly attenuated by choline administration. Follow-up analyses confirmed that the ethanol-exposed females that received vehicle took path lengths significantly longer than all other groups. There was no effect of ethanol or choline among the males. EtOH = ethanol group; GC = gastrostomy controls; SC = suckle controls. ***Significantly different from all other treatment groups.

103.1, $p < .001$; and Day \times Trial, $F(12, 1368) = 2.5, p < .01$, due to a reduction in path length during acquisition. Finally, there was an effect of treatment, $F(5, 114) = 3.5, p < .01$, and a Treatment \times Day interaction, $F(20, 456) = 1.8, p < .05$.

Follow-up analyses conducted on data from each sex confirmed that there were no treatment effects in males. However, as seen in Figures 5 and 6A, spatial learning was impaired in females exposed to alcohol and treated with vehicle. Indeed, all other groups showed a steady decline in path length with continued training, including all ethanol-exposed subjects treated with choline. By the end of the training phase, females in the EtOH + 0 mg/kg group swam longer distances to find the platform compared with all other groups, producing a significant effect of treatment, $F(5, 60) = 5.1, p < .001$, as well as interactions of Treatment \times Day, $F(20, 240) = 1.8, p < .05$, and Treatment \times Trial, $F(15, 180) = 2.5, p < .01$. In fact, on the last day of training, 5 EtOH + 0 mg/kg female subjects traveled over 10 m before finding the escape platform, whereas no more than 1 subject from any other group (control or EtOH + choline) swam as far. Follow-up analyses confirmed that the EtOH + 0 mg/kg group was significantly different from all other groups and that there were no significant differences among controls and ethanol-treated subjects that received choline supplementation.

A similar pattern was seen for the latency to find the platform, as seen in Figure 6B. Ethanol induced spatial learning deficits in females, and this effect was mitigated by administration of all choline doses, producing a significant main effect of treatment, $F(5, 114) = 3.7, p < .01$, and sex, $F(1, 114) = 6.3, p < .01$, as well as Treatment \times Sex, $F(5, 114) = 3.4, p < .01$; Treatment \times Day, $F(20, 456) = 1.6, p < .05$; and Treatment \times Sex \times Trial, $F(15, 342) = 2.0, p < .05$, interactions. There were also main effects of day, $F(4, 456) = 104.4, p < .001$, and trial, $F(3, 342) = 69.0, p < .001$, as well as an interaction of Day \times Trial, $F(12, 1368) = 2.4, p < .01$, due to improved performance over training. Follow-up comparisons for each sex illustrated no treatment ef-

MORRIS MAZE

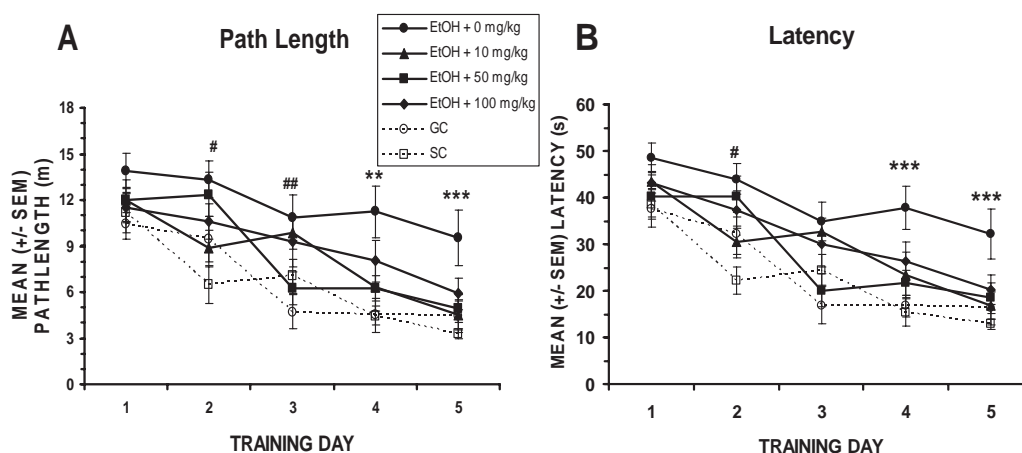


Figure 6. Path length (A) and latency (B) to find the hidden platform in the Morris water maze, over days among female subjects. As shown, performance of subjects improved over days; however, ethanol-exposed females that did not receive choline showed little improvement over days. Error bars represent standard error. EtOH = ethanol group; GC = gastrostomy controls; SC = suckle controls. #Significantly different from controls and EtOH + 10 mg/kg subjects. ##Significantly different from controls and EtOH + 50 mg/kg subjects. ***Significantly different from all other groups except the EtOH + 100 mg/kg group. ****Significantly different from all other treatment groups.

fects in the males. However, there were significant treatment effects, $F(5, 60) = 5.7, p < .001$, in the females, as well as significant interactions of Treatment \times Day, $F(20, 240) = 1.6, p < .05$, and Treatment \times Trial, $F(15, 180) = 2.7, p < .001$. The ethanol group that did not receive choline treatment exhibited significantly longer latencies to find the platform compared with all other groups. Indeed, by the last day of training, the EtOH + 0 mg/kg group took longer than all other groups, and the EtOH groups that received choline did not differ significantly from controls.

Discussion

Choline administration reduced the severity of overactivity and spatial learning deficits related to alcohol exposure during the third-trimester-equivalent brain growth spurt. It is important to note that choline administration was effective even when administered after the ethanol treatment was complete and during a developmental period that would be equivalent to postnatal development in humans. Moreover, the ability of choline to attenuate ethanol's effects on spatial learning was evident months after choline treatment, suggesting that choline's effects are long lasting. These data suggest that choline can attenuate the adverse effects of prenatal alcohol exposure, even after an alcohol-induced insult has occurred.

Alcohol exposure during the early neonatal period produced significant increases in both locomotor activity and the amount of time spent in the center of the chamber. Hyperactivity is a commonly described symptom of prenatal alcohol exposure (Mattson & Riley, 1998; Steinhausen, Williams, & Spohr, 1993) and has been reported even in alcohol-exposed children who exhibit average intelligence levels (Shaywitz, Cohen, & Shaywitz, 1980). Numerous studies using animal models have further illustrated that alcohol exposure during development can alter activity levels (Abel, 1984; Bond, 1981; Thomas, Garrison, & O'Neill, 2004). Similarly, alcohol disrupted performance on a spatial learning task, the Morris water maze, a finding consistent with both clinical (Hamilton, Koditwakku, Sutherland, & Savage, 2003) and animal model studies (Berman & Hannigan, 2000; Cronise, Marino, Tran, & Kelly, 2001; Kelly, Goodlett, Hulsether, & West, 1988; Marino et al., 2004; Tomlinson, Wilce, & Bedi, 1998).

Of interest, consistent significant alcohol-related alterations on both tasks were found only among the female subjects. Sex differences in vulnerability to developmental alcohol exposure have been reported on a variety of behavioral measures (Barron & Riley, 1990; Kelly, Mahoney, Randich, & West, 1991; Weinberg, Zimmerberg, & Sonderegger, 1982), including changes in activity level (Grant, Choi, & Samson, 1983) and Morris maze spatial learning (Kelly et al., 1988). In our previous study, we reported hyperactivity in both male and female subjects (Thomas, Garrison, & O'Neill, 2004). In that study, a slightly higher ethanol treatment was used (6.6 g/kg/day), and higher blood alcohol levels were achieved (370–410 mg/dl, as compared with 320–350 mg/dl in the present study). The present data suggest that the threshold for ethanol-related hyperactivity may be lower for females than for males. Most important, the sex effects observed in this study were related to the alcohol effects and not to the choline effects, and when we have observed ethanol-induced hyperactivity in male

subjects, choline was effective in reducing that hyperactivity (Thomas, Garrison, & O'Neill, 2004).

Administration of all doses of choline from PD 10 to PD 30 reduced the severity of alcohol-related changes in activity, with the two highest doses (50 and 100 mg/kg) significantly affecting both total distance traveled and center time. We also found that all doses effectively mitigated spatial learning deficits associated with developmental alcohol exposure. Previously, we found that administration of doses as low as 10 mg/kg/day choline chloride from PD 4–30 was effective in reducing the severity of reversal learning deficits associated with neonatal alcohol exposure (Thomas et al., 2002), so the findings in the present study are consistent with our earlier results, and as little as 10 mg/kg/day can be effective in reducing the severity of ethanol's effects on some behaviors. It is notable that there was, essentially, no dose–response effect, suggesting that once sufficient levels are achieved, additional choline supplementation is not effective.

In most of the previous choline supplementation studies, choline has been administered during either the gestational period or the combined gestational and early postnatal period and administered via either saccharin-enhanced drinking water, food, or intragastric or subcutaneous injection. In seminal work by Williams and colleagues, choline was administered in the drinking water during gestation, leading to maternal choline supplementation levels estimated from 250 to 300 mg/kg/day (Cheng et al., 2006; Williams, Meck, Heyer, & Loy, 1998). Levels of choline supplementation continued into postnatal development vary greatly, as some studies have continued maternal supplementation while also supplementing the pups (Brandner, 2002), administered a single volume of the choline solution independent of subject weight (i.e., Meck et al., 1989), or provided a constant dose (i.e., 250 mg/kg/day; Williams et al., 1998). Based on Meck et al. (1989), in our initial studies we administered a single volume of a choline chloride solution each day so that the dose of choline chloride decreased as subject body weight increased (with doses ranging from 188 mg/kg for a 10-g rat to 18.8 mg/kg for a 100-g rat), via either subcutaneous injection (PD 4–21; Thomas, Garrison, & O'Neill, 2004) or intragastric administration (PD 2–21; Thomas et al., 2000). The doses used in the present study were chosen to represent this range. At present, the lowest effective choline level has not yet been determined; neither is it clear how administration route or pattern of administration (one injection vs. 24-hr consumption) influences outcome.

Although alterations in activity level can be caused by dysfunction of a variety of brain regions, hyperactivity and spatial learning deficits following neonatal alcohol exposure are consistent with cholinergic hypofunctioning in the hippocampus (see Riley, Barron, & Hannigan, 1986, for review). First, numerous studies have reported that alcohol exposure during either the prenatal or the early neonatal period can disrupt development of cholinergic systems (Brodie & Vernadakis, 1992; Kelly, Black, & West, 1989; Nagahara & Handa, 1999a, 1999b; Pick, Cooperman, Trombka, Rogel-Fuchs, & Yanai, 1993; Rawat, 1977; Schambra, Lauder, Petrusz, & Sulik, 1990). Second, hippocampal cholinergic dysfunction, via lesions or pharmacological manipulations, can impair spatial learning ability (Berger-Sweeney et al., 2001) and also produce hyperactivity (Lamberty, Gower, Gobert, Hanin, & Wulfert, 1992; Waite et al., 1995; Waite & Thal, 1995). Previously, we reported that choline supplementation from PD 4–30 mitigated the effects of neonatal alcohol on activity level and

reversal learning but not on motor coordination, which supports the hypothesis that choline is preferentially affecting forebrain cholinergic systems (Thomas, Garrison, & O'Neill, 2004; Thomas, O'Neill, & Dominguez, 2004), at least during this period of postnatal development.

Prenatal choline supplementation in control rats has been shown to induce enduring morphological, neurochemical, and electrophysiological changes in the CNS and, specifically, in the hippocampus and cortex. For example, choline supplementation during development can lead to morphological changes in cholinergic cells of the basal forebrain (Loy, Heyer, Williams, & Meck, 1991; Williams et al., 1998), as well as in hippocampal pyramidal cells (Li et al., 2004). Choline supplementation can also lead to enhanced efficiency of cholinergic functioning in the hippocampus and cortex (Blusztajn, Cermak, Holler, & Jackson, 1998; Cermak et al., 1999; Cermak, Holler, Jackson, & Blusztajn, 1998; Coutcher, Cawley, & Wecker, 1992; Meck et al., 1989; Montoya et al., 2000) and can lead to enhanced hippocampal long-term potentiation (Jones, Meck, Williams, Wilson, & Swartzwelder, 1999; Pyapali, Turner, Williams, Meck, & Swartzwelder, 1998), a mechanism of plasticity believed to underlie some learning and memory. Recently, it was reported that prenatal choline supplementation also enhances mitogen-activated protein kinase and cyclic-AMP response-activated binding protein activation in hippocampal slices (Mellott, Williams, Meck, & Blusztajn, 2004). Thus, several lines of evidence illustrate that early choline supplementation can affect hippocampal function, a finding that is consistent with the behavioral consequences of choline supplementation (Brandner, 2002; Meck et al., 1988, 1989; Meck & Williams, 1997a, 1997b, 1997c, 1999, 2003; Tees & Mohammadi, 1999). It is important to note, however, that none of these studies examined the effects of choline supplementation during the developmental period in the present study, and only two (Meck et al., 1989; Ricceri & Berger-Sweeney, 1998) limited choline supplementation to the postnatal period. To date, we have not yet evaluated neuronal changes associated with the choline supplementation during this postnatal period; thus, at this time, we can only speculate on the underlying neural bases of choline's beneficial effects.

We have also yet to elucidate the mechanisms by which choline mitigates ethanol's adverse effects on behavioral development. First, choline's effects may be related to its actions as a precursor to acetylcholine, although choline may also act directly as an agonist of the alpha-7 nicotinic receptor (Alkondon, Pereira, Cortes, Maelicke, & Albuquerque, 1997). Meck and Williams (2003) suggested that acetylcholine availability during development leads to long-lasting adaptations in choline metabolism, which has enduring effects on cholinergic neurotransmission. This "metabolic imprinting" may lead to an increased availability of choline from membrane phosphatidylcholine stores. In addition, choline may alter brain organization and function by serving as a precursor to other membrane constituents or as a source of biologically labile methyl groups (Zeisel & Niculescu, 2006), independent of action on cholinergic systems. Although the effective mechanisms have yet to be identified, it is likely that the consequences of choline supplementation on brain organization may depend on the developmental timing of administration.

Choline's therapeutic potential is strengthened by the present evidence that it is effective during a period of development equivalent to early postnatal development in humans, a period during

which a treatment could be most successfully administered. One issue is how late, developmentally, choline can be administered and still produce beneficial effects. A recent study (Holmes et al., 2002) suggested that choline supplementation can effectively mitigate the effects of a CNS insult even when administered after PD 35. Specifically, Holmes and colleagues (2002) found that 4 weeks of choline supplementation initiated immediately after kainic-acid-induced status epilepticus on PD 35 improved performance on a Morris water maze task and reduced the severity of hippocampal damage induced by seizures, although the authors did not include a nonseizure control group. If choline is effective even when administered during adolescence and adulthood, the therapeutic potential for alcohol-exposed individuals would be even greater.

In summary, these data provide evidence that choline supplementation may alter CNS functioning, even after ethanol-induced damage is complete. Moreover, the effects of choline are enduring, evident even after the choline supplementation administration has been terminated. These findings have important implications for children exposed to alcohol prenatally and suggest that dietary interventions implemented after birth may reduce the severity of some fetal alcohol effects.

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