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Maternal supplementation of nucleotides improves the behavioral development of prenatal ethanol-exposed mice

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Abstract Maternal ethanol consumption during pregnancy can induce learning deficits in the offspring. The objective of this study was to assess whether supplementation of exogenous nucleotides during pregnancy and lactation would ameliorate prenatal ethanol-induced learning and memory deficits in the offspring of mice, and to explore the possible mechanisms. In the present study, pregnant C57BL/6J mice were exposed to ethanol (5 g/kg body weight) intragastrically from gestational day (GD) 6 to GD15. The dams in exogenous nucleotide intervention groups were fed with feed containing 0.01 %, 0.04 %, or 0.16 % nucleotide powder, with control and ethanol groups receiving normal feed. The dams were allowed to deliver naturally and to breast feed their offspring. After weaning, behavioral tests were carried out in the offspring of each group. Serum oxidation indexes were analyzed, and the hippocampus of each offspring was collected and detected for acetyl cholinesterase (AChE) activity and the expression of p-CREB, CREB, and BDNF. The results showed that maternal supplementation with exogenous nucleotides during pregnancy could ameliorate prenatal ethanol-induced learning and memory deficits in the offspring of mice, through improving their antioxidant capacity, reversing hippocampus AChE levels, and allowing the expression of some proteins related to learning and memory. However, different sensitivities were found between the two sexes.

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Prenatal ethanol exposure is one of the main risk factors that result in a series of birth defects and developmental retardation, the most serious of which is termed fetal alcohol syndrome (FAS) (Detering, Reed, Ozand, & Karahasan, 1979; Ouellette, Rosett, Rosman, & Weiner, 1977; Welch-Carre, 2005). FAS patients often have more than one neurodevelopmental disorder that have far-reaching impacts on human life, including substantial, lifelong impairments in intellectual, cognitive, and psychosocial functioning. Although it is well known that alcoholic beverage drinking during gestation may jeopardize the offspring, the prevalence of FAS is still increasing worldwide. Especially in some developing countries where the social status of women is improving and females are attending more social activities than ever before, the odds of unintentional drinking during early pregnancy (the first trimester, when many women may not be aware of their pregnancy) are increasing rapidly.

Not only does the disease cause heavy psychological pressure to families, it also brings heavy economic burdens to both families and governments. In Canada, the total adjusted annual costs associated with FAS were estimated at $14,342 per child (Naha, Lee, Naser, Park, Kim, & Kim, 2009); in the US, estimates of the annual economic impact of FAS reached up to $9.7 billion (Yeaney, He, Tang, Malouf, O'Riordan, Lemmon, & Bearer, 2009), and it would cost $2, 342 per year to prevent one case of FAS (Goodlett & Horn, 2001). Considering the great loss and cost that we have to sacrifice, it is of great significance to find measures to ameliorate the detrimental effects of prenatal ethanol exposure.

Our previous research showed that 5.0 g/kg body weight ethanol consumption during the organogenesis period in mice

could jeopardize the development of the fetal central nervous system, causing excessive cell apoptosis, decreased adenosine 5′-triphosphate content in the fetal brain, and microcephaly (Xu, Liu, & Li, 2005), and could also cause fetal brain proteomic changes (Xu, Tang, & Li, 2008). Meanwhile, maternal supplementation with one-carbon unit, which are necessary materials for the de novo synthesis of nucleic acid, can improve ethanol-induced developmental toxicity in mouse fetuses (Xu et al., 2008). Considering the limitation of de novo synthesis of nucleic acid under stress conditions—such as impaired immunity, liver injury, and rapid growth periods (Kvigne, Leonardson, Neff-Smith, Brock, Borzelleca, & Welty, 2004)—we hypothesized that enhancement of the salvage pathway of nucleic acid production by supplementing exogenous nucleotides might be an effective and economical way to reverse prenatal ethanol-induced learning and memory deficits.

Materials and method

Chemicals

5′-Nucleotide (NT) powder of analytical grade (>99 % pure), derived from brewer's yeast RNA, was provided by Zhen-Ao Biotechnology (Dalian, China). The proportions of 5′AMP:5′CMP:5′GMPNa_2:5′UMP Na_2 in the powder were 22.8 %:26.6 %:30.2 %:20.4 %. A multi-generation assessment of the safety of this NT powder had been done in rats, and no behavior disorder and organ pathology related to toxic symptoms were observed (Xu, Ma, Xu, Xu, & Li, 2012). Anhydrous ethanol of analytical grade was purchased from Beijing Chemical Company (Beijing, China). Primary and secondary antibodies used in western blot were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horse radish peroxidase (HRP) conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Animals and treatment

Specific pathogen-free (SPF) 10-week-old C57BL/6J mice were provided by the Department of Laboratory Animal Science of Peking University. The animals were quarantined for 7 days after shipping and were maintained in a temperature- and humidity-controlled animal facility with a 12-h:12-h light:dark cycle (lights on 7:00 AM). Mice were provided with food and clean water ad libitum throughout the whole study. After the quarantine period, female mice were mated with male mice overnight and were checked for vaginal plugs the next morning at 7:00 AM. The presence of a vaginal plug signified gestational day (GD) 0.

The plug-positive females were randomly divided into six groups (ten dams/group): control, nucleotide, and ethanol groups, as well as NT groups receiving a low, medium, or high dose of the nucleotide intervention. Dams in the control group received commercial pregnancy forage for mice (total nucleotides content about 0.4 mg/g) and were administered 5.0 g/kg body weight (BW) twice-distilled water daily from GD6 to GD15. Dams in the ethanol group were fed with pregnancy forage and administered 5.0 g/kg BW ethanol intragastrically as a 25 % (v/v) solution at 8:00 AM every day from GD6 to GD15 (mid- to late-first-trimester human equivalent) in order to cover organogenesis. This ethanol dose was selected on the basis of a previous study (Xu et al., 2008). Dams in the three NT intervention groups were also administered equal volumes of ethanol from GD6 to GD15, but they were also fed with specially made NT-fortified pregnancy forage—which contained 0.01 %, 0.04 %, or 0.16 % of NT powder—during pregnancy and lactation. Dams in the nucleotide group received 0.16 % NT fortified pregnancy forage during pregnancy and lactation and were administered 5.0 g/kg BW twice-distilled water daily from GD6 to GD15. The body weight and food consumption of each dam were recorded weekly throughout the gestation period. On GD15, maternal tail blood samples were collected into heparinized capillary tubes at 1 h after the ethanol administration. The blood ethanol concentrations were analyzed by headspace capillary gas chromatography according to the method described by Livy, Parnell, and West (2003). The lowest detectable concentration of ethanol in the mouse blood was 0.4 mg/ml. The use of animals in this research was approved by, and conducted in compliance with, the guidelines for animal care and research of Peking University.

Offspring manipulations

Pregnant mice were allowed to deliver and nurse their offspring freely. On the 4th day after delivery, litter size was adjusted to six, with three of each sex. On the 21th day after delivery, the offspring was weaned, housed separately, and fed with basic forage. The body weight gain of each offspring was recorded weekly throughout the lactation period.

Behavioral tests of the offspring

The offspring of each sex in one litter were numbered "1," "2," or "3." Postweaning behavioral tests, including the open-field test, Morris water maze test, and shuttle-box avoidance test, were evaluated in both male and female mice from each litter. Since the training procedures of some behavioral tests, such as Morris water maze test, could change the expression of some proteins related to learning and memory (Hosseini-Sharifabad, Mohammadi-Eraghi, Tabrizian, Soodi,

Khorshidahmad, Naghdi, . . . Sharifzadeh, 2011; Oh, Kim, Choi, Lee, Choi, & Hyeon, 2012; Orsetti, Casamenti, & Pepeu, 1996; Tan, 2009), we carried out such tests (Morris water maze test and shuttle-box avoidance test) in offspring with different numbers. For each test, the males and females were tested separately on different apparatuses with the same instrument model, to ensure minimal impact of mixed-sex behavioral testing.

Open-field test The open-field test was evaluated at 8:00 AM on postweaning Day 3 (24 days old) under dark and quiet surroundings. All of the offspring were included in this test, and each animal was tested just once. The apparatus used in this test was a 40 × 40 × 40 cm box made of organic glass. A total of 16 little square cells (10 × 10 cm) were located on the undersurface; the central four cells were called the *central grid*, and the other 12 cells were called the *peripheral grid*. At the beginning of the test, each animal was put at the center of the undersurface and was allowed to move freely for 3 min. The animal's behavior during this period including time staying in the central grid, number of grid crossings, frequency of rearing (rearing up on haunch with forelimbs ~3–4 cm off the floor), frequency of grooming (including washing face, pluming, and licking tails), and time of grooming were recorded by the same researcher through the whole test.

Morris water maze test The Number 1 animals in each litter were submitted to this test at 4 weeks old. The Morris water maze was a circular black pool with a diameter of 120 cm and a height of 60 cm. The water was made opaque with skim milk powder. The maze was geographically divided into four equal quadrants, and a hidden circular platform was located at the center of one of the quadrants and submerged 1.0 cm. The platform remained at the same place in order to help animals memorize and locate it throughout the whole test. The test last 7 consecutive days and was divided into two sessions: the training session in the first 6 days (begun at 8:00 AM every day) and the test session on the 7th day (begun at 8:00 AM). During the training session, animals were first put on the platform facing the wall for 15 s, then each was tested four times per day—from four start positions in each of the four quadrants. A test commenced the moment that an animal was dropped in the water and finished the moment that it firmly sat on the platform, and a maximum of 120 s were allowed for each test. For animals that failed to locate the platform in 120 s, a 10-s rest on the platform were allowed before a new test commenced. Each test was videotaped with a camera mounted directly above the center of the pool, and the time that a mouse took in locating the hidden platform was recorded as the latency. On the 7th day, the platform was removed and each mouse was placed into water in the same quadrant, and the number of the times that it crossed the original location of the platform in 120 s was recorded.

Shuttle-box avoidance test The Number 2 animals in each litter were submitted to this test at 4 weeks old, beginning at 8:00 AM every day. Animals were tested in a shuttle-box, a 50 × 16 × 18 cm box with a floor consisted of parallel stainless steel bars and walls with a photo-sensory detector at each end. We expected the animals to learn to actively avoid electric shocks when they heard the signal, a beep sound, and to run toward the end of the box after training. At first, each animal was given 5 min to explore freely in the box without getting electric shocks. In the training session, ten consecutive electric shocking cycles were administered each day for each animal for four consecutive days. A cycle began with 5-s of beeps, followed by 10-s electric shock (100 V, 0.2 mA, 50 Hz, AC) and a 10-s interval. On the 5th day, animals were tested again in the box for ten cycles in order to measure their learning results. During the whole test, the time for which animals actively avoided electric shocks (called the *active avoidance latency*), the time that they got shocked (*shock time*), and the frequency with which they got shocked (shock times) were recorded every day.

Measurement of serum oxidation indexes

Biochemical parameters and protein expression were analyzed in the Number 3 animals in each litter in order to avoid possible confounding factors induced by the behavioral tests. After fasting overnight, animals were put into an airtight jar filled with diethyl ether at 8:00 AM when they were 4 weeks old, the same age as the Number 1 and Number 2 animals when they were tested with the Morris water maze or shuttle-box. After deep anesthesia, blood samples were collected from the orbital sinus by removing eyeballs. Then after 30 minutes' clotting at room temperature, the blood samples were centrifuged at 3, 000 rpm for 15 min, and the serum was transferred to new tubes and preserved at –20 °C. The serum oxidation indexes of super oxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px) were measured with commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by colorimetric methods, according to the manufacturer's instruction.

Acetyl cholinesterase (AChE) level in hippocampus

The Number 3 animals were sacrificed after blood sampling. The whole brain of each animal was separated immediately. Then the hippocampus was isolated on ice as soon as possible and preserved at –80 °C. The AChE level was analyzed with a commercially available kit (Nanjing Jiancheng

Bioengineering Institute, Nanjing, China) according to the manufacture's instructions.

Western blots

Hippocampus expression of cAMP response element binding protein (*CREB*), phosphorylated CREB (*p-CREB*), and brain-derived neurotrophic factor (*BDNF*) was detected using the western blot technique. The hippocampus was placed into a tube with a cold extraction buffer. The mixture was ultrasound blended three times, for 3 s each time. The slurry was then left to react for 30 min at 4 °C, and spun in a centrifuge at 12,000 rpm at 4 °C for 15 min. Then the liquid supernatant was transferred into new tubes. The Bradford method was used to determine the concentration of protein in samples before running a western blot. Samples (70 μg) were separated on 12 % SDS-PAGE gels, then electro-transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5 % nonfat milk in Tris-buffered saline 0.05 % Tween (TBST) for 2 h and then incubated with primary antibodies (1:1000 dilution), followed by redetection with secondary antibodies (1:1000 dilution) in TBST-5 % milk overnight at 4 °C. Detection of a specific binding was performed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Specific signals were exposed in a darkroom using electrochemical luminescence (ECL) (Sunbio, Beijing China). The images were scanned for densitometry using the Quantity One software package.

Statistics analysis

The data were expressed as means ± *SD*s. The SPSS 17.0 for Windows software was used for all of the statistical analyses. In comparing the difference between groups, a one-way analysis of variance (ANOVA) was used to analyze all data of the open-field test, the 7th day's data from the Morris water maze test, the data in the test session of the shuttle-box test, and the data of all molecular biology tests; a one-way ANOVA with repeated measures was used to analyze the data of the first six days in the Morris water maze test and the data in the training session of the shuttle-box avoidance test. Statistical significance was accepted at $p < .05$.

Results

Maternal blood ethanol levels, body weight changes, and food utilization during gestation

The mean maternal blood ethanol concentrations on GD15 in the dams of the ethanol group, 0.01 % NT group, 0.04 % NT group, and 0.16 % NT group were 2.58 ± 0.24, 2.49 ± 0.27, 2.53 ± 0.28, and 2.48 ± 0.26 mg/ml, respectively. The four groups were not statistically different ($p > .05$). Ethanol was not detected in the blood of the dams of the control group and the nucleotide group on GD15. No significant between-group difference was found in the body weight gains during gestation (Fig. 1a); however, the calculated food utilization rates in the ethanol group were significantly lower than those in the control group ($p < .01$), and the rates in the NT supplementation groups were significantly higher than in the ethanol group (Fig. 1b).

Body weight changes of the offspring after birth

We observed a time-dependent increase in the body weights of the offspring in each group, and no significant difference was found in the mean body weights of the offspring between the six groups among either female or male animals (Fig. 2).

Performances in the behavioral tests of the offspring

Open-field test For females, offspring in the ethanol group stayed much longer in the central cells and crossed fewer grids than did animals in the control group. The rearing times were also less in the ethanol than in the control group. Animals in the NT administration groups showed better performance and paralleled the control performance (Table 1).

For males, offspring in the ethanol group stayed much longer in the central cells than did animals in the control group. Animals in the 0.16 % NT administration group showed better performance. Meanwhile, animals in the 0.04 % and 0.16 % NT administration groups crossed more grids than did animals in the ethanol group (Table 1).

Morris water maze test As is shown in Fig. 3, the mean latencies to find the submerged platform declined progressively during the 6 consecutive training days in all animals. However, it took longer for the offspring in the ethanol group to locate the platform than for the control group in both female and male animals. NT supplementation during pregnancy improved the performance of the offspring with prenatal ethanol exposure; however, female and male animals showed different effects. For female offspring, the 0.04 % NT group showed the best effect, and the performance of the female animals in the 0.04 % NT group even paralleled that of the control group. However, the higher-dosage (0.16 %) group showed poorer performance. For male offspring, the 0.16 % NT group showed the best performance. Animals in the 0.16 % NT group were much quicker to locate the platform than were the ethanol group ($p < .01$), and even quicker than those in the control group ($p < .05$). We found no significant difference between the nucleotide group and the control group in escape latencies in both female and male animals.

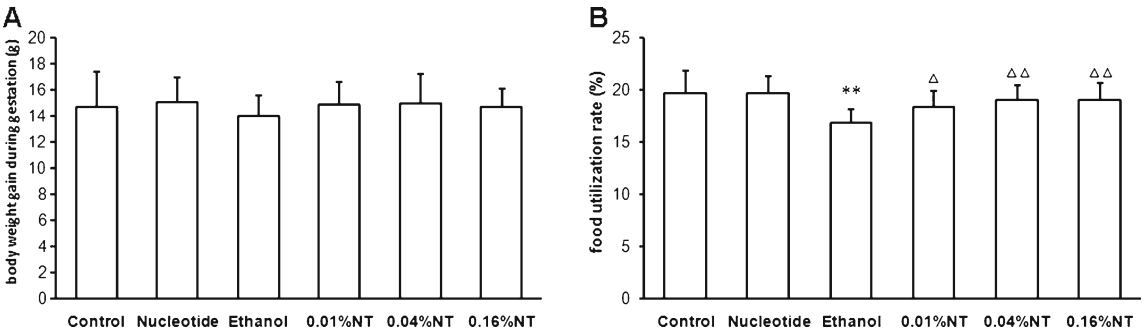

Fig. 1 Effects of maternal nucleotide supplementation on body weight gains and food utilization of the dams during gestation. **a** body weight gain. **b** Food utilization. **Significantly different from the control group, $p < .01$. △Significantly different from the ethanol group, $p < .05$. △△Significantly different from the ethanol group, $p < .01$

On the 7th day of the test, as compared with the control group, the platform-crossing times of the ethanol group were significantly fewer in the female offspring ($p < .05$). The NT intervention groups showed a trend toward an improvement, but the difference between the NT intervention groups and the ethanol group was not statistically significant ($p > .05$). For male offspring, no statistical difference was found between the five groups ($p > .05$). The platform-crossing times of the nucleotide group were not significantly different from those of the control group in both female and male animals (Fig. 4).

Shuttle-box avoidance test As is shown in Fig. 5, the mean active avoidance latencies increased progressively during the four consecutive training days in all of the offspring. However, for female offspring, the mean active avoidance latency of the ethanol group was significantly shorter than that of the control group on the first day of training ($p < .01$). The performance of the three NT intervention groups was all improved ($p < .05$), without a significant difference being found between the NT intervention groups ($p > .05$). No statistical difference was found between the nucleotide group and the control group in the mean active avoidance latencies ($p > .05$). On the 5th day, no statistical difference was found in shock times between any of the six groups (Fig. 6).

For male offspring, unlike in the female ones, we found no statistical difference in the mean active avoidance latencies between all groups on the first day of training. However the performance of animals in the 0.16 % NT group improved faster than that in the other groups. On 4th and 5th days, the mean active avoidance latency of the 0.16 % NT group was significantly longer than that in the ethanol group ($p < .05$) (Fig. 5). On the 5th day, animals in the ethanol group got significantly more shocks than did those in the control group ($p < .01$). However, the shock times of animals in the 0.16 % NT group were not statistically different from control ($p > .01$), although no statistical difference was also found between the 0.16 % NT and ethanol groups (Fig. 6). There was no statistical difference between the nucleotide group and the control group in either active avoidance latencies or shock times on the 5th day.

Serum oxidation indexes

As is shown in Table 2, prenatal ethanol exposure resulted in decreased activity of serum SOD and GSH-Px, and elevated MDA levels in both female and male animals. NT supplementation during pregnancy showed some protective effects. For female offspring, SOD activity was significantly elevated in the 0.04 % NT group as compared with the ethanol

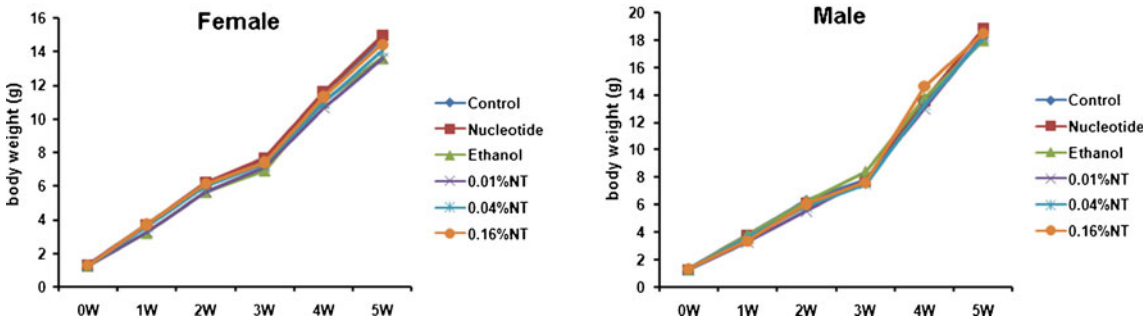

Fig. 2 Effects of maternal nucleotide supplementation on the body weights of offspring with prenatal ethanol exposure

Table 1 Effects of maternal nucleotide supplementation during pregnancy and lactation on the performance of offspring with prenatal ethanol exposure in the open-field test

Sex	Group	*N*	Time Spent in Central Grids (s)	Number of Grid Crossings	Frequency of Rearing	Frequency of Grooming	Time Grooming (s)
Female	Control	30	14.73±8.89	67.86±14.53	22.73±6.20	1.77±0.87	13.50±9.46
	Nucleotide	30	13.52±9.01	69.97±17.49	20.88±8.74	1.61±0.59	12.96±8.65
	Ethanol	30	29.60±24.15**	61.12±14.42*	16.81±9.66*	1.24±0.93	7.84±6.57
	0.01 % NT	30	16.60±10.05$^{\triangle}$	62.19±24.49	23.04±8.29$^{\triangle}$	1.22±0.74	11.39±10.35
	0.04 % NT	30	14.05±7.37$^{\triangle\triangle}$	73.74±19.41$^{\triangle}$	21.84±7.79	2.24±1.26$^{\triangle\triangle}$	15.38±8.96$^{\triangle}$
	0.16 % NT	30	13.05±7.56$^{\triangle\triangle}$	74.30±18.90$^{\triangle}$	20.25±6.94	1.70±1.17	10.95±9.90
Male	Control	30	16.44±6.14	72.70±23.79	20.65±8.40	1.78±1.04	18.30±13.76
	Nucleotide	30	17.25±8.74	71.63±24.89	19.88±10.21	1.82±1.21	18.95±12.14
	Ethanol	30	23.42±14.62*	64.79±27.14	19.25±9.20	1.71±1.33	13.33±13.28
	0.01 % NT	30	17.65±9.53	68.83±23.47	24.85±11.43	1.60±0.99	14.55±11.78
	0.04 % NT	30	17.13±5.80	80.25±24.20$^{\triangle\triangle}$	20.96±8.70	1.88±0.85	15.13±11.25
	0.16 % NT	30	15.87±5.07$^{\triangle}$	80.30±20.41$^{\triangle\triangle}$	21.13±9.59	1.43±0.73	10.87±7.78

Data are presented as means ± *SD*s. * Significantly different from the control group for animals of the same sex, $p < .05$. ** Significantly different from the control group for animals of the same sex, $p < .01$. $^{\triangle}$ Significantly different from the ethanol group for animals of the same sex, $p < .05$. $^{\triangle\triangle}$ Significantly different from the ethanol group for animals of the same sex, $p < .01$

group ($p<.05$), and MDA levels were significantly decreased in the 0.04 % ($p < .05$) and 0.16 % ($p < .01$) NT groups, as compared with the ethanol group. For male offspring, GSH-Px activity was significantly elevated in 0.04 % ($p < .05$) and 0.16 % ($p < .05$) NT group as compared with ethanol group, and MDA level was significantly decreased in all three NT intervention groups as compared with the ethanol group ($p < .05$). No significant differences were found in any of the three parameters between the nucleotide and control groups in both female and male animals ($p > .05$).

Hippocampal AChE level

Prenatal ethanol exposure resulted in increased hippocampal AChE level in both female and male offspring (Fig. 7). AChE levels were reversed by NT supplementation. In female offspring, the hippocampal AChE levels of all three NT intervention groups were significantly lower than that of the ethanol group. In male offspring, hippocampal AChE activity of the 0.04 % and 0.16 % NT groups was significantly lower than that of ethanol group. No significant difference emerged in AChE levels between the nucleotide and control groups in both female and male animals ($p > .05$).

Hippocampus expression of p-CREB, CREB, and BDNF

Prenatal ethanol exposure inhibited the expression of BDNF and decreased the p-CREB/CREB ratio in the hippocampus. Maternal supplementation of nucleotides reversed the expression of those proteins to some extent. For female animals, the p-CREB/CREB ratio was significantly increased in all three of the nucleotide intervention groups, and the BDNF expression increased in the 0.04 % and 0.16 % nucleotide intervention groups (Fig. 8). For male animals, the p-CREB/CREB ratio was significantly increased in the 0.16 % nucleotides intervention group, and the BDNF

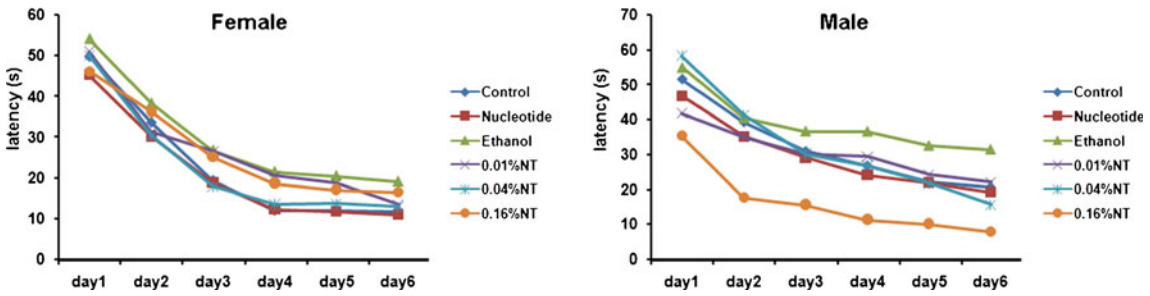

Fig. 3 Effects of maternal nucleotide supplementation during pregnancy and lactation on the escape latencies of offspring with prenatal ethanol exposure in a Morris water maze test

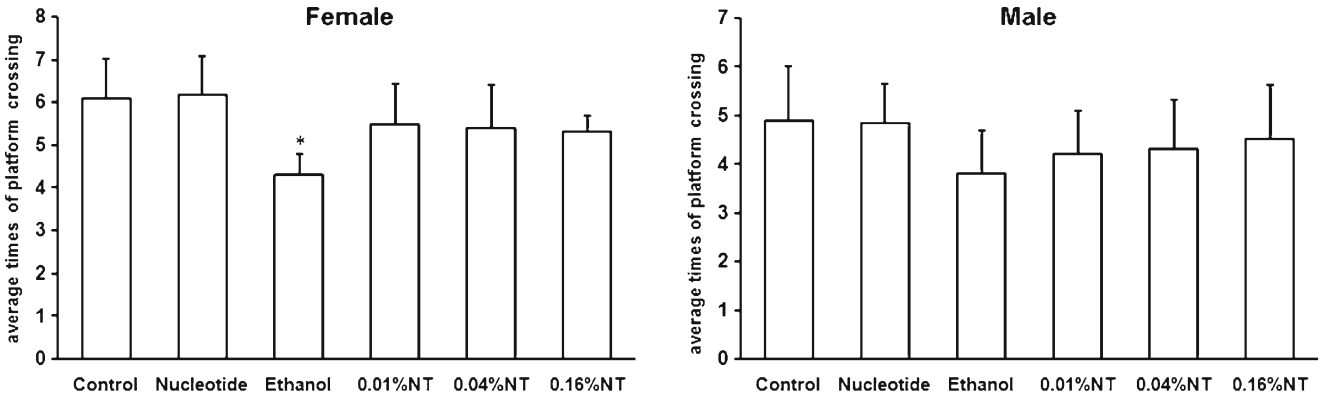

Fig. 4 Effects of maternal nucleotide supplementation during pregnancy and lactation on platform crossing numbers for offspring with prenatal ethanol exposure in a Morris water maze test. *Significantly different from the control group for animals of the same sex, $p < .05$

expression increased in both the 0.04 % and 0.16 % nucleotide intervention groups (Fig. 8). We found no statistical difference in the protein expression of p-CREB/CREB and BDNF between the nucleotide and control groups in both female and male offspring.

Discussion

In this study, we investigated the effect of exogenous nucleotide supplements during pregnancy and lactation on the learning and memory ability of mice offspring prenatally exposed to ethanol. Ethanol may damage the developing nervous system of the fetus in different ways (Welch-Carre, 2005; Xu et al., 2008), such as inhibiting the proliferation and differentiation of brain cells and inducing excessive apoptosis, down-regulating brain-derived neurotrophic factor levels, increasing oxidative stress levels, and so forth. Nucleotides and their related metabolic products play an essential role in cell replication and metabolism. They serve as the monomeric units of RNA and DNA, store cellular energy as adenosine and guanosine triphosphates, mediate intracellular metabolic processes as cyclic adenosine monophosphate and cyclic guanosine monophosphate, and support metabolism and protein synthesis as components of vitamin-derived cofactors such as flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD; Tressler, Ramstack, White, Molitor, Chen, Alarcon, & Masor, 2003). In recent years, adenosine 5′-triphosphate (ATP) has been recognized as a cotransmitter in most nerves in both the peripheral and central nervous systems (Burnstock, 2007, 2008, 2009). ATP acts as both a fast excitatory neurotransmitter and a neuromodulator, and it has potent long-term (trophic) roles in cell proliferation, differentiation, and death in development and regeneration (Burnstock & Verkhratsky, 2010). The pyrimidines cytidine (as CTP) and uridine (which is converted to UTP and then to CTP) have also been recognized as contributing to brain phosphatidylcholine and phosphatidylethanolamine synthesis via the Kennedy pathway (Cansev, 2006). All of these data indicate the important role of nucleotides and their derivatives in normal behavior.

It is well known that pregnant women need more nucleotides to meet the extra needs of embryo development. Two pathways of nucleotide synthesis are available in the human body: the de novo pathway and the salvage pathway, which are complementary in keeping the nucleotide pool in balance. When the salvage pathway is enhanced, the de novo pathway can be spared, to some extent. This can explain the result of the present research that the offspring of the nucleotide group, whose mother only received additional

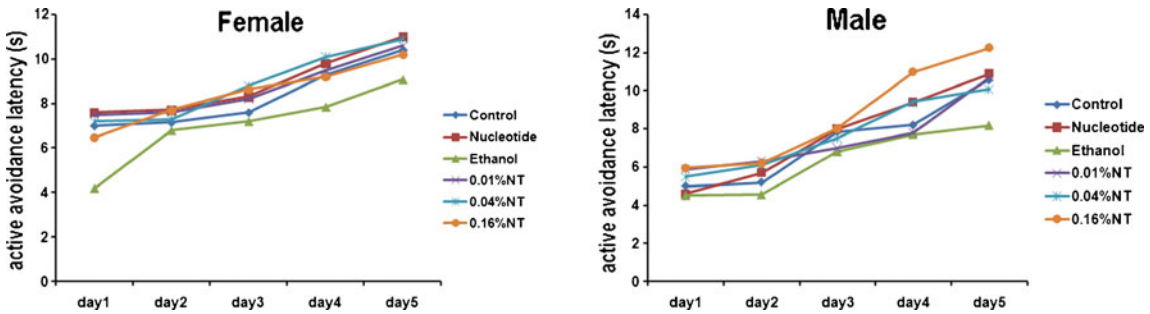

Fig. 5 Effects of maternal nucleotide supplementation during pregnancy and lactation on active avoidance latencies of offspring with prenatal ethanol exposure in a shuttle-box avoidance test

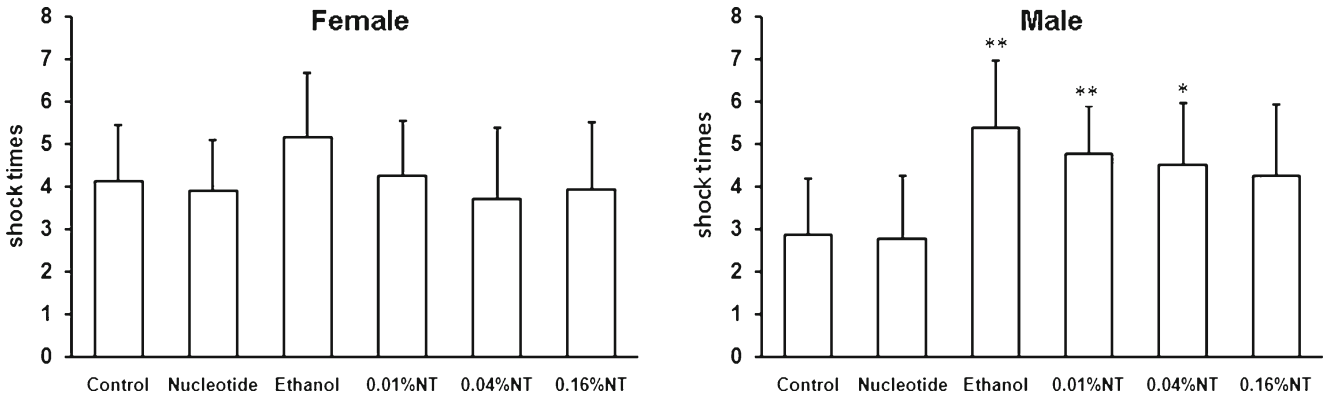

Fig. 6 Effects of maternal nucleotide supplementation during pregnancy and lactation on shock times of offspring with prenatal ethanol exposure on 5th day of a shuttle-box avoidance test. *Significantly different from the control group for animals of the same sex, $p < .05$. **Significantly different from the control group for animals of the same sex, $p < .01$

nucleotides during gestation and lactation, did not significantly differ in their performance in behavioral tests, serum chemistry, and hippocampal protein expression from the control group. However, ethanol intake will accelerate nucleotide degradation and increase urinary excretion of related substances, disturbing the balance of the nucleotide pool (Yamamoto, Moriwaki, & Takahashi, 2005). On the other hand, ethanol can traverse the placenta barrier, directly disturb the nucleotide metabolism of the developing fetus, especially the central nervous system. This may be an explanation of the abnormal behavioral performance of FAS patients. Therefore, extra supplementation of nucleotides to pregnant women with alcohol exposure (especially alcoholic pregnant women) makes some sense.

Dietary nucleotide supplementation has been found to be beneficial in enhancing immunity (Prut & Belzung, 2003; Sato, Murakami, Nakano, Sugawara, Kawakami, Idota, & Nakajima, 1995), promoting physical development (Caldwell, Sheema, Paz, Samudio-Ruiz, Laughlin, Spence, . . . Allan, 2008), improving learning abilities (Morris, 1981), and so forth. Their functions may be particularly important in rapidly growing infants. In this research, we found that maternal supplementation of exogenous nucleotides could also reverse prenatal ethanol-induced learning and memory deficits in the mice. Three behavioral tests were carried out in the offspring with in-utero ethanol exposure in the present study. The open-field test was used to evaluate spontaneous locomotor activity and adaptation ability in a novel environment, the Morris water maze test to assess spatial learning and memory ability, and the shuttle-box avoidance test to evaluate active danger-avoiding ability. All three of the behavioral tests indicated beneficial effects of maternal nucleotide supplementation in protecting offspring against in-utero ethanol exposure. Maternal blood ethanol concentrations analyzed 1 h after

Table 2 Effect of maternal supplementation of nucleotides during pregnancy and lactation on serum oxidation indexes of the offspring with prenatal ethanol exposure

Sex	Group	*N*	GSH-Px (U/ml)	SOD (U/ml)	MDA (nmol/ml)
Female	Control	10	1,147.00±455.51	146.60±36.66	5.40±1.10
	Nucleotide	10	1,099.86±397.82	150.23±39.76	4.82±0.73
	Ethanol	10	742.98±309.75*	92.18±18.17**	6.58±1.71*
	0.01 % NT	10	881.09±572.42	119.61±33.44	5.57±1.21
	0.04 % NT	10	988.70±408.56	124.20±41.58△	5.43±0.69△
	0.16 % NT	10	1,083.60±429.45	116.20±20.15	4.79±0.67△△
Male	Control	10	1,152.60±405.85	144.42±39.87	5.08±0.45
	Nucleotide	10	1,139.24±378.54	152.98±31.11	4.88±0.53
	Ethanol	10	682.87±345.41*	101.85±19.19*	6.40±0.55**
	0.01 % NT	10	865.98±430.18	113.52±36.54	5.26±0.77△
	0.04 % NT	10	1,023.30±347.31△	114.51±42.95	5.22±0.79△
	0.16 % NT	10	982.22±288.26△	118.44±25.73	5.19±0.68△

Data are presented as means ± *SD*s. * Significantly different from the control group for animals of the same sex, $p < .05$. ** Significantly different from the control group for animals of the same sex, $p < .01$. △ Significantly different from the ethanol group for animals of the same sex, $p < .05$. △△ Significantly different from the ethanol group for animals of the same sex, $p < .01$

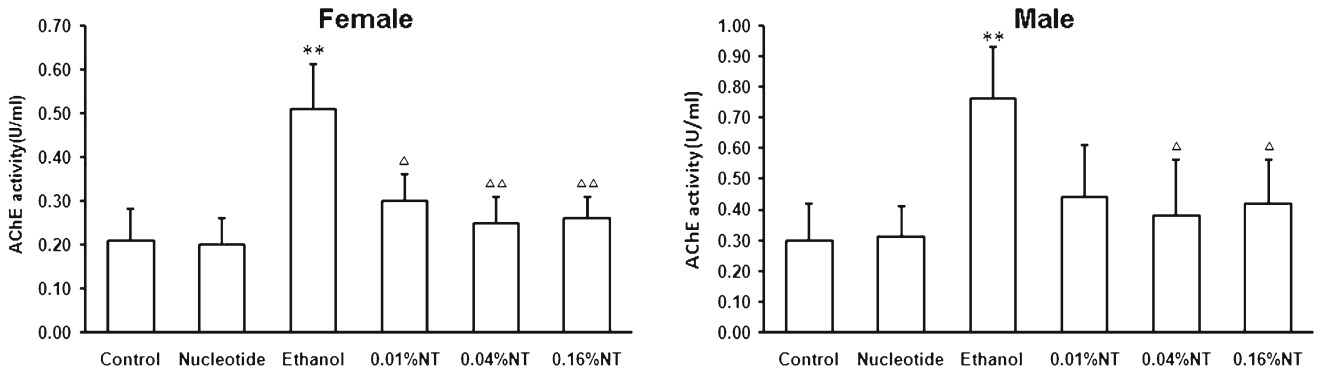

Fig. 7 Effects of maternal nucleotide supplementation during pregnancy and lactation on hippocampus acetyl cholinesterase (AChE) levels of offspring with prenatal ethanol exposure. **Significantly different from the control group for animals of the same sex, $p < .01$. △Significantly different from the ethanol group for animals of the same sex, $p < .05$. △△Significantly different from the ethanol group for animals of the same sex, $p < .01$

ethanol administration revealed that the protective effect of additional nucleotide supplementation was not due to decreased maternal ethanol absorption, since no significant difference was found in blood ethanol levels between the four ethanol administration groups. We thought the protective effects might be partially explained by the observed changes of hippocampal AChE levels, since acetylcholine (ACh) is widely distributed in the nervous system and it plays an essential role in structural and functional remodeling of the brain by establishing synaptic contacts in networks of cells that will subserve complex cognitive functions. ACh released by the cholinergic nerve terminals innervates specific brain areas during spontaneous behaviors or during acquisition and performance of learned responses. Previous studies have revealed that when a rat is placed in a novel environment, a large increase in extracellular ACh levels in the cerebral cortex and hippocampus, associated with active exploratory activity, is detected (Giovannini, Rakovska, Benton, Pazzagli, Bianchi, & Pepeu, 2001). Cholinergic system is also involved in spatial memory formation. A large cortical ACh increase is detected

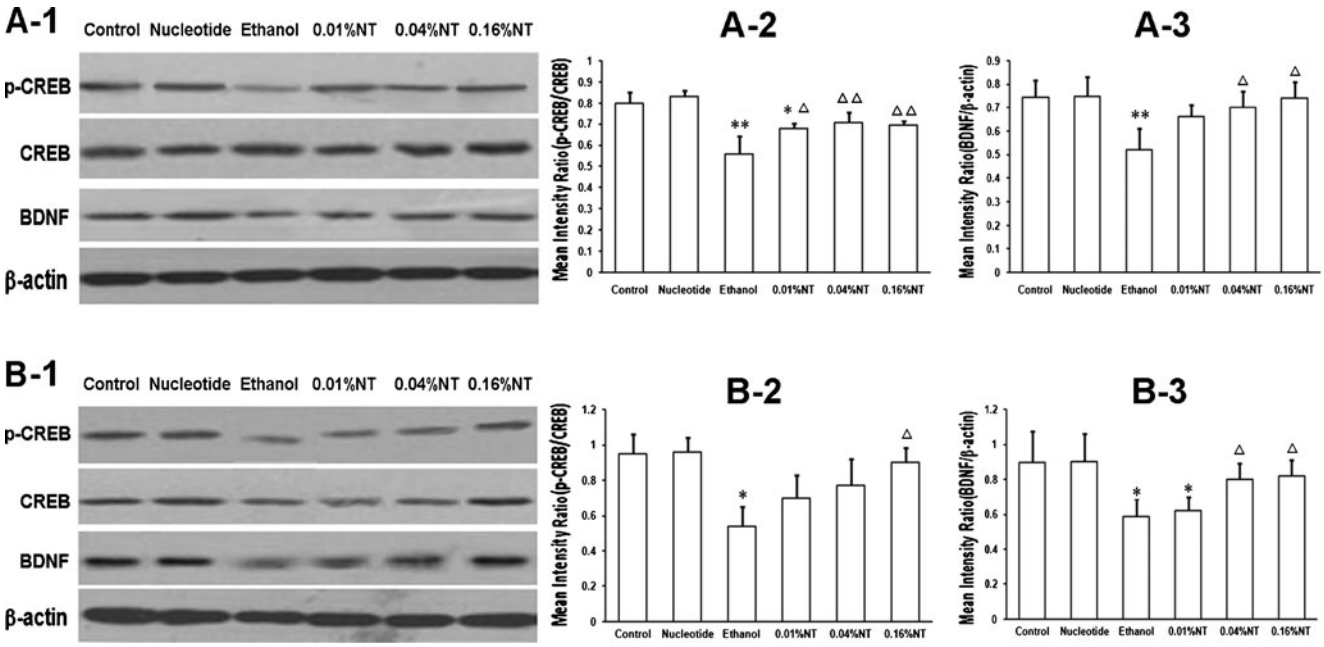

Fig. 8 Effects of maternal nucleotide supplementation during pregnancy and lactation on hippocampus expression of cAMP response element binding protein (CREB), phosphorylated CREB (p-CREB), and brain-derived neurotrophic factor (BDNF) in offspring with prenatal ethanol exposure. In the panel labels, A is for female and B is for male animals. The numeral 1 is representative of western blots of the protein levels of p-CREB, CREB, and BDNF in the different experimental groups; 2 is the mean intensity ratio of p-CREB/CREB; and 3 is the mean intensity ratio of BDNF to β-actin. *Significantly different from the control group for animals of the same sex, $p < .05$. **Significantly different from the control group for animals of the same sex, $p < .01$. △Significantly different from the ethanol group for animals of the same sex, $p < ;.05$. △△Significantly different from the ethanol group for animals of the same sex, $p < .01$

when rats alternate spontaneously in a Y maze (Giovannini et al., 2001), and an increase in hippocampal ACh release occurs during spontaneous alternation in a cross maze (Anzalone, Roland, Vogt, & Savage, 2009; Ragozzino, Pal, Unick, Stefani, & Gold, 1998). However ACh can be hydrolyzed by AChE. In the present investigation, prenatal ethanol exposure resulted in elevated AChE levels in the hippocampus in both female and male mice, which was paralleled by impaired performance of the animals in the behavioral tests. NT supplementation during pregnancy reversed the AChE levels to some extent.

Drever, Yin, Kechichian, Costantine, Longo, Saade, and Bytautiene (2012) recently reported that maternal ethanol consumption caused a decrease in SOD, GSH-Px, and catalase mRNA expression in the fetal brain, which might explain the long-term neurologic findings in FAS. Similar to Drever et al., we found in this study that prenatal ethanol exposure resulted in decreased activity of serum SOD and GSH-Px as well as elevated levels of MDA in both female and male animals. Certain doses of NT supplementation reversed the activity of antioxidase, which might be due to the reversed mRNA expression of those genes.

Previous studies have pointed out that BDNF might have a protective effect on animals under stress. In the research of Feng, Yan, and Yan (2005), prenatal ethanol-exposed rats had a lower BDNF protein level in the cortex and hippocampus. Similarly, we found in this investigation that BDNF expression in the hippocampus was inhibited by in-utero ethanol exposure, too, which paralleled the performance of the offspring in the behavioral tests. CREB is a transcription factor that binds to cAMP-response-element-containing promoters. It is activated by phosphorylation on Ser-133 through the action of several kinases. CREB activation has been considered essential during hippocampal long-term potentiation (LTP; Schulz, Siemer, Krug, & Hollt, 1999) and long-term memory (Viola, Furman, Izquierdo, Alonso, Barros, de Souza, . . . Medina, 2000), habituation to a novel environment (Vianna, Alonso, Viola, Quevedo, de Paris, Furman, . . . Izquierdo, 2000), and spatial learning (Bourtchuladze, Frenguelli, Blendy, Cioffi, Schutz, & Silva, 1994). In this study, hippocampal activation of CREB was significantly inhibited by prenatal ethanol exposure, which might be another explanation of impaired performance in the behavioral tests of the offspring. Certain dosages of 5′-nucleotide supplementation during pregnancy reversed the expression of BDNF and the activation of CREB; however, differences were noticed between the offspring of different sexes, which will be discussed below. A limitation was that, according to Close et al. (1996), the usage of diethyl ether in sacrificing might have had some impact on the hippocampus, although in this study all of the animals received the same manipulation, and the impact from diethyl ether could be considered a systematic error. Western blot was carried out with six samples of each sex per group, and the significant differences found in protein expression between groups should make sense.

Substantial studies have shown sex differences in cognitive and learning abilities (Newcombe, 1983; Voyer, Voyer, & Bryden, 1995; Williams, Barnett, & Meck, 1990): Women generally show advantages in verbal fluency, perceptual speed, and accuracy, as well as in fine motor skills, whereas men generally show advantages in spatial and mathematical abilities. Additionally, males appear to have spatial abilities superior to those of females in a number of mammalian species, including human and rats (Cahill, 2006; Yao, Hu, & Shi, 2011), but the reason lying behind this has not yet been fully understood—genetics, gonadal secretions, lateralization, experience, and so forth, may all contribute to sex differences (Begliuomini, Casarosa, Pluchino, Lenzi, Centofanti, Freschi, . . . Genazzani, 2007). The role that sex hormones play in learning and behavior is not only controversial, but also complicated: Undeniably, sex hormones matter much in neuroscience (Healy, Braham, & Braithwaite, 1999), play a part in maintaining the normal function of the central nervous system (Young, Neiss, Samuels, Roselli, & Janowsky, 2010), and may also influence plasma BDNF levels (Andreano & Cahill, 2009); however, some other studies have found no sex differences in spatial working memory among rats (Kerns & Berenbaum, 1991), and in Young et al.'s study, exogenous hormone treatment unexpectedly did not affect or modify cognition in men (Horvath, Hartig, Van der Veen, Keijser, Mulder, Ziegert, . . . Luiten, 2002). Cahill points out that even if no significant sex difference is found in female and male behavior, sex is still an important factor that should not be ignored (El-Bakri, Islam, Zhu, Elhassan, Mohammed, Winblad, & Adem, 2004), because males and females may use different neural paths to reach the same behavioral end point for some tasks.

An interesting phenomenon found in this study was that low-dose nucleotide supplementation during pregnancy showed a protective effect against in-utero ethanol exposure in female offspring, but not so much in the males. The male offspring were best protected by the highest dose of nucleotides. This might be related to the different hormone levels in the different sexes. Since many researchers have pointed out that estrogen is an extremely significant factor for the central nervous system and may also be related to the higher sensitivity of females to some chemicals. Studies have indicated that estrogen can increase the release of ACh (Horvath et al., 2002), enhance the function of NMDA receptors (El-Bakri et al., 2004), improve the function of dopaminergic neurons (Zuo, Sui, & Ge, 2002), and so forth. All of these functions are closely related to animals' learning abilities. In the present study, we found that the activation of

hippocampal CREB of female offspring was reversed in the lowest nucleotide dose group (0.01 % NT); however, among the male offspring, only the highest-dose group (0.16 % NT) showed beneficial effects. This might partially explain why low-dose nucleotides were more effective to female offspring, whereas male offspring—with little help from estrogen—might delay their response to the intervention measures. Another interesting result was that in the training section of the Morris water maze test, male offspring in the ethanol + 0.16 % NT group showed even better performance than did the control group, which was not noticed in the female offspring. We thought the better performance of male offspring in the ethanol +0.16 % NT group might partly be due to the fact that nucleotides participate in formation of the cellular energy substances ATP and GTP and mediate such intracellular metabolic processes as cAMP and cGMP, which contribute to physical performance in sports. In-utero ethanol exposure might stimulate the potential of the offspring's formation and storage of energy substances, since ethanol could accelerate nucleotide degradation and increase urinary excretion of related metabolites. This could also explain why the offspring of the nucleotide group, whose mothers only received equal doses of NT without ethanol, did not perform better than the control group, since they did not receive equal stimulation from ethanol. Still, a sex difference was also noticed, owing to different hormone conditions.

In conclusion, in this research we found that maternal supplementation of exogenous nucleotides could ameliorate prenatal ethanol-induced learning and memory deficits in C57BL/6J mice to some extent. However, differences existed between the different sexes. It is worth mentioning that, since the study modeled the human equivalent of women, ethanol intake happens in the first trimester, and NT supplementation covers all three trimesters, which should be taken in mind when extrapolated to humans.

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References

Andreano, J. M., & Cahill, L. (2009). Sex influences on the neurobiology of learning and memory. *Learning and Memory, 16,* 248–266.

Anzalone, S., Roland, J., Vogt, B., & Savage, L. (2009). Acetylcholine efflux from retrosplenial areas and hippocampal sectors during maze exploration. *Behavioural Brain Research, 201,* 272–278.

Begliuomini, S., Casarosa, E., Pluchino, N., Lenzi, E., Centofanti, M., Freschi, L., . . . Genazzani, A. R. (2007). Influence of endogenous and exogenous sex hormones on plasma brain-derived neurotrophic factor. *Human Reproduction*, *22*, 995–1002.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., & Silva, A. J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell, 79,* 59–68.

Burnstock, G. (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiological Reviews, 87,* 659–797.

Burnstock, G. (2008). Purinergic signalling and disorders of the central nervous system. *Nature Reviews Drug Discovery, 7,* 575–590.

Burnstock, G. (2009). Purinergic cotransmission. *Experimental Physiology, 94,* 20–24.

Burnstock, G., & Verkhratsky, A. (2010). Long-term (trophic) purinergic signalling: Purinoceptors control cell proliferation, differentiation and death. *Cell Death and Disease, 1,* e9.

Cahill, L. (2006). Why sex matters for neuroscience. *Nature Reviews Neuroscience, 7,* 477–484.

Caldwell, K. K., Sheema, S., Paz, R. D., Samudio-Ruiz, S. L., Laughlin, M. H., Spence, N. E., . . . Allan, A. M. (2008). Fetal alcohol spectrum disorder-associated depression: Evidence for reductions in the levels of brain-derived neurotrophic factor in a mouse model. *Pharmacology, Biochemistry, and Behavior*, *90*, 614–624.

Cansev, M. (2006). Uridine and cytidine in the brain: Their transport and utilization. *Brain Research Reviews, 52,* 389–397.

Close, B., Banister, K., Baumans, V., Bernoth, E. M., Bromage, N., Bunyan J., . . . Warwick, C. (1996). Recommendations for euthanasia of experimental animals: Part 1. *Laboratory Animals*, *30*, 293–316. doi:10.1258/002367796780739871

Detering, N., Reed, W. D., Ozand, P. T., & Karahasan, A. (1979). The effects of maternal ethanol consumption in the rat on the development of their offspring. *Journal of Nutrition, 109,* 999–1009.

Drever, N., Yin, H., Kechichian, T., Costantine, M., Longo, M., Saade, G. R., & Bytautiene, E. (2012). The expression of antioxidant enzymes in a mouse model of fetal alcohol syndrome. *American Journal of Obstetrics and Gynecology, 206*(358), e319–e322.

El-Bakri, N. K., Islam, A., Zhu, S., Elhassan, A., Mohammed, A., Winblad, B., & Adem, A. (2004). Effects of estrogen and progesterone treatment on rat hippocampal NMDA receptors: Relationship to Morris water maze performance. *Journal of Cellular and Molecular Medicine, 8,* 537–544.

Feng, M. J., Yan, S. E., & Yan, Q. S. (2005). Effect of prenatal alcohol exposure on brain-derived neurotrophic factor and its receptor tyrosine B in offspring. *Brain Research, 1042,* 125–132.

Giovannini, M. G., Rakovska, A., Benton, R. S., Pazzagli, M., Bianchi, L., & Pepeu, G. (2001). Effects of novelty and habituation on acetylcholine, GABA, and glutamate release from the frontal cortex and hippocampus of freely moving rats. *Neuroscience, 106,* 43–53.

Goodlett, C. R., & Horn, K. H. (2001). Mechanisms of alcohol-induced damage to the developing nervous system. *Alcohol Research and Health, 25,* 175–184.

Healy, S. D., Braham, S. R., & Braithwaite, V. A. (1999). Spatial working memory in rats: No differences between the sexes. *Proceedings of the Royal Society B, 266,* 2303–2308.

Horvath, K. M., Hartig, W., Van der Veen, R., Keijser, J. N., Mulder, J., Ziegert, M., . . . Luiten, P. G. (2002). 17beta-estradiol enhances cortical cholinergic innervation and preserves synaptic density following excitotoxic lesions to the rat nucleus basalis magnocellularis. *Neuroscience*, *110*, 489–504.

Hosseini-Sharifabad, A., Mohammadi-Eraghi, S., Tabrizian, K., Soodi, M., Khorshidahmad, T., Naghdi, N., . . . Sharifzadeh, M. (2011). Effects of training in the Morris water maze on the spatial learning acquisition and VAChT expression in male rats. *Daru: Journal of Faculty of Pharmacy, Tehran University of Medical Sciences*, *19*, 166–172.

Kerns, K. A., & Berenbaum, S. A. (1991). Sex differences in spatial ability in children. *Behavior Genetics, 21,* 383–396.

Kvigne, V. L., Leonardson, G. R., Neff-Smith, M., Brock, E., Borzelleca, J., & Welty, T. K. (2004). Characteristics of children who have full

or incomplete fetal alcohol syndrome. *Journal of Pediatrics, 145,* 635–640.

Livy, D. J., Parnell, S. E., & West, J. R. (2003). Blood ethanol concentration profiles: A comparison between rats and mice. *Alcohol, 29,* 165–171.

Morris, R. G. M. (1981). Spatial localization does not require the presence of local cues. *Learning and Motivation, 12,* 239–260.

Naha, N., Lee, H. Y., Naser, M. I., Park, T. J., Kim, S. H., & Kim, M. O. (2009). Ethanol inhibited apoptosis-related RNA binding protein, Napor-3 gene expression in the prenatal rat brain. *Medical Science Monitor, 15,* BR6–BR12.

Newcombe, N. (1983). Sex differences in spatial ability and spatial activities. *Sex Roles, 9,* 377–386.

Oh, D. H., Kim, B. W., Choi, M., Lee, G., Choi, J. S., & Hyeon, S. (2012). Changes in vascular endothelial growth factor (VEGF) induced by the Morris water maze task. *Molecules and Cells, 33,* 295–300.

Orsetti, M., Casamenti, F., & Pepeu, G. (1996). Enhanced acetylcholine release in the hippocampus and cortex during acquisition of an operant behavior. *Brain Research, 724,* 89–96.

Ouellette, E. M., Rosett, H. L., Rosman, N. P., & Weiner, L. (1977). Adverse effects on offspring of maternal alcohol abuse during pregnancy. *New England Journal of Medicine, 297,* 528–530.

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. *European Journal of Pharmacology, 463,* 3–33.

Ragozzino, M. E., Pal, S. N., Unick, K., Stefani, M. R., & Gold, P. E. (1998). Modulation of hippocampal acetylcholine release and spontaneous alternation scores by intrahippocampal glucose injections. *Journal of Neuroscience, 18,* 1595–1601.

Sato, N., Murakami, Y., Nakano, T., Sugawara, M., Kawakami, H., Idota, T., & Nakajima, I. (1995). Effects of dietary nucleotides on lipid metabolism and learning ability of rats. *Bioscience, Biotechnology, and Biochemistry, 59,* 1267–1271.

Schulz, S., Siemer, H., Krug, M., & Hollt, V. (1999). Direct evidence for biphasic cAMP responsive element-binding protein phosphorylation during long-term potentiation in the rat dentate gyrus in vivo. *Journal of Neuroscience, 19,* 5683–5692.

Tan, S. E. (2009). Activation of hippocampal nitric oxide and calcium/calmodulin-dependent protein kinase II in response to Morris water maze learning in rats. *Pharmacology, Biochemistry, and Behavior, 92,* 260–266.

Tressler, R. L., Ramstack, M. B., White, N. R., Molitor, B. E., Chen, N. R., Alarcon, P., & Masor, M. L. (2003). Determination of total potentially available nucleosides in human milk from Asian women. *Nutrition, 19,* 16–20.

Vianna, M. R., Alonso, M., Viola, H., Quevedo, J., de Paris, F., Furman, M., . . . Izquierdo, I. (2000). Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. *Learning and Memory*, *7*, 333–340.

Viola, H., Furman, M., Izquierdo, L. A., Alonso, M., Barros, D. M., de Souza, M. M., . . . Medina, J. H. (2000). Phosphorylated cAMP response element-binding protein as a molecular marker of memory processing in rat hippocampus: Effect of novelty. *Journal of Neuroscience*, *20*, RC112.

Voyer, D., Voyer, S., & Bryden, M. P. (1995). Magnitude of sex differences in spatial abilities: A meta-analysis and consideration of critical variables. *Psychological Bulletin, 117,* 250–270.

Welch-Carre, E. (2005). The neurodevelopmental consequences of prenatal alcohol exposure. *Advances in Neonatal Care, 5,* 217–229.

Williams, C. L., Barnett, A. M., & Meck, W. H. (1990). Organizational effects of early gonadal secretions on sexual differentiation in spatial memory. *Behavioral Neuroscience, 104,* 84–97.

Xu, M., Ma, Y., Xu, L., Xu, Y., & Li, Y. (2012). Multigenerations assessment of dietary nucleotides consumption in weaned rats. *Birth Defects Research Part B, 95,* 460–466.

Xu, Y., Liu, P., & Li, Y. (2005). Impaired development of mitochondria plays a role in the central nervous system defects of fetal alcohol syndrome. *Birth Defects Research (Part A), 73,* 83–91.

Xu, Y., Tang, Y., & Li, Y. (2008). Effect of folic acid on prenatal alcohol-induced modification of brain proteome in mice. *British Journal of Nutrition, 99,* 455–461.

Yamamoto, T., Moriwaki, Y., & Takahashi, S. (2005). Effect of ethanol on metabolism of purine bases (hypoxanthine, xanthine, and uric acid). *Clinica Chimica Acta, 356,* 35–57.

Yao, S., Hu, Z., & Shi, Q. (2011). Effects of phytoestrogen on learning and memory in ovariectomized rats and the underlying mechanism. *Zhejiang Journal of Integrated Traditional Chinese and Western Medicine, 21,* 153–158.

Yeaney, N. K., He, M., Tang, N., Malouf, A. T., O'Riordan, M. A., Lemmon, V., & Bearer, C. F. (2009). Ethanol inhibits L1 cell adhesion molecule tyrosine phosphorylation and dephosphorylation and activation of pp 60(src). *Journal of Neurochemistry, 110,* 779–790.

Young, L. A., Neiss, M. B., Samuels, M. H., Roselli, C. E., & Janowsky, J. S. (2010). Cognition is not modified by large but temporary changes in sex hormones in men. *Journal of Clinical Endocrinology and Metabolism, 95,* 280–288.

Zuo, P. P., Sui, Y. P., & Ge, Q. S. (2002). Effects of estrogen on the central nigral–striatal dopamine neuron system in ovariectomized rats. *Journal of Reproductive Medicine, 11,* 140–144.