Protective Effect of Vitamin E Against Ethanol-Induced Hyperhomocysteinemia, DNA Damage, and Atrophy in the Developing Male Rat Brain

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Background: Chronic alcoholism leads to elevated plasma and brain homocysteine (Hcy) levels, as demonstrated by clinical investigations and animal experiments. It has been posited that elevated levels of Hcy mediate DNA damage, brain atrophy, and excitotoxicity. The current study sought to elucidate the effect of vitamin E on ethanol-induced hyperhomocysteinemia, DNA damage, and atrophy in the developing hippocampus and cerebellum of rats.

Methods: Pregnant Wistar rats received ethanol with or without vitamin E from gestation day 7 throughout lactation. Weight changes in the brain, hippocampus and cerebellum, DNA damage, and Hcy levels in the plasma, hippocampus, and cerebellum of male offspring were measured at the end of lactation.

Results: The results revealed that along with a significant decrease in brain, cerebellum, and hippocampus weights in animals that received alcohol, the levels of DNA damage and Hcy significantly increased. Significant amelioration of brain atrophy and DNA damage as well as restoration of the elevated level of Hcy to that of controls were found in vitamin E-treated rats.

Conclusions: These findings strongly support the idea that ethanol intake by dams during pregnancy and lactation induces Hcy-mediated oxidative stress in the developing hippocampus and cerebellum of offspring rats, and that these effects can be alleviated by vitamin E as an antioxidant.

Key Words: Fetal Alcohol Spectrum Disorders, Developing Brain, Homocysteine, DNA Damage, Vitamin E.
before or during alcohol exposure may protect the developing fetus against the teratogenic effects of alcohol.

The current study aimed to investigate the possible hyperhomocysteinemia mediated in utero and preweaning ethanol exposure-induced oxidative DNA damage and brain atrophy in the developing hippocampus and cerebellum of rats and neuroprotective and ameliorative effects of vitamin E.

MATERIALS AND METHODS

Subjects

All procedures utilizing rats were performed according to the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985), as well as the specific rules provided by the "Animal Care and Use Committee," National Medical and Health Service. Adult female Wistar rats weighing 220 ± 20 g were maintained under standard laboratory conditions, with a 12-hour light/dark cycle and food and water provided ad libitum. The rats were then mated with males previously tested as fertile, and were checked for the presence of vaginal plugs the next morning. The presence of a vaginal plug was considered to be indicative of conception and was designated as day 0 of gestation (GD0). On day GD7 of gestation, pregnant rats were randomly divided into 3 groups: (i) control, (ii) ethanol, and (iii) ethanol–vitamin E groups.

Treatment Procedure

The control group was treated with vehicle only (normal sterile saline). Rats assigned to the alcohol group received 4.5 g/kg of ethanol (Merck KGaA, Darmstadt, Germany) solution in saline (20% w/v) subcutaneously once per day. In the ethanol–vitamin E group, each rat received 300 mg of water-miscible vitamin E (Merck KGaA, Darmstadt) in 20 ml of tap water in addition to ethanol. Regular daily diet, i.e., food and water were provided ad libitum. Treatment was initiated on GD7 and continued through postnatal (PN) day 21 (lactation period).

Sample Collection

Following birth, litters were culled to 3 or 4 male pups/mother to decrease possible nutritional deficiencies due to competition within litters. Because of important sex differences in response to ethanol (Barron et al., 2008; Kono et al., 2000; Sayal et al., 2007), only male pups were used in the present study. After 35 days (14 prenatal and 21 PN days) of dam treatment, 8 male pups from each group were anesthetized with ether. Pups were first weighed, and then the thoracic cavity was opened. Blood samples were collected directly from the hearts of litters with a syringe, mixed with ethylenediamine tetraacetic acid (EDTA) as an anticoagulant, and centrifuged at 10,000 g for 10 minutes. The plasma was then separated and stored at −20°C until analysis.

Pups were sacrificed by decapitation and the brain was immediately removed to an ice-cold surface and weighed. The hippocampus and cerebellum were dissected from the brain and weighed on a digital balance with 0.0001 g sensitivity. The unilateral hippocampus and cerebellum from each pup was homogenized in 10 mM Tris–HCl (pH 7.4), 5 mM EDTA. Two-hundred micro liters of plasma or tissue homogenates were subjected to acid deproteinization with an equal volume of perchloric acid (7%), and the mixture was centrifuged at 10,000g for 20 min at 4°C. The remaining parts of the hippocampus and cerebellum were immediately frozen using liquid nitrogen and stored at −80°C.

The levels of Hcy in the serum and tissues of the subjects were measured by means of a high-pressure liquid chromatography method using the ClinRep® complete Kit (Recipe Chemical and Instruments GmBH, Munich, Germany), as described by the manufacturer. The flow rate was 1.0 ml/min and the column temperature was 30°C. The retention times obtained for cysteine, internal standard, cysteinylglycine, and Hcy were 2.07, 2.41, 2.76, and 3.29 minutes, respectively. The fluorescence detector was set at 385 and 515 nm excitation and emission wavelengths, respectively. Twenty milliliters of sample or calibrator (ClinCal® and ClinChcek®, Recipe Chemical and Instruments GmBH) was injected. Calculation of unknown samples was performed using the internal standard method via peak areas.

For the comet assay, small pieces of tissue were washed with an excess volume of ice-cold Hank’s balanced salt solution (HBSS), and then quickly minced into approximately 1–3 mm pieces with a pair of stainless steel scissors while immersed in HBSS. After several washes with cold phosphate-buffered saline (PBS), the minced tissues were dispersed into single cells by pipetting and resuspended at 1 × 105 cells/ml in ice-cold 1× PBS (Cu++, and Mg2+ free). The comet assay was performed as described by Singh et al. (1995) using Trevigen’s CometAssay™ kit (AMS Biotechnology GmbH, Wiesbaden, Deutschland) as a simple and effective method to evaluate the DNA damage present in cells.

The principle of the assay was based on the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates at a slower rate and remains within the confines of the nucleoid during current application. Briefly, the cells were adjusted to 105 cell/ml and mixed with molten LM Agarose (at 37°C; AMS Biotechnology GmbH) at a ratio of 1:10 (v/v). Finally, 75 μl of the mixture was immediately transferred onto the CometSlide™ (AMS Biotechnology GmbH), which was placed flat at 4°C in the dark for 30 minutes and immersed in prechilled lysis solution for 30 minutes at 4°C. Slides were immersed in alkaline solution for 60 minutes at room temperature in complete darkness. Eventually, the slides were transferred to a horizontal electrophoresis apparatus and electrophoresis was carried out at a voltage of 1 V/cm (300 mA) for 20 minutes using alkaline electrophoresis solution (pH > 13, 300 mM NaOH, 1 mM EDTA). They were then treated with 70% ethanol for 5 minutes and air-dried. Finally, the slides were stained with SYBR® Green I Staining Solution (AMS Biotechnology GmbH), diluted 1:10,000 (10,000× concentrate in dimethylsulfoxide), and examined by epifluorescence microscopy (fluorescein filter, excitation and emission of 494 and 521 nm, respectively). Three blinded independent observers analyzed 100 cells from each slide, and the percentage of comet-positive cells were expressed as the “mean ± SD”. All cells exhibiting any tail size were considered as comet-positive cells. Hydrogen peroxide-treated cells were used as a positive control.

Data Analyses

ANOVA was used to compare the Hcy levels among the groups. In each test, the data are expressed as the mean ± SEM. p < 0.05 was accepted as statistically significant. The percentage of comet-positive cells was also analyzed by ANOVA and the χ2 test. Tukey’s post hoc test was used to clarify the results obtained by ANOVA.

RESULTS

Weights

Table 1 summarizes the effect of alcohol and vitamin E treatment on several parameters of litters. The body weights of ethanol-treated rats were significantly lower than those of the control group (p < 0.005), although there were no significant differences between the control group and vitamin E-treated group (p < 0.7).
Similarly, a significant decrease in hippocampus and cerebellum weights was observed in the ethanol group compared with the control group ($p < 0.001$), but the weights of these organs in vitamin E-treated animals were found to be similar to those of controls ($p < 0.1$).

In comparison to controls, the brain/body weight ratio in ethanol-treated rats was significantly high ($p < 0.001$); however, no significant differences were obtained in the vitamin E-treated group ($p < 0.9$). The ratios of the hippocampus/body and cerebellum/body weights in the ethanol group were significantly lower than those obtained for control rats ($p < 0.001$), but no significant differences were found between the vitamin E and control groups ($p < 0.9$).

**Homocysteine Levels**

Plasma Hcy concentrations in the ethanol-treated rats were significantly higher than in controls ($p < 0.001$), and administration of vitamin E decreased the elevated levels compared with those exhibited by the control group ($p < 0.15$) (Fig. 1A). As shown in Fig. 1B and 1C, the levels of Hcy in the hippocampus and cerebellum of the ethanol group were also significantly higher than in controls ($p < 0.001$), but no significant differences were detected between the vitamin E and control groups ($p < 0.9$).

**DNA Damage**

As shown in Fig. 2A, the percentage of comet-positive, DNA-damaged cells was significantly increased in the hippocampal and cerebellar tissues of rats that received ethanol ($p < 0.01$). The numbers of comet-positive cells were significantly decreased in the vitamin E-treated rats ($p < 0.01$), but the number of comet-positive cells was still significantly higher than in the control group ($p < 0.05$). Figure 2B shows a comparison of comet-positive cells with comet-negative cells.

**DISCUSSION**

The present study shows that male litters obtained from mothers receiving 4.5 g/kg body weight of ethanol during gestation and lactation displayed a significant decrease in overall body weight as well as in brain, cerebellum, and hippocampus weights. Because of important sex differences in response to ethanol (Barron et al., 2008; Kono et al., 2000; Sayal et al., 2007), only male pups were used in the present study. FASD-related neuronal degenerative changes ranging from minor structural changes in neuronal cell death to the central nervous system (CNS) malfunctions were also demonstrated in several studies (Burke et al., 2009; Fryer et al., 2008; Guerri et al., 2009; Heaton et al., 2003; Jaatinen and Rintala, 2008; Lebel et al., 2008; Livy and Elberger, 2008).

We found that the litters had significantly increased plasma, hippocampus, and cerebellum Hcy concentrations. Similar chronic alcoholism-related hyperhomocysteinemia has only been found in clinical studies (Harper, 1998). Increased levels of Hcy may be considered to trigger a pathological increase in receptor activity and subsequent reactive oxygen species (ROS)-related excitotoxic damage (Matsumoto et al., 2001; Streck et al., 2003).

Alternatively, Hcy may potentiate the toxic effects of ROS via inhibition of the expression and synthesis of antioxidant enzymes or radical scavengers (Badger et al., 1993). Oxidation of its sulfhydryl group resulted in the production of superoxide and hydrogen peroxide (Fryer et al., 2008), which disturbed glutathione synthesis via competitive antagonism of cysteine transport and provoked the generation of free radicals during thiol oxidation (Hegg, 1999). This compromised the activity of glutathione peroxidase and decreased the bioavailability of nitric oxide (Upchurch et al., 1997). It also decreased the reducing ability of the tissues by diminishing the reserves of antioxidants, such as vitamins A, C, and E (Badger et al., 1993).

In the current study, the significant apoptotic DNA damage observed in the hippocampus and cerebellum of developing rats was probably caused by one or several mechanisms mentioned earlier and supported reported Hey-related neurodegeneration with apoptotic and excitotoxic characteristics (Blech et al., 2003). The authors recently reported that oxidative stress played a crucial role in ethanol-induced brain damage (Shirpoor et al., 2009). This was in agreement with previous reports in which ethanol-induced DNA breaks were observed in ethanol-treated cultured rat hepatocytes as well as in rat brain cells following a single oral dose of ethanol (Rajasinghe et al., 1990; Singh et al., 1995).
Immunohistochemical analyses had revealed that Hcy was not distributed uniformly in the brain but accumulated in specific regions, including the cerebellum, hippocampus, and sub-ventricular zone lining the lateral ventricle (Chung et al., 2003). In addition to the high accumulation of Hcy in the hippocampus and cerebellum, these regions of the CNS have also been reported to exhibit low endogenous levels of vitamin E (an important biochemical antioxidant), and are therefore particularly susceptible to oxidative stress (Abel and Hannigan, 1995). This could justify why the developing cerebellum and hippocampus of rats were examined as vulnerable to the consequences of ethanol in the current study. Interestingly, the results of the present study also demonstrated that the hippocampus and cerebellum brain weight ratios in the ethanol group were significantly lower than those assessed in the control group, and revealed their greater susceptibility compared with other parts of the brain.

We found that nutritional supplementation of the ethanol-treated animals with vitamin E restored the reduced weights and elevated Hcy levels compared with those of control rats. Mechanistically, vitamin E is a scavenger of peroxyl radicals and quenches other free radicals, such as singlet O₂, superoxide, and hydroxyl radicals (Davis et al., 1990). Recently, we demonstrated that vitamin E supplementation decreased alcohol-induced oxidative stress and apoptosis in the developing hippocampus and cerebellum (Shirpoor et al., 2009). Since then, alleviation of ethanol-induced neurotoxicity was observed after nutritional supplementation with alpha-lipoic acid, another potent antioxidant (Shirpoor et al., 2008a).

![Fig. 1](image1.png)  
**Fig. 1.** Ethanol significantly increases homocysteine (Hcy) concentrations in the plasma (A), hippocampus (B), and cerebellum (C). Homocysteine concentrations in vitamin E-treated rats were restored to those of controls. “a” significant at $p < 0.001$ with control. “b” significant at $p < 0.001$ with ethanol. Data are presented as group means ± SEM.

![Fig. 2](image2.png)  
**Fig. 2.** (A) Percentage of comet-positive cells in the hippocampus and cerebellum of the different groups. (B) A comparison of comet-positive cells with comet-negative cells is presented, (a: comet-negative, b: comet-positive). “a” significant at $p < 0.01$ with control, “b” significant at $p < 0.01$ with ethanol. Data are presented as group means ± SEM.
Oxidative stress seemed to play a crucial role in ethanol-induced neurotoxicity, most likely via hyperhomocysteinemia.

Although our findings revealed that vitamin E significantly decreased the level of DNA damage in the hippocampus and cerebellum of offspring rats, it was still significantly higher than that observed in controls. For this reason, additional mechanisms further to oxidative stress should be contributed to ethanol-induced DNA damage. Similar findings were also reported for the alleviative effect of vitamin E on oxidative stress-triggered cell death in diabetic cardiomyopathy (Shirypoole et al., 2008b). Further investigation is required to elucidate why vitamin E therapy is not able to restore normal physiologic status.

To our knowledge, this is the first report of a combined in utero and preweaning experiment that indicates hyperhomocysteinemia-induced oxidative stress plays a crucial role in pathogenesis of fetal alcohol syndrome and ameliorates by vitamin E supplementation. However, further research is still required to elucidate the comprehensive details of FASD including fetal alcohol syndrome.

REFERENCES


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