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## Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats

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### Abstract

Prenatal alcohol exposure can lead to a range of physical, neurological, and behavioral alterations referred to as fetal alcohol spectrum disorders (FASD). Variability in outcome observed among children with FASD is likely related to various pre- and postnatal factors, including nutritional variables. Choline is an essential nutrient that influences brain and behavioral development. Recent animal research indicates that prenatal choline supplementation leads to long-lasting cognitive enhancement, as well as changes in brain morphology, electrophysiology and neurochemistry. The present study examined whether choline supplementation during ethanol exposure effectively reduces fetal alcohol effects. Pregnant dams were exposed to 6.0 g/kg/day ethanol via intubation from gestational day (GD) 5-20; pair-fed and lab chow controls were included. During treatment, subjects from each group received choline chloride (250 mg/kg/day) or vehicle. Physical development and behavioral development (righting reflex, geotactic reflex, cliff avoidance, reflex suspension and hindlimb coordination) were examined. Subjects prenatally exposed to alcohol exhibited reduced birth weight and brain weight, delays in eye opening and incisor emergence, and alterations in the development of all behaviors. Choline supplementation significantly attenuated ethanol's effects on birth and brain weight, incisor emergence, and most behavioral measures. In fact, behavioral performance of ethanol-exposed subjects treated with choline did not differ from that of controls. Importantly, choline supplementation did not influence peak blood alcohol level or metabolism, indicating that choline's effects were not due to differential alcohol exposure. These data indicate early dietary supplements may reduce the severity of some fetal alcohol effects, findings with important implications for children of women who drink alcohol during pregnancy.

### Keywords

fetal alcohol spectrum disorders; ethanol; physical development; reflex development; treatment

### 1. Introduction

The consequences of prenatal alcohol exposure range from physical anomalies and growth retardation [35] to CNS dysfunction and behavioral alterations [59,67]. In the U.S., it is

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#### Conflict of Interest Statement for Authors

None of the authors have any conflicts of interest related to this project.

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estimated that around 0.5–3.1 per 1000 births [13,45] suffer from a full blown fetal alcohol syndrome and an estimated 1 in 100 live births exhibit at least some adverse effects of prenatal alcohol exposure [68]. It is clear that fetal alcohol spectrum disorders (FASD), the term used to describe the range of fetal alcohol effects, constitute a serious problem throughout the world [86].

Given that women continue to drink alcohol during pregnancy, it is important to identify methods to reduce the severity of FASD. Both clinical and animal studies suggest that postnatal behavioral and environmental factors can mitigate some of ethanol's adverse effects, and may improve outcome following prenatal alcohol exposure [28,38,40,72]. We have reported that choline supplementation during early postnatal development can also reduce the severity of some behavioral alterations associated with developmental alcohol exposure. Specifically, choline supplementation from postnatal days (PD) 2-21 reduced the severity of working memory deficits in adult rats induced by prenatal alcohol exposure [78]. Choline supplementation from PD 4-30 also significantly reduced the severity of spatial learning deficits [77], overactivity [77], trace eyeblink conditioning [81] and trace fear conditioning deficits [84], but not motor coordination deficits [79], associated with alcohol exposure during the 3<sup>rd</sup> trimester equivalent brain growth spurt (PD 4-9 in the rat is commonly accepted as a model of the 3<sup>rd</sup> trimester equivalent [17], although there is some variability among models used to compare development between rats and humans (see [www.translatingtime.net](http://www.translatingtime.net))). More recently, we reported that choline supplementation from PD 10-30, during a period equivalent to postnatal development in humans, reduces the severity of learning deficits and hyperactivity associated with 3<sup>rd</sup> trimester alcohol exposure [74]. Collectively, these data suggest that choline supplementation during late gestation and/or the early postnatal period may effectively attenuate ethanol's adverse effects on behavioral development.

Ideally, one would intervene at the time of alcohol exposure, preventing or reducing the amount of alcohol-related CNS damage. A number of potential therapeutics have been identified, primarily based on putative mechanisms of alcohol-induced teratogenesis, including neurotrophic agents [9,18,30,31,46], neuroactive peptides [10,82,91,92,99], antioxidants [11,14,29,44,55] and NMDA receptor antagonists [75,76]. The present study examines whether choline supplementation would be effective when administered during prenatal alcohol exposure. This is the first study, to our knowledge, to examine the effects of choline supplementation coincident with prenatal alcohol exposure.

Choline is recognized as an essential nutrient by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences [8] and is necessary for fetal development [95]. In fact, choline deficiency is associated with increased neural tube defects and CNS dysfunction [3,16,19,20,48,51,71]. A growing literature indicates that prenatal choline supplementation in typically developing (non alcohol-exposed) rats leads to long-lasting enhancements in cognitive functioning, as well as changes in CNS structure and function [47,50]. Given that choline serves not only as a precursor to the neurotransmitter acetylcholine, but also as a precursor to cell membrane constituents phosphatidylcholine and sphingomyelin, and can act as a methyl donor and epigenetic factor, its effects may be quite broad. In the present study, we examined whether prenatal choline supplementation, during the ethanol exposure period, would affect a range of physical and behavioral outcomes.

## 2. Materials and Methods

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

## 2.1 Treatment

Subjects were derived from timed births at the Center for Behavioral Teratology, San Diego State University Animal Care facilities. A Sprague-Dawley male and female were housed together overnight and the presence of a seminal plug in the morning indicated mating and designated gestational day (GD) 0. Pregnant females were then randomly assigned to one of six treatment groups in a 3 (ethanol-exposed (EtOH), yoked pair-fed (PF), or ad lib control (LC))  $\times$  2 (choline supplementation, control) design. Each group included 10–14 dams (n's are shown in Table I). Subjects were singly housed in a temperature- and humidity-controlled room with access to food (LabDiet® 5001, Richmond, IN, which contains 2.25 g choline chloride/kg diet) and water. On GD 5–20, all dams were orally gavaged once a day. Ethanol-exposed dams received 6.0 g/kg/day in a 28.5% (v/v) ethanol solution (0.02675 ml/g of body weight), whereas PF dams received isocaloric maltose dextrin, and LC dams received vehicle (saline). Daily food intake was measured for EtOH dams; each pair-fed dam was matched to an EtOH dam of similar weight and food intake was yoked. Choline chloride (250 mg/kg/day) [12,93] or saline control was added to the intubation formula. Dams were weighed daily.

Beginning on GD 20, dams were monitored each morning for birth of pups. The day of birth (usually GD 22) was recorded as PD 0 and dams were not disturbed that day. On PD 1, litters were pseudorandomly culled to 10 pups (5 males and 5 females, if possible). Brain tissue from one extra male and one extra female pup were collected on PD 1 when possible (depending on number of pups born and male/female ratio from each litter).

## 2.2 Blood Alcohol Level

Blood alcohol levels were measured in a separate group of adult pregnant rats, to determine if choline supplementation influenced blood alcohol level. Subjects were treated with 6.0 g/kg ethanol with or without 250 mg/kg choline supplementation. Forty microliters of blood were drawn from a tail clip from each subject at 1.0, 2.0, 4.0, 8.0 and 24 hours after the alcohol gavage on GD 5 and 20. Blood samples were analyzed using the Analox Alcohol Analyzer (Model AM1, Analox Instruments; Lunenburg, MA).

## 2.3 Physical development

On PD 1–21, physical signs of development were monitored and recorded daily for all pups. The physical signs included body weight, onset of eye opening, ear unfurling and tooth eruption. Body weight was recorded in grams (g). Eye opening was defined as a full slit length break in the membrane covering the eyes. Ear unfurling was defined as unfolding of external pinnae of both ears to the fully erect position and tooth eruption was recorded at the first break in gum for both upper incisors and lower incisors [22,39].

## 2.4 Behavioral testing

To reduce the possibility of carryover effects and excessive handling [21], different sex pairs within each litter were tested on the various behavioral tasks. One sex pair was tested on the reflex battery and was sacrificed on PD 21. The other sex pairs underwent testing on other behavioral tasks (motor coordination and balance, open field activity, spatial learning, working memory), which will be presented in a separate report.

## 2.5 Reflex development

On PD 2–18, one sex pair per litter was tested on a series of reflex development tasks to examine sensorimotor maturation [22]: righting reflex, geotactic reflex, cliff avoidance, grip strength and hindlimb coordination. Righting reflex was evaluated on PD 2–7. On a warm heating pad, each pup was placed in the supine position and the latency to right, defined by the four paws touching the floor, was recorded. A maximum of 30 seconds was provided for task completion;

pups were tested for two trials per day. Maturation of this response was reached when the pup successfully righted itself in less than five seconds during both trials.

Subjects were tested for geotactic reflexes on PD 7-15. During this task, each pup was placed facing downward on a rough board held at a 45° slant. Latency to rotate 180 degrees was measured with a maximum of 3 minutes given per trial, with two trials per day. Success was considered when the pup performed a 180-degree turn within the allotted time.

Cliff avoidance was evaluated on PD 6-12, with two trials per day. Each rat pup was placed with its head and front paws over a ledge. The latency to retract the body 1.5 cm from the edge was recorded. Subjects were given a maximum of 30 sec per trial.

Finally, on PD 12-20, grip strength and hindlimb coordination were measured. Each pup was suspended by its forefeet from a wire, 2-mm in diameter. A cage filled with bedding was placed under the wire should the pup fall. Subjects were given a maximum of 30 seconds per trial, for 2 trials per day. A successful grasp trial was recorded if the rat pup was able to hold on to the wire for 30 sec. A successful coordination trial was recorded if the pup was able to place one of its hindlimbs on the wire.

Developmental evaluations were sequentially performed on the same sex pair from each litter. Subjects were first evaluated in the earlier reflexes and each new test was added at the end of the sequence. The intertrial interval between the two trials in each test was 5–10 sec. Time between reflex tests varied from 5–30 mins, depending on the number of subjects evaluated on a particular day (5–30 min).

## 2.6 Data Analyses

Data were analyzed with ANOVAs, using SPSS software. Prenatal treatment (Ethanol, Pair-fed, Lab Chow), choline (choline, vehicle), and sex served as between-subject factors on all measures. Dependent variables recorded across time were analyzed with repeated measures. Because within-litter variability is different from between-litter variability [34], for body weight and physical development data, the mean from each litter was determined and used as one data point. In addition, these data were also analyzed using litter as a nested factor. Body weight data were analyzed with day as a repeated measure. Follow-up comparisons were conducted with LSD post-hoc analyses with  $p < .05$ . For the reflex development measures where a criterion was established, the percent of subjects per group reaching criterion was calculated day by day. Fisher exact probability tests for independent samples were performed to compare percentage of subjects reaching criterion each day ( $p < .05$ ).

## 3. Results

### 3.1 Gestation and birth variables

Maternal body weight during gestation was significantly affected by the prenatal ethanol treatment. No significant differences were observed in the body weight of the dams on gestational day 0 (GD 0) ( $p > 0.1$ ). Analysis of the percent body weight gained during pregnancy showed that LC dams gained significantly more weight than PF and EtOH-treated dams relative to their initial body weight (data collapsed across choline treatment) [ $F(2, 66) = 28.61$ ;  $p < .005$ ] (Table I). Choline treatment did not significantly affect dam body growth. As seen in Table I, neither litter size nor sex ratio of pups were significantly affected by ethanol or choline treatments.

### 3.2 Blood Alcohol Concentrations (BAC)

Choline administration during prenatal ethanol treatment did not significantly affect the peak BAC or alcohol metabolism within the parameters of the present experiment. Data were statistically analyzed using a mixed ANOVA with choline treatment as a between subject factor and gestational day and time as repeated measures. The analysis revealed a significant main effect of Time [ $F(4,36) = 74.6$ ;  $p < .001$ ], as alcohol concentration reached a peak within 2–4 hours after the intubation, declined significantly after 8 hours and approached 0 24 hrs after the ethanol intubation. There were no significant main or interactive effects of gestational day, although peak blood alcohol levels tended to be higher on GD 20 (see Table I for peak blood alcohol concentrations). Importantly, there were no significant effects of choline on BAC at any time, indicating that choline did not alter ethanol metabolism.

### 3.3 Body Weight

All pups in the litter were weighed daily from PD 1-21 and the means for males and females within each litter were used as the unit of analyses (Table II). An overall ANOVA revealed a significant main effect of EtOH [ $F(2, 132) = 11.5$ ;  $p < .001$ ], as pups in the Ethanol and PF groups had significantly lower body weights than LC controls, as well as EtOH  $\times$  Day [ $F(40, 2640) = 3.3$ ;  $p < .001$ ] and EtOH  $\times$  Choline  $\times$  Day [ $F(40, 2640) = 2.0$ ;  $p < .001$ ] interactions. There were also effects of Day [ $F(20, 2640) = 13881$ ;  $p < .001$ ], Sex [ $F(1, 132) = 8.0$ ;  $p < .01$ ], and a Day  $\times$  Sex [ $F(20, 2640) = 5.4$ ;  $p < .001$ ] interaction due to faster growth among males compared to females.

Follow-up analyses examined the EtOH  $\times$  Choline  $\times$  Day interaction. Analyses of saline-treated subjects demonstrated a significant effect of EtOH [ $F(2, 68) = 13.5$ ], day [ $F(20, 1360) = 8216$ ,  $p < .001$ ] and an EtOH  $\times$  day interaction [ $F(40, 1360) = 5.1$ ,  $p < .01$ ]. At birth, ethanol-exposed and pair-fed controls weighed significantly less than lab chow controls. By PD 2, the ethanol-exposed subjects weighed less than pair-feds, which weighed significantly less than lab chows and this trend continued through PD 21. In contrast, there was no effect of ethanol exposure on body weights among choline-treated subjects, only an effect of day [ $F(20, 1280) = 5930$ ,  $p < .001$ ]. Two-way ANOVAs (Choline  $\times$  Day) were also used to determine the choline effect within each ethanol treatment group. Analysis of the ethanol-exposed groups showed significant main effects of Day [ $F(20, 880) = 3424$ ;  $p < .001$ ], and a Day  $\times$  Choline interaction [ $F(20, 880) = 2.18$ ;  $p < .005$ ]. The main effect of Choline approached significance [ $F(1, 44) = 3.23$ ;  $p = .08$ ]. Daily analyses of body weights of the pups prenatally exposed to ethanol revealed that on PD 1, EtOH pups weighed significantly less than EtOH + Choline pups [ $F(1, 44) = 5.9$ ;  $p < .05$ ] (Table II). On subsequent days, this tendency was maintained, although, with the exception of PD 13, the difference between these two groups did not reach statistical significance.

Similar analyses for each of the control groups failed to show any effect of choline on the body weight of the pups. As expected, a significant main effect of day was evident in both PF and LC treatment groups, revealing the pups' growth across days ( $F(20, 920) = 5103$ ;  $p < .001$  and  $F(20, 960) = 5817$ ;  $p < .001$ , respectively).

### 3.4 Brain Weight

Brains were collected from approximately 50–75% litters from each treatment group (EtOH  $n = 8/12$ , EtOH + Choline  $n = 7/13$ , PF  $n = 7/14$ , PF + Choline  $n = 6/10$ , LC  $n = 6/11$ , LC + Choline  $n = 10/12$ ). Prenatal ethanol exposure significantly reduced brain weight, whereas choline supplementation significantly increased brain weight on PD 1 pups (see Figure 1). The ANOVA revealed significant main effects of EtOH [ $F(2,90) = 5.8$ ;  $p < .05$ ] and Choline [ $F(1,90) = 14.3$ ;  $p < .05$ ] treatments. When collapsed across choline treatment, brain weights of ethanol-exposed subjects were significantly lower than controls. Overall, brain weights of

choline-treated pups were heavier than vehicle treated controls. No significant interactions were revealed in this analysis.

### 3.5 Physical Development

The day of emergence of physical features is illustrated in Table III. Data on eye opening from 2 litters (one PF and one EtOH + C) were not collected due to experiment error. Prenatal alcohol exposure significantly delayed the eye opening of the pups compared to both control groups. The ANOVA revealed a significant main effect of EtOH [ $F(2,128) = 17.8$ ;  $p < .001$ ], but no Choline effect was observed. Similarly, prenatal ethanol delayed the emergence of incisors.

The ANOVA revealed that the lower incisors emerged significantly earlier than upper incisors for all pups [ $F(1,132) = 31.6$ ;  $p < .001$ ]. There were also significant main effects of EtOH [ $F(2,132) = 18.9$ ;  $p < .001$ ] and Choline [ $F(1,132) = 10.4$ ;  $p < .05$ ] on incisor emergence. Follow-up analyses illustrated that both upper and lower incisors emerged significantly later in pups prenatally exposed to alcohol, compared to both control groups. In contrast, incisors emergence was observed earlier in subjects that received prenatal choline supplementation. Finally, ear unfolding was not significantly affected by either prenatal EtOH or Choline treatments. There were no main or interactive effects of sex on any measures. Similar results were obtained when analyzing the data with litter as a nested factor on all dependent variables.

### 3.6 Reflex Ontogeny

**3.6.1 Surface righting reflex**—Prenatal ethanol exposure significantly delayed the age of maturation of the surface righting reflex (Figure 2), an effect that was mitigated with choline supplementation. On PD 4, the percent of EtOH + Vehicle subjects showing a mature surface righting reflex (able to right themselves within 5 sec for 2 trials) began to lag and was significantly lower than that of the LC + Choline group. By PD 5, the percent of EtOH + Vehicle subjects was significantly lower than all other groups, including the EtOH + Choline group, with the exception of the LC group [Fisher's  $p$ 's  $< .05$ ]. In fact, EtOH + Choline subjects showed a maturation rate similar to and not significantly different from that observed in control groups. There were no longer significant differences among groups by PD 6.

**3.6.2 Cliff avoidance**—Prenatal ethanol exposure significantly affected the latency to retract from the edge at the beginning of testing. On PD 6, ethanol-exposed subjects treated with vehicle were more successful in retracting compared to other groups [Fisher's  $p$ 's  $< .05$ ] (Figure 3A). In addition, prenatally alcohol-exposed subjects treated with vehicle exhibited shorter latencies than all other groups, whereas prenatally alcohol-exposed subjects that received choline supplementation performed similar to control subjects (Figure 3B). An overall ANOVA on latency to retract revealed significant main effects of EtOH [ $F(2, 132) = 7.7$ ;  $p < .001$ ], Day [ $F(6, 792) = 59.4$ ;  $p < .001$ ], and the interactions EtOH  $\times$  Choline  $\times$  Day [ $F(12, 792) = 1.9$ ;  $p < .01$ ] and Choline  $\times$  Day [ $F(6, 792) = 2.9$ ;  $p < .01$ ]. Follow-up analyses were conducted for each testing day to further investigate these interactions. On PD 6, performance of the EtOH + Vehicle group was significantly different from all other groups, producing an EtOH  $\times$  Choline [ $F(2,138) = 2.9$ ,  $p < .05$ ] interaction and main effect of EtOH [ $F(2,138) = 3.9$ ,  $p < .05$ ]. Follow-up analyses confirmed that ethanol-exposed subjects treated with vehicle were faster than those treated with choline [ $F(1,44) = 8.0$ ;  $p < .01$ ], whereas ethanol-exposed subjects treated with choline did not differ from controls. There were also main effects of ethanol treatment on PD 7 [ $F(2,138) = 3.2$ ;  $p < .05$ ], PD 8 [ $F(2,138) = 3.0$ ;  $p = .05$ ], and PD 9 [ $F(2,138) = 3.7$ ,  $p < .05$ ], as subjects prenatally exposed to ethanol responded faster than LC controls. Additionally, choline-treated animals showed significantly shorter latencies than vehicle-treated subjects on PD 9 [main effect of choline;  $F(1, 138) = 6.1$ ,  $p < .05$ ].

**3.6.3 Negative Geotaxis**—Prenatal ethanol exposure significantly affected the number of subjects that exhibited the negative geotaxis reflex on PD 7. The percent of EtOH + Vehicle subjects showing successful responses on PD 7 was significantly lower than the percentage of subjects in the EtOH + Choline group as well as all control groups (Fisher's  $p < .05$ ) with the exception of the LC + Vehicle group (see Figure 4). No significant differences were observed during the following days of testing.

**3.6.4 Grip Strength**—The ANOVA revealed that the age when the ethanol-exposed pups acquired the ability to hold onto the wire was significantly delayed compared to the control groups ( $F(2,130) = 3.01$ ;  $p < .05$ ). As can be observed in Figure 5, this effect of EtOH treatment was mainly driven by the EtOH + Vehicle subjects. Nevertheless, no significant EtOH  $\times$  Choline interaction was evident on this measure. In contrast, when examining the percent of subjects able to hold onto the wire for 2 consecutive trials/day over the course of testing, ethanol-exposed subjects treated with vehicle were significantly impaired compared to all other groups, including the ethanol-exposed group treated with choline [Fisher's exact probabilities,  $p < .05$ ].

**3.6.5 Hindlimb coordination**—Prenatal exposure to ethanol significantly delayed the age of first success for hindlimb coordination (Figure 6). Four subjects were never successful (1 EtOH, 1 EtOH + Choline, 1 PF + Choline, and 1 LC + Choline). The ANOVA of the age to first success for the hindlimb coordination test revealed a significant main effect of EtOH [ $F(2,126) = 5.71$ ;  $p < .05$ ]. Although prenatal choline supplementation showed a tendency to reduce this deficit in ethanol exposed subjects, no significant interaction was observed.

## 4. Discussion

The present study is the first report that prenatal choline supplementation can prevent some of the adverse consequences of prenatal alcohol exposure on early physical and behavioral development. Specifically, prenatal choline reduced the severity of alcohol-related birth and brain weight deficits, delays in incisor emergence, and alterations in reflex development. These data indicate that nutritional variables may modify the expression of fetal alcohol effects.

First, prenatal choline supplementation significantly attenuated alcohol-related birth weight reductions, but did not significantly influence body weights among controls, consistent with the findings of Meck and colleagues [49]. Interestingly, although there was a trend, choline did not significantly increase body weights among the pair-fed controls, suggesting that choline was not simply mitigating a nutritional deficiency related to food intake, but rather influenced ethanol's specific effects on physical development. Prenatal choline also increased brain weight at birth among all groups, suggesting that choline influences brain development in both alcohol-exposed and control subjects. One important caveat to this finding is that brain weights at birth were only collected from litters with more than 10 pups. Although there were no differences in the percent of litters sampled from each treatment condition, this finding does overrepresent large litters and it is possible that choline only has this effect when there are a larger number of pups competing for prenatal nutritional factors.

Choline was also effective in mitigating many of ethanol's effects on physical and reflex development. First, prenatal alcohol led to delays in eye opening and incisor emergence, consistent with previous reports [24,43,65,83]. Although choline did not influence eye opening, it did advance incisor emergence across all groups, ethanol-exposed and control. To our knowledge, this is the first report of changes in physical maturation associated with prenatal choline supplementation. Similar to previous reports, prenatal alcohol exposure also delayed righting reflex [24,56,62], negative geotaxis [27,36], grip strength [27], and motor coordination. In contrast, prenatal alcohol advanced and enhanced cliff avoidance. This differs

from some reports of delayed maturation of this reflexive response [56], but it is likely related to increased arousal and/or activity level. Notably, ethanol-exposed subjects eventually performed at control levels. While it is possible that repeated testing and/or handling masked alcohol effects after the first days of testing, many studies suggest that alcohol alters maturation on these tasks, rather than leading to long-lasting impairments. Importantly, choline normalized behavioral development among ethanol-exposed subjects on all measures, with the exception of hindlimb coordination.

Most studies investigating the interactions of nutrition and alcohol have examined the effects of undernutrition as a provocative factor. Several animal studies have shown that the teratogenic effects of alcohol, including low birth weight [90], physical anomalies [88], brain damage [85] and reduced IGF levels [70], are more severe when consumed with suboptimal nutrition; however, often blood alcohol levels are higher among malnourished subjects. Conversely, nutritional supplements 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 [87]. The present study indicates that choline is among these supplements. Importantly, choline supplementation did not influence blood alcohol levels, so effects were not related to reductions in fetal alcohol exposure. However, it is not yet known if ethanol leads to changes in choline levels, either by reducing nutritional intake or by altering choline absorption or metabolism. It is possible that ethanol exposure creates a choline deficiency, which could impair brain and behavioral development. Nevertheless, the growing literature on the effects of choline supplementation among non-alcohol-exposed subjects suggests that choline can have beneficial effects on CNS and behavioral development, even when there is no choline deficiency.

Choline plays a number of important roles during development. First, choline contains three methyl groups and, therefore, acts as a methyl donor, influencing the methionine-homocysteine cycle [98]. Choline methylates homocysteine to form methionine; reductions in choline lead to increased homocysteine concentrations, which are associated with increased risk for birth defects [32]. Choline also serves as an epigenetic factor by influencing DNA methylation and subsequent gene expression [41,63]. In addition, choline acts as a precursor to phosphatidylcholine and sphingomyelin, major constituents of cell membranes, allowing choline to influence the structural integrity and signaling functions of cell membranes [96, 97]. Finally, choline also acts as a precursor to acetylcholine which not only serves as a neurotransmitter but also a neurotrophic factor. In fact, choline can act directly as a nicotinic receptor agonist [5,6,53]. Thus, choline has several potential modes of action on the developing fetus. Interestingly, prenatal alcohol may influence methionine absorption [64] and DNA methylation [23], can alter neuronal membrane phospholipids profiles [89] and disrupt cholinergic functioning [15,61,73], although choline does not necessarily need to be influencing the same targets as alcohol to alter the course of physical and behavioral development among alcohol-exposed subjects.

Much of what is known of prenatal choline supplementation on brain development is focused on the hippocampus and cortex, areas also known to be adversely affected by prenatal alcohol [7,54]. Choline supplementation from GD 11-17 increases cell division in the neuroepithelial layer of the hippocampus and septum, changes the timing of migration, and increases differentiation of hippocampal neurons [1,2,4,16], leading to long-lasting increases in cell size and basal dendritic arborization in CA1 pyramidal neurons [42]. Prenatal choline supplementation also increases nerve growth factor (NGF) in both the hippocampus and cortex [69], as well as insulin-like growth factor (IGF) 2 and calcium/calmodulin-dependent protein kinase (CaMK) I gene expression in the cortex [52]. Long-lasting functional changes are also evident. Perinatal choline supplementation enhances N-methyl-D-aspartate (NMDA) receptor neurotransmission [57], excitability of CA1 pyramidal cells [42], and long-term potentiation



(LTP) [37,66] in adult rats. 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 [47].

Unfortunately, the effects of prenatal choline supplementation on other brain regions or the peripheral nervous system have not been extensively examined. The present study suggests that choline may influence neural systems outside the hippocampus, at least in the alcohol-damaged CNS. Although the neural substrates of the reflexes tested in the present study have not been fully elucidated, they do involve motor systems. Interestingly, we have previously reported that choline supplementation during early postnatal development (PD 4-30) reduces the severity of behavioral alterations that rely on the functional integrity of the hippocampus, but not the cerebellum. Specifically, choline failed to improve performance on a parallel bar motor coordination task, in which subjects must traverse two parallel steel rods to a safety platform [79]. However, a study using a mouse model of Rett syndrome, a neurodevelopmental disorder characterized by severe motor dysfunction, reported that choline supplementation in the drinking water of the nursing dams from PD 1-22 improved motor coordination on a rotarod task in males and grip strength in females (there were no effects of choline on controls) [60]. It is likely that the sites of choline's action depend on the developmental timing of administration and may also depend on the functional integrity of neural systems.

Neuroprotective effects of choline supplementation have been reported with other insults as well. Choline supplementation mitigates the effects of early postnatal maternal separation and undernutrition in rats on emotional and cognitive development [80]. Using slice culture, it has also been shown that choline protects against hippocampal toxicity induced by NMDA as well as cortisol-related exacerbation of NMDA-induced toxicity [58]. Not only is choline neuroprotective during an insult, but perinatal choline has also been shown to protect against neuropathology even when administered weeks before the insult. For example, prenatal choline supplementation protects against neurotoxicity induced by MK-801, an NMDA receptor antagonist, administered during adolescence [25] or adulthood [26], as well as seizure-induced neurotoxicity in 40-day-old rats [94]. Conversely, choline supplementation may also have beneficial effects when administered after an insult. Holmes et al (2002) [33] demonstrated that four weeks of choline supplementation was neuroprotective when administered immediately after kainic acid-induced status epilepticus. Similarly, we have shown that choline supplementation that commences after early alcohol exposure reduces the severity of cognitive deficits [74,81]. Thus, it is clear that choline supplementation can protect against a variety of insults at various developmental stages.

In sum, these data illustrate that prenatal choline supplementation may effectively reduce the severity of some fetal alcohol effects, both physical and behavioral. These findings suggest that choline supplementation may effectively protect against ethanol's teratogenic effects, an important finding in the search to prevent the adverse effects of prenatal alcohol exposure.

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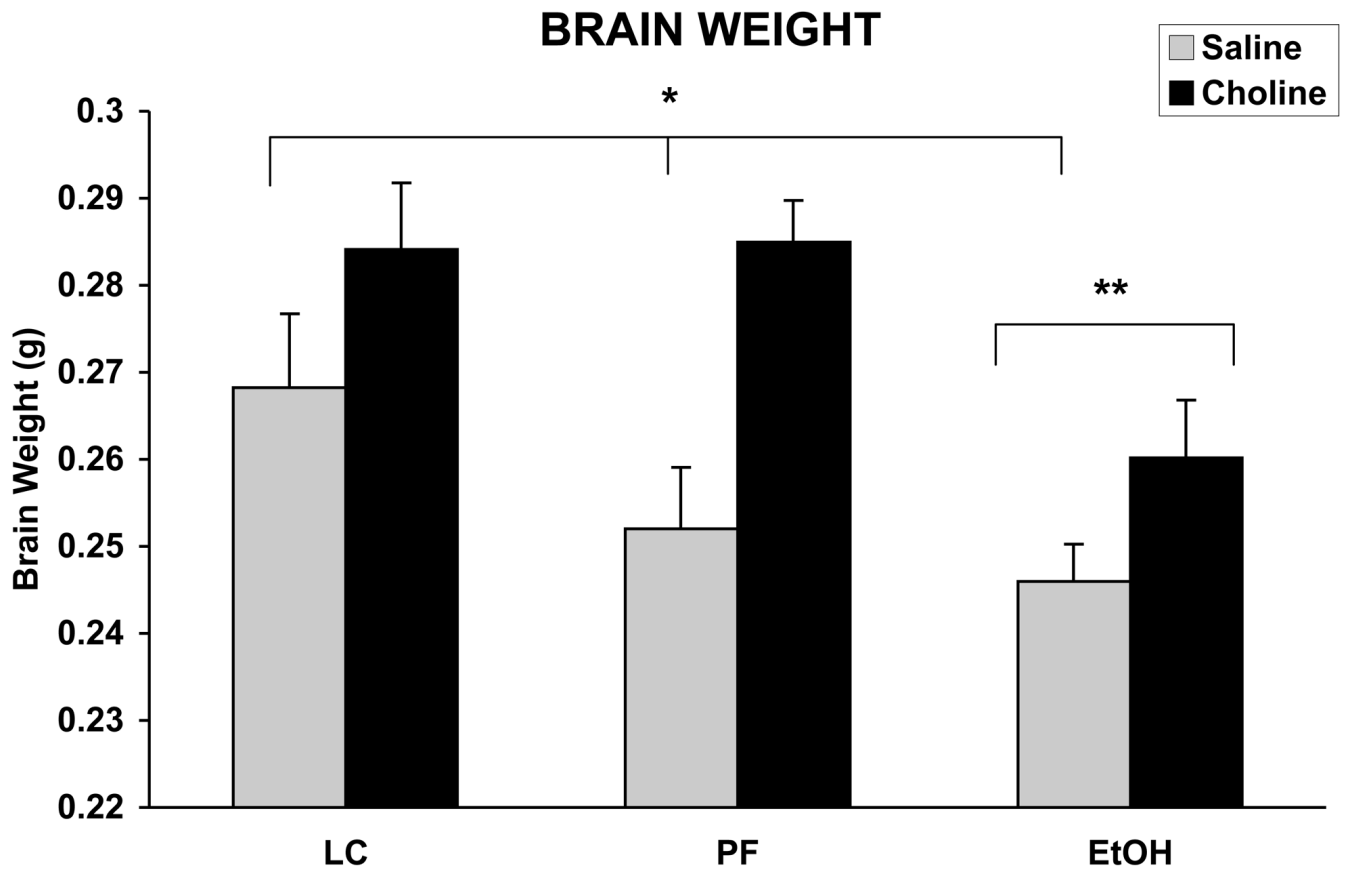
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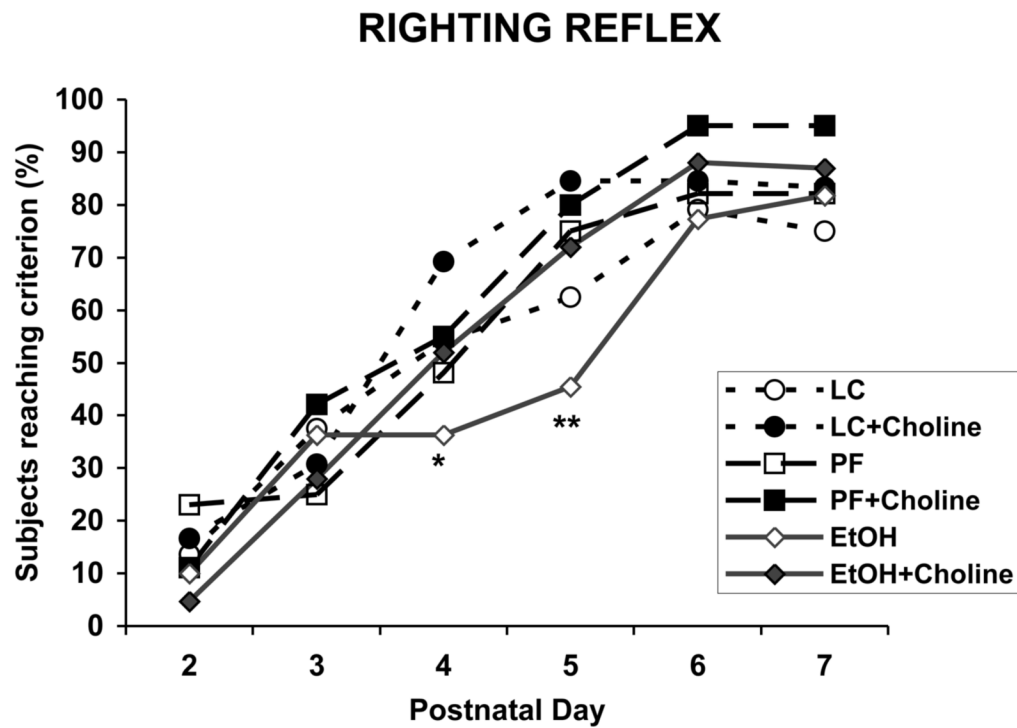
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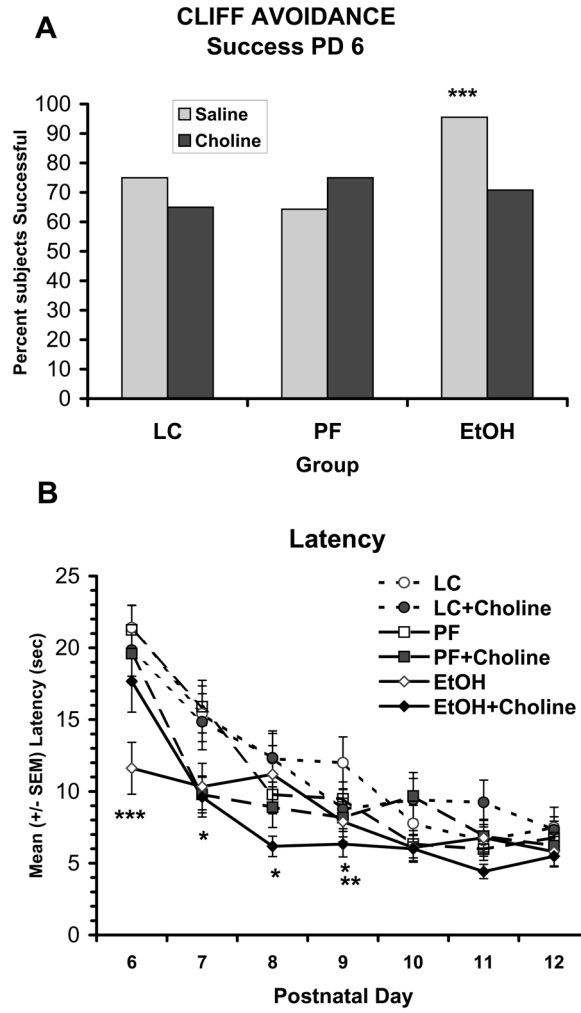


**Figure 1.** Mean (+ SEM) brain weight on PD 1. Brains of ethanol-exposed weighed significantly less than both control groups and choline significantly increased brain weight in all groups. There was no significant interaction between ethanol and choline treatment. \* = vehicle treated groups significantly different from choline treated groups, \*\* = EtOH significantly different from both control groups

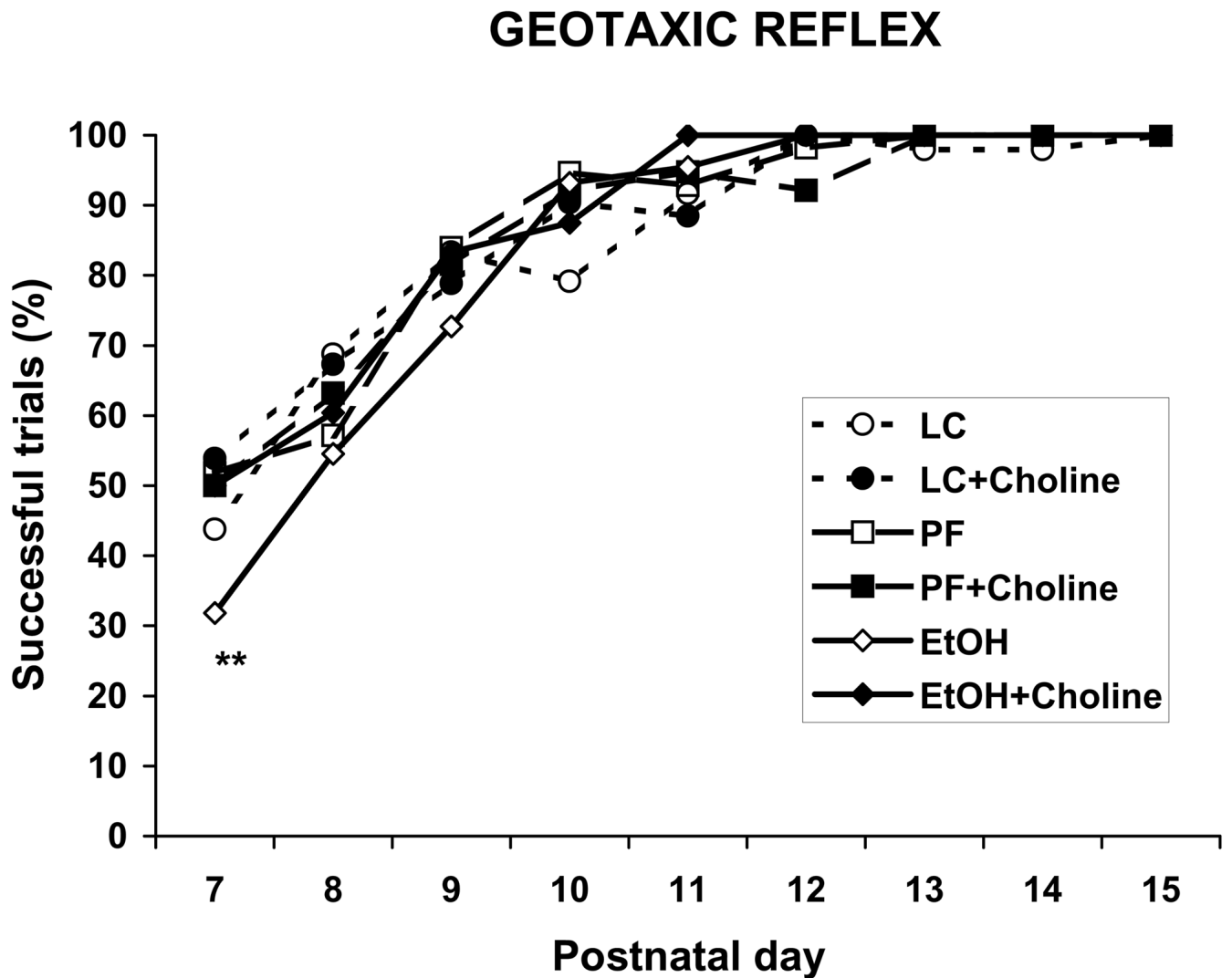


**Figure 2.** Percent of subjects who exhibited a mature surface righting reflex across PD 2-7. Fewer EtOH + Vehicle subjects showed a mature response compared to the LC + Choline subjects on PD 4 and compared to all other groups except the LC + Vehicle group on PD 5. \* = significantly different from LC + Choline; \*\* = significantly different from all other groups except LC + Vehicle group

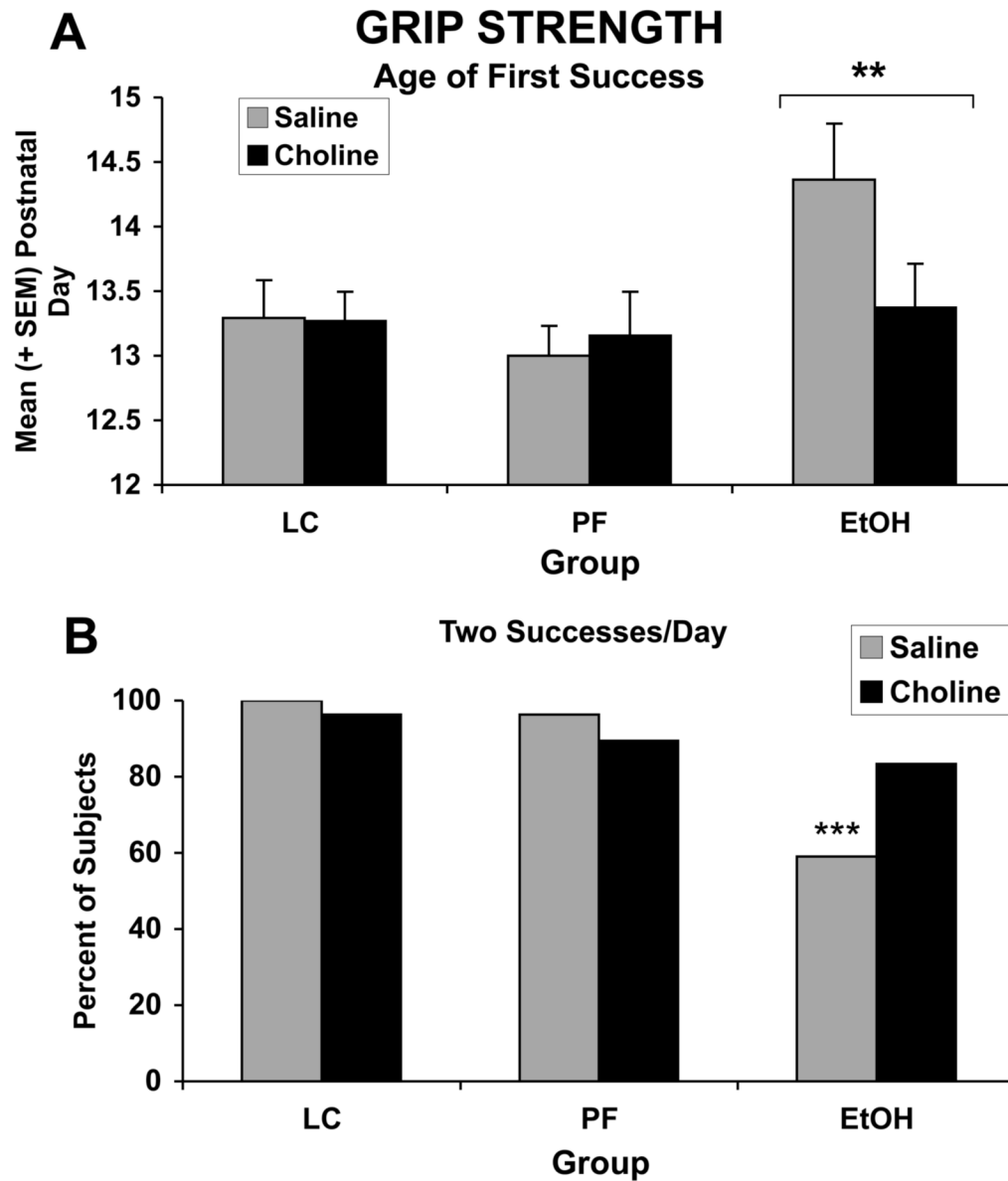




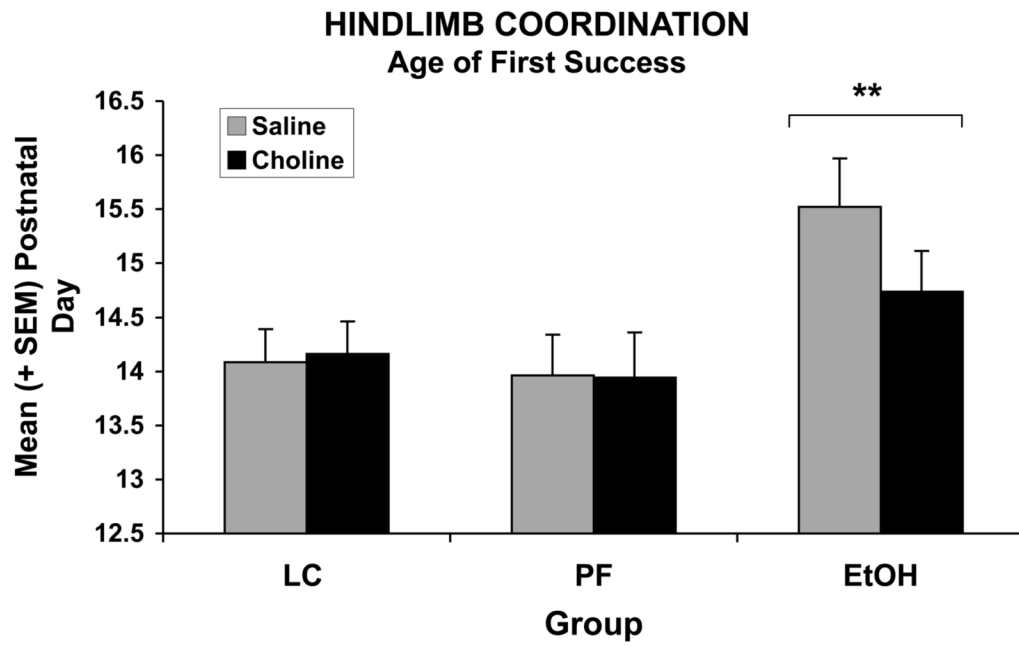
**Figure 3.** Percent of subjects successful on PD 6 (A) and mean (+/- SEM) latency to retract from the cliff over testing days (B). On PD 6, subjects exposed to prenatal alcohol and treated with vehicle were more successful and had significantly shorter latencies compared to all other groups. On subsequent days (PD 7-9), ethanol-exposed subjects were faster than LC controls and on PD 9, choline-treated subjects were faster than vehicle-treated subjects (Panel B). \*\*\* = EtOH + Vehicle significantly different from all other groups, \* = EtOH significantly different from LC, \*\* = choline significantly different from vehicle



**Figure 4.** Percentage of successful responses on the geotaxic reflex task over testing days. On PD 7, ethanol-exposed subjects treated with vehicle were less successful compared to all groups except the LC + vehicle group.  
 \*\* = significantly different from all groups except LC + vehicle



**Figure 5.** Age of first successful grip (A) and percent of subjects successful for 2 trials/day over the course of training (B). Ethanol delayed the ability to grip the bar for 30 seconds. Although ethanol-exposed subjects treated with choline exhibited this behavior earlier, there was no significant interaction of alcohol and choline. In contrast, fewer subjects exposed to ethanol and treated with vehicle were successful for 2 trials/day compared to all other groups, including the ethanol-exposed subjects treated with choline. \*\*\* = significantly different from all other groups, \*\* = significantly different from both control groups



**Figure 6.** Ethanol exposure significantly delayed hindlimb coordination and choline did not significantly mitigate this effect.  
\*\* = EtOH significantly different from both control groups

Peak BAC, and Delivery Statistics: Percent of maternal body weight gained during pregnancy, length of gestation, male/female ratio, and number of litters for each prenatal treatment group

**Table 1**

|                                    | Prenatal treatment |      |                |      |                |      |                |      |                |      |                |      |
|------------------------------------|--------------------|------|----------------|------|----------------|------|----------------|------|----------------|------|----------------|------|
|                                    | LC                 |      |                |      | PF             |      |                |      | EtOH           |      |                |      |
|                                    | Vehicle (n=12)     |      | Choline (n=13) |      | Vehicle (n=14) |      | Choline (n=10) |      | Vehicle (n=11) |      | Choline (n=12) |      |
|                                    | Mean               | SEM  | Mean           | SEM  | Mean           | SEM  | Mean           | SEM  | Mean           | SEM  | Mean           | SEM  |
| <i>BAC</i>                         | —                  | —    | —              | —    | —              | —    | —              | —    | 191            | 26   | 221            | 19   |
| <i>† GD 5</i>                      | —                  | —    | —              | —    | —              | —    | —              | —    | 258            | 26   | 239            | 12   |
| <i>† GD 20</i>                     | —                  | —    | —              | —    | —              | —    | —              | —    | 36.7           | 3.0  | 34.7           | 2.7  |
| <i>Maternal % Body Weight Gain</i> | * 49.6             | 2.9  | 52.8           | 2.3  | 33.9           | 1.8  | 36.5           | 1.1  | 14.5           | 0.9  | 12.8           | 0.9  |
| <i>Litter Size</i>                 | 13.7               | 0.9  | 13.7           | 0.9  | 14.3           | 0.6  | 14.4           | 0.6  | 21.5           | 0.2  | 21.8           | 0.2  |
| <i>Gestational Length (days)</i>   | 21.5               | 0.1  | 21.4           | 0.1  | 21.4           | 0.2  | 21.5           | 0.2  | 0.50           | 0.04 | 0.53           | 0.03 |
| <i>Male Ratio</i>                  | 0.43               | 0.03 | 0.55           | 0.05 | 0.50           | 0.04 | 0.46           | 0.05 | 0.50           | 0.05 | 0.53           | 0.03 |

Symbols:

\* LC significantly different from PF and EtOH, collapsed over choline treatment ( $p < .05$ )

*†* Additional pregnant rats: EtOH-Vehicle (n=6); EtOH-Choline (n=5)

Notes: EtOH= Ethanol; PF= Pair-Fed; LC= Lab Chow; n= number of litters, SEM= Standard Error of the Mean, BAC= Blood Alcohol Concentration, GD= Gestational day

**Table II**

Mean and SEM for body weights during lactation.  
Data collapsed across sex and presented at three-day intervals

| Postnatal Day | Prenatal treatment |     |         |     |         |      |         |     |         |       |         |         |
|---------------|--------------------|-----|---------|-----|---------|------|---------|-----|---------|-------|---------|---------|
|               | LC                 |     |         |     | PF      |      |         |     | EtOH    |       |         |         |
|               | Vehicle            |     | Choline |     | Vehicle |      | Choline |     | Vehicle |       | Choline |         |
|               | Mean               | SEM | Mean    | SEM | Mean    | SEM  | Mean    | SEM | Mean    | SEM   | Mean    | SEM     |
| 1             | 7.3                | 0.2 | 7.5     | 0.1 | 6.7     | 0.1* | 6.9     | 0.1 | 6.5     | 0.1*  | 6.9     | 0.1**** |
| 3             | 10.1               | 0.2 | 10.2    | 0.2 | 9.1     | 0.1* | 9.5     | 0.2 | 8.4     | 0.2** | 8.8     | 0.2     |
| 6             | 15.1               | 0.3 | 15.3    | 0.3 | 14.1    | 0.2* | 14.5    | 0.3 | 13.0    | 0.2** | 13.6    | 0.3     |
| 9             | 21.3               | 0.4 | 21.3    | 0.5 | 19.8    | 0.3* | 20.4    | 0.4 | 18.8    | 0.4** | 19.8    | 0.5     |
| 12            | 27.9               | 0.5 | 27.9    | 0.6 | 26.5    | 0.3* | 26.7    | 0.5 | 25.1    | 0.5** | 26.4    | 0.6     |
| 13            | 30.1               | 0.5 | 29.7    | 0.6 | 28.5    | 0.4* | 28.9    | 0.6 | 27.0    | 0.4** | 28.7    | 0.6**** |
| 15            | 34.5               | 0.5 | 33.8    | 0.6 | 32.8    | 0.4* | 33.1    | 0.7 | 31.3    | 0.5** | 32.9    | 0.7     |
| 18            | 40.9               | 0.6 | 40.2    | 0.7 | 39.2    | 0.5* | 39.0    | 0.8 | 37.3    | 0.6** | 39.0    | 0.9     |
| 21            | 52.5               | 0.8 | 52.0    | 0.9 | 50.6    | 0.8* | 50.6    | 0.8 | 47.1    | 0.9** | 49.6    | 1.1     |

\* significantly different from LC vehicle;

\*\* significantly different from LC and PF vehicle,

\*\*\* significantly different from EtOH vehicle

Physical Development. Days of age for the appearance of the following physical features: ears unfolded, upper and lower incisors cutting the gums and eyes opening

**Table III**

| Physical Feature      | Prenatal treatment |      |         |      |         |      |         |      |         |      |         |     |
|-----------------------|--------------------|------|---------|------|---------|------|---------|------|---------|------|---------|-----|
|                       | LC                 |      |         |      | PF      |      |         |      | EtOH    |      |         |     |
|                       | Vehicle            |      | Choline |      | Vehicle |      | Choline |      | Vehicle |      | Choline |     |
|                       | Mean               | SEM  | Mean    | SEM  | Mean    | SEM  | Mean    | SEM  | Mean    | SEM  | Mean    | SEM |
| <i>Ears Unfolded</i>  | 3.1                | 0.2  | 2.7     | 0.2  | 3.0     | 0.1  | 3.0     | 0.2  | 3.3     | 0.2  | 3.1     | 0.2 |
| <i>Lower Incisors</i> | ***                | 10.3 | 0.4     | 9.6  | 0.4     | 9.8  | 0.3     | 9.8  | 0.3     | **   | 11.1    | 0.3 |
| <i>Upper Incisors</i> | ***                | 10.6 | 0.2     | 10.1 | 0.2     | 10.6 | 0.2     | 10.4 | 0.2     | **   | 11.6    | 0.3 |
| <i>Eyes Opened</i>    | 14.1               | 0.2  | 14.1    | 0.2  | 14.2    | 0.2  | 14.4    | 0.1  | **      | 14.9 | 0.2     | 0.2 |

\*\* EtOH significantly different from both control groups,

\*\*\* choline treatment significantly different from vehicle