Vitamin E protects developing rat hippocampus and cerebellum against ethanol-induced oxidative stress and apoptosis

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ABSTRACT

It has been suggested that developmental alcohol-induced brain damage is mediated through increases in oxidative stress. Current research suggests that antioxidant therapy may afford some level of protection against the toxicity of alcohol and alcoholic beverage in cellular and genomic levels. Seven day pregnant wistar rats were randomly divided into three groups: control, ethanol and vitamin E treated alcoholic groups. Apoptosis, lipid peroxidation and protein oxidation amounts, as well as, catalase and superoxide dismutase (SOD) activities were compared in male offspring hippocampus and cerebellum in the end of lactation. Offspring from ethanol-exposed animals showed significant increase in apoptosis, protein carbonyl and lipid hydroperoxide (LPO) in hippocampus and cerebellum than controls. Vitamin E treatment significantly decreased apoptosis in ethanol group and restored the increased protein carbonyl and LPO contents and catalase activity to the level of controls. Our findings strongly support oxidative nature of alcohol-induced cellular stress in developing hippocampus and cerebellum, prove that oxidative stress induced cell apoptosis plays a crucial role in pathogenic consequences, and imply that a strong protective effect could be achieved using vitamin E as an antioxidant.

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1. Introduction

Alcoholic beverages are used universally and alcohol is the world's most widely used psychoactive drug, but chronic, excessive alcohol consumption leads to permanent organ damage or death. Alcohol-induced brain damage produces some of the most insidious effects of alcoholism, including cognitive deficits such as learning and memory impairment (Butterfield et al., 1999). The neurotoxic effects of chronic ethanol consumption on the developing and mature nervous system have been well documented in both human beings and experimental animals (Diamond & Messing, 1994; Rouach, Houze, Gentil, Orfanelli, & Nordmann, 1994). The term alcohol-related neurodevelopmental disorder (ARND) is used to describe the central nervous system (CNS) dysfunction that may result from ethanol exposure during pregnancy (Iqbal, Dringenberg, Brien, & Reynolds, 2004). The typical features of alcoholic neuropathology are neuronal loss and apoptosis (Ikegami et al., 2003). While early fetal alcohol research has focused on identifying specific deficits, much of the recent attention has emphasized the underlying mechanisms through which alcohol affects the developing brain, partic-

ularly during the vulnerable period of development known as the brain growth spurt (Ikegami et al., 2003). The mechanism behind ethanol-induced neuronal damage is not well understood, but several explanations have been proposed. These include excitotoxicity associated with excessive neurotransmitter release, oxidative stress leading to free radical damage (Andreoli & Mallett, 1997; Eskay, Chautard, Torda, Daoud, & Hamelink, 1995) and edema caused by alterations in cellular control of ion transport (Crews et al., 2004). Recently, several studies have examined the role of oxidative stress on developmental alcohol-mediated neurotoxicity, possibly via the formation of free radicals (Chen & Sulik, 1996; Collins, Zou, & Neafsey, 1998; Heaton, Mitchell, & Paiva, 2000). Free radicals, and reactive oxygen species (ROS), are generated during oxidative metabolism and can inflict damage on all classes of cellular macromolecules (e.g. mitochondria, endoplasmic reticulum, etc), eventually leading to cell death (Tran, Jackson, Horn, & Goodlett, 2005). Oxidative stress is attractive as a possible mechanism for the alcohol-induced brain damage for many reasons. The brain processes large amounts of O_2 in relatively small mass, and has a high content of substrates available for oxidation (i.e. polyunsaturated fatty acids and catecholamines) in conjunction with low antioxidant activities, making it extremely susceptible to oxidative damage (Bergamini, Gambetti, Dondi, & Cervellati, 2004). The

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developing brain, which has only a fraction of the antioxidant enzyme activity of the adult brain, is perhaps even more vulnerable to the neurotoxic effects of oxidative stress than the adult brain (Bergamini et al., 2004). In addition, certain regions of CNS, such as the hippocampus and cerebellum, may be particularly sensitive to oxidative stress because of their low endogenous levels of vitamin E, an important biochemical antioxidant, relative to other brain regions (Henderson, Chen, & Schenker, 1999). Such a depressed defense system may be adequate under normal circumstances. However, in pro-oxidative conditions, such as during alcohol exposure, these low antioxidant defenses can predispose the fetal brain to oxidative damage. During the metabolism of alcohol, acetaldehyde is formed as the principal metabolite by alcohol dehydrogenase enzyme in the liver. The acetaldehyde must be further oxidized to acetate by acetaldehyde dehydrogenase enzyme, which is present in the brain and is capable of producing reactive oxygen species (Abel & Hannigan, 1995). In addition, alcohol has been shown to cause an increase in the production of nitric oxide (Schlorff, Husain, & Somani, 1999), a potent free radical known to be cytotoxic to neurons and glial cells (Baraona, Zeballos, Shoichet, Mak, & Lieber, 2002). The role of oxidative stress in alcohol-induced neurotoxicity is also supported by studies showing beneficial effects of antioxidant therapy during alcohol exposure (de la Monte & Wands, 2002). It has been reported that the protective capacity of melatonin against ethanol-induced cerebral damage in adult rats where its pretreatment either prevented or greatly ameliorated the damage inflicted by aggressive radical species induced by ethanol (El-Sokkary, Reiter, Tan, Kim, & Cabrera, 1999). Vitamin E is a major antioxidant in biological systems acting as a powerful chain-breaking agent through the scavenging of peroxyl radicals (Beyer, 1994). Vitamin E terminates the chain reaction of lipid peroxidation in membranes and lipoproteins. Thus a number of studies have been carried out of determine the protective effects of vitamin E in different biological models of injury (Ernster & Dallner, 1995). Pathogenic dysfunction of tissues due to cell death via apoptosis is one of the important consequences of oxidative stress that could be diminished using antioxidant such as vitamin E (Shirpoor et al., in press). The objective of this study was to evaluate the potential effect of vitamin E on the attenuation of neuronal apoptosis and oxidative stress in the hippocampus and cerebellum of rat under alcoholic conditions, *in vivo*.

2. Material and methods

2.1. Animals and treatments

All procedures on rats were followed according to "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985), as well as specific rules of "Animal Care and Use Committee", National Medical and Health Service. Female Long-Evans rats were placed with a proven breeder male overnight for breeding. Vaginal smears were done the next morning to check for the presence of sperm. Once sperm was detected, that day was assigned as gestational day 1 (GD). On day 7 of gestation, pregnant rats were randomly divided into three groups namely control, ethanol and ethanol–vitamin E groups. The control group received only vehicle treatment (normal sterile saline). Ethanol and ethanol–vitamin E groups were treated subcutaneously with 4.5 g/kg ethanol (Merck-Darmstadt, Germany) once a day. Ethanol was prepared as a 20% solution in sterile normal saline. Rats in ethanol–vitamin E group received 300 mg vitamin E (Merck-Darmstadt, Germany) in tap water beside ethanol and regular diet daily. The treatment began on GD7 and continued trough 21 day after postnatal (PN) days (lactation period). Following birth, litters culled to 3, 4 male pups/l to decrease possible nutritional deficiencies due to within litter competition. Because of important sex differences in response to ethanol, we used only male pups for this study. After 35 days (14 days prenatal and 21 days postnatal) treatment of dams, 8 male pups from each group, were anesthetized with ether.

2.2. Sample preparation

The pups were killed by decapitation and the brain was immediately removed. The hippocampus and cerebellum were dissected for analyzing the oxidative stress parameters and apoptosis. The part of hippocampus and cerebellum was dissected and fixed in buffered formalin for apoptosis evaluation. The other part of tissue samples was homogenized in 10 mM Tris–HCl (pH 7.4), 5 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 10,000g for 15 min at 4 °C; supernatant were collected and stored at −80 °C until determination of requested parameters.

2.3. Biochemical markers

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Catalase activity was determined in the supernatant using the catalase assay kit (Cayman Chemical, Ann Arbor, USA). The method is based on the reaction of the enzyme with methanol in the presence of optimal concentration (35.2 mM) of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen, at 540 nm (Johansson and Borg, 1988). Catalase activity calculated as nmol/min/ml and converted to the specific activity by dividing to protein content of samples. Tissue superoxide dismutase (SOD) activity was determined using a Cayman assay Kit (Cayman Chemical, Ann Arbor, USA). The method uses tetrazolium salt to quantify superoxide radicals generated by xanthine oxidase and hypoxanthine. The standard curve was generated by using a quality-controlled SOD standard. Protein carbonyl content was measured in the supernatant using a protein carbonyl assay kit (Cayman Chemical, Ann Arbor, USA); briefly, 2,4-dinitrophenylhydrazine (DNPH) reacts with protein carbonyl forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically. Because of the methodology of the assay, the protein carbonyl assay would react with carbonyls from both protein and lipid. To discriminate between the two, the protein carbonyl assay uses a trichloroacetic acid (TCA) precipitation step to precipitate the proteins, which are pelleted by centrifugation, followed by two ethanol/ethyl acetate wash steps that would effectively remove lipids from the protein pellet. So the signal from the assay would only come from protein carbonyls, not from lipid carbonyls. Lipid hydroperoxides (which reflects the degree of lipid peroxidation better than other peroxidation products such as malondialdehyde) was measured by enzyme immunoassay using specific Kits (Cayman Chemical, Ann Arbor, USA). Briefly, the LPO assay is based on redox reactions between hydroperoxides and ferrous ions to produce ferric ions, and the resulting ferric ions are detected using thiocyanate ion as the chromogen. Tissue preparations (500 μl) were mixed with 50 μl chromogen reagent (2.3 mM ferrous sulfate in 0.2 M HCl and 1.5% methanolic solution of ammonium thiocyanate), and 300 μl samples were transferred to a glass 96-well plate, and absorbance was determined at 500 nm. This assay used 13-hydroperoxy-octadeacadienoic acid as a lipid hydroperoxide standard to construct a standard curve (linearity from 0.5 to 5-nmol hydroperoxide).

2.4. Assessment of apoptosis

Tissue sections from formalin fixed paraffin-embedded cerebellum and hippocampus, 5 μm thick, were deparaffinized by

immersing them in xylene, rehydrated, and washed in PBS. Subsequently, the sections were permeabilized using proteinase K (30 μg/ml, 30 min, 37 °C), and were washed in PBS. The terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) reaction performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). An optimized terminal transferase (TdT) was used to label free 3′ OH ends in genomic DNA with fluorescein-dUTP, followed by the detection of incorporated fluorescein with an anti-fluorescein antibody POD conjugate that was visualized by diaminobenzine (DAB) as a substrate. Briefly, the slides were incubated with TUNEL reaction mixture for 60 min (humid chamber, 37 °C), and then were washed twice in PBS. Sections were covered by anti-fluorescein-POD and incubated for 30 min at 37 °C. After multiple washing steps, they visualized by adding diaminobenzine (DAB) for 10 min at room temperature, and then washed in phosphate buffer saline (PBS), counterstained using hematoxylin staining and finally mounted for light microscopic observation. As a positive control, DNase I was used to induce DNA strand breaks. The number of TUNEL positive cardiac myocytes was determined by counting them using 400× microscopic magnifications. The averages of 10 histological sections

were reported as mean ± S.E.M. All morphometric measurements were carried out by at least three independent expert examiners in a blinded manner and expressed in comparison to controls.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the activities of SOD, catalase, LPO and protein carbonyl levels among groups. In each test, the data are expressed as the mean ± S.E.M. and $p < 0.05$ is accepted as statistically significant. The percentage of comet-positive cells were analyzed using the x_2 test and analysis of variance (ANOVA).

3. Results

3.1. Biochemical markers

The protein carbonyl content was increased in both regions of brain in ethanol group compared control ($p < 0.005$), but it diminished to the level of controls in the ethanol–vitamin E group ($p < 0.04$) (Fig. 1a). Level of lipid hydroperoxides (Fig. 1b) was also
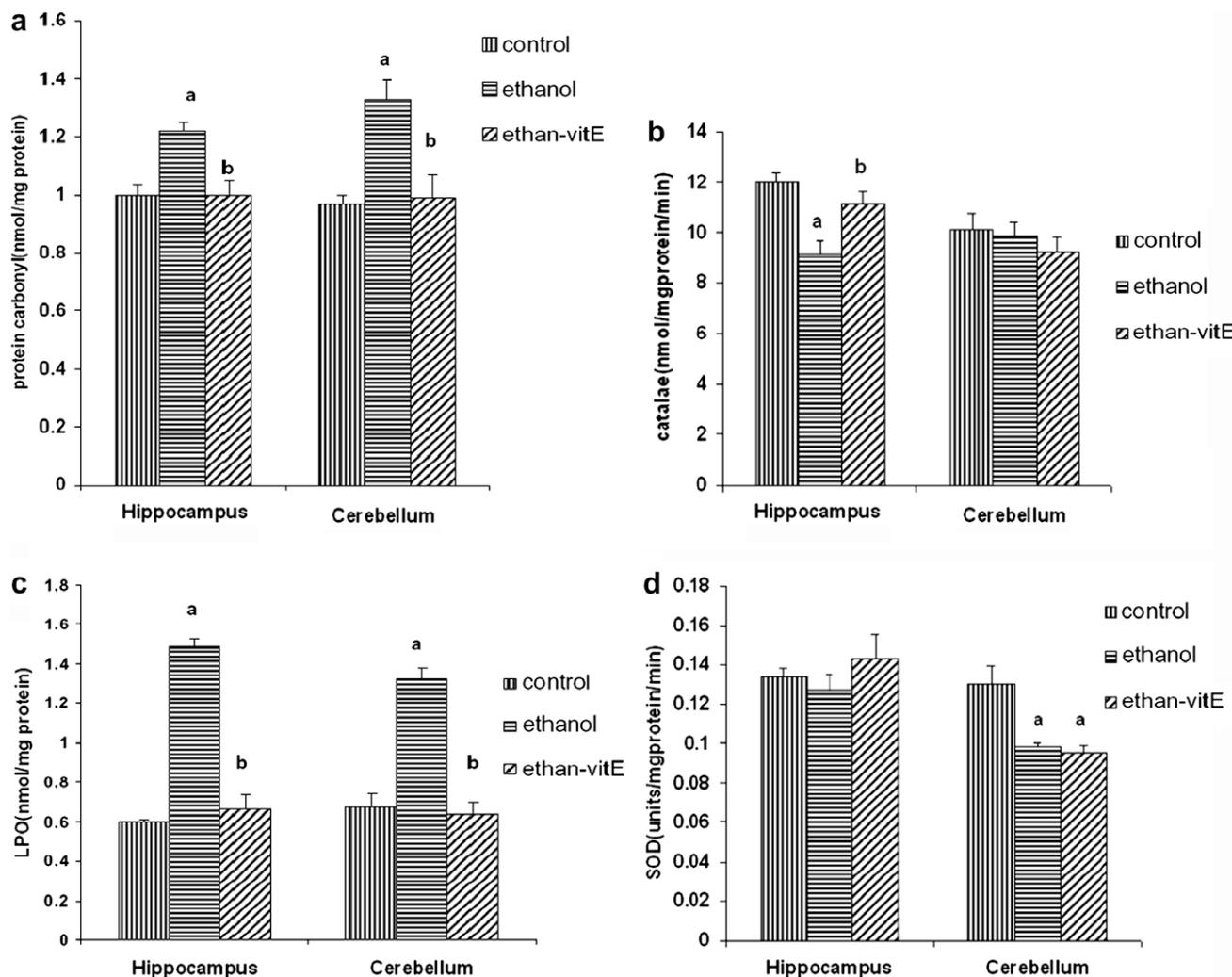

Fig. 1. Status of protein carbonyl content, level of lipid hydroperoxides and activity of catalase and superoxide dismutase in hippocampus and cerebellum of rat brain. (a) Level of protein carbonyl increased in hippocampus and cerebellum of brain in ethanol group, but it diminished to the level of controls in the ethanol–vitamin E group. (b) LPO levels in hippocampus and cerebellum of ethanol group were significantly higher then controls but it decreased in vitamin E treated group. (c) In contrast to cerebellum, catalase activity significantly decreased in hippocampus of ethanol group and restored to normal status in vitamin E treated. (d) In ethanol and ethanol–vitamin E treated groups, the activity of SOD of cerebellum were significantly lower then controls but no significant difference found between them. No significant differences in SOD activity of hippocampal tissues found among groups. Results are presented as mean ± S.E.M. "a" significant at $p < 0.05$ with control. "b" significant at $p < 0.05$ with ethanol.

significantly higher in hippocampus and cerebellum of ethanol group compared to the control group ($p < 0.005$) that decreased to level of controls in ethanol–vitamin E group too ($p < 0.5$). As shown in Fig. 1c, catalase activity was significantly decreased in hippocampus of ethanol group compared to control ($p < 0.009$), but it was restored to normal status in ethanol–vitamin E group ($p < 0.25$). There were no significant differences in catalase activity of cerebellum among control, ethanol and ethanol–vitamin E treated groups ($p < 0.25$). The activity of superoxide dismutase (SOD) of cerebellum were significantly lower in ethanol and ethanol–vitamin E treated groups compared to the control ($p < 0.05$). There was no significant difference between the ethanol–vitamin E treated and ethanol groups ($p < 0.5$). There were insignificant differences in SOD activity of hippocampus among different groups ($p < 0.5$) (Fig. 1d).

3.2. Apoptosis

3.2.1. In cerebellum

In comparison to control rats, apoptotic cells were significantly increased in alcohol receiving rats. The numbers of apoptotic cells in Vitamin E treated rats were significantly lower then alcohol receiving group (Fig. 2a). DNase I treated section was used as positive control. Slides were counterstained with hematoxylin and examined using 100× and 400× microscopic magnification. For the quantitative measurement of the number of cells that underwent apoptosis, 100 cells counted randomly in these different

areas and the percentage of the apoptosis cells calculated. As shown in Fig. 2a, compared to the granular and molecular cells, Purkinje cells were significantly resistant to the alcohol-induced apoptosis ($p < 0.05$).

3.2.2. In hippocampus

Apoptotic cells spread in all hippocampal parts, including DG, CA1, CA2 and CA3 of alcohol receiving rats were significantly higher then control rats. Treatment with vitamin E significantly reduces apoptotic cell death in all parts of hippocampus compared with alcohol receiving group. The percentage of apoptotic cells in different parts of hippocampus among different groups presented in Fig. 2b.

4. Discussion

Epidemiologic studies indicate that fetal alcohol syndrome is a major public health problem that concerns all industrialized countries (Cornelius, Goldshmidt, Taylor, & Day, 1999). Neuronal vulnerability to ethanol coincides with a period of intense synaptogenesis that, in human, starts during the third trimester of pregnancy (Ikonomidou et al., 2000). However, the mechanisms underlying the deleterious effects of ethanol on the developing brain remain largely unknown and no efficient treatment is currently available. The major findings in this study are firstly, maternal ethanol intake during gestation and lactation induce lipid peroxidation and protein oxidation in developing hippocampus and cerebellum in litters that give rise to apoptosis as an important cause of brain dysfunction. Secondly, vitamin E treatment coincide alcohol intake, alleviated apoptosis and oxidative stress induced by alcohol in hippocampus and cerebellum of offspring's. Although a limited number of studies have shown maternal alcohol intake-induced alterations in some measures of oxidative stress in the developing brain, such as lipid peroxidation and oxidative DNA damage, this model of study is the first in vivo study to show that alcohol exposure during pregnancy increases protein carbonyl formation in the developing hippocampus and cerebellum. Since, the methodology of the assay discriminates between the carbonyls from protein and lipid, the signal from the assay would only come from protein carbonyls, not from lipid carbonyls. In fact, alcohol-induced oxidative stress in the developing hippocampus, cerebellum has been reported in only a few studies such as current study, to best of our knowledge, this is the only one that reveals coincidence of oxidative stress and apoptosis in the developing hippocampus and cerebellum. A few clinical studies have shown increases in protein carbonyls in blood serum and plasma from human alcoholics (Grattagliano, Vendemiale, Sabba, Buonamico, & Altomare, 1996; Mutlu-Turkoglu et al., 2000). Increased protein carbonyls have also been in the hippocampus of neonatal rats exposed to alcohol actually (Marino, Aksenov, & Kelly, 2004). In addition, we have also shown that maternal alcohol exposure caused an increase in LPO levels in developing hippocampus and cerebellum. Recently, lipid peroxidation and protein oxidation has become the subject of interest and has been investigated as markers of oxidative damage in vitro, in vivo, and in human studies (Orhan et al., 2004; Salonen, 2000). It has been suggested that ethanol may cause tissue damage through lipid peroxidation (Mi, Mak, & Lieber, 2000). Ethanol can enhance reactive oxygen species formation through induction of cytochrome p4502E1 (CYP2E1), which is widely distributed in the brain (Montoliu et al., 1995). It has been reported that the metabolic activation of high concentration of alcohol are mediated predominantly by the microsomal ethanol oxidizing system (MEOS), because this cytochrome has a high Km value for ethanol oxidation (Montoliu et al., 1995). Elevated levels of reactive oxyradicals caused by ethanol may also arise as
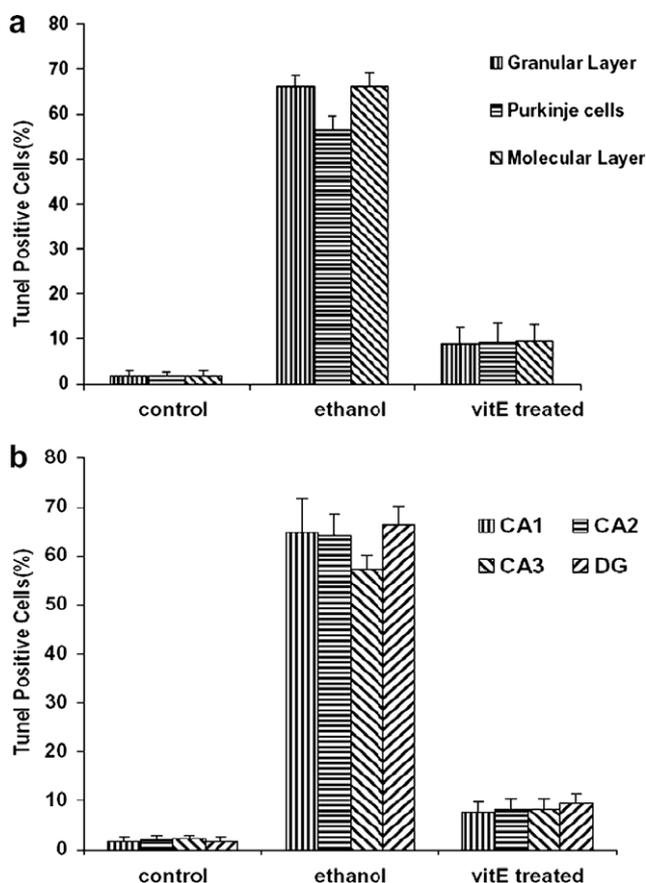
Fig. 2. Status of TUNEL positive apoptotic nuclei in cerebellar and hippocampal section of pups. (a) In cerebellum, the number of TUNEL positive neurocytes in ethanol group was significantly higher than controls and a significant decrease of apoptotic cells found after treatment with vitamin E. (b) In hippocampus, the number of TUNEL positive neurocytes in ethanol group was also significantly higher than controls. Treatment with vitamin E decreased the number of apoptotic neurons of all regions of hippocampus. Results shown are mean ± S.E.M.

a consequence of an increased in the NADH/NAN⁺ redox ratio or as a result of the release of iron (Kukielka & Cederbaum, 1994), which has also been associated with an increased cellular NADH/NAN⁺ ratio (Singh, Lai, & Khan, 1995). Chronic ethanol administration has been shown to decrease antioxidant enzymes, such as glutathione peroxidase, and alter glutathione homeostasis (Coleman, Eason, & Bailey, 2001). In the current study, we found that vitamin E treatment alleviated the increase in protein carbonyl and LPO formation associated with ethanol exposure. Although a few studies exist on the effect of antioxidant treatment on alcohol-induced protein carbonyl formation (or alcohol-induced oxidative stress in general), our previous study and others have shown that vitamin E treatment decrease the production of protein carbonyls in small intestine (Shirpoor, Ansari, Salami, Pakdel, & Rasmi, 2007), liver (Swierczynski & Mayer, 1998) and plasma (Lee, Lee, & Kim, 1998) in response to various oxidative stressor. Thus, current results support the antioxidant role that has been attributed to vitamin E by previous studies. This effect of vitamin E may be related to its antioxidative property, which consequently holds back accumulation of free radicals or other toxic materials and induction of neurodegeneration. Data from current study also indicate that ethanol-induced massive apoptosis in developing hippocampus and cerebellum. Previous studies have suggested that ethanol disrupts brain development in both rodent and humans by apoptotic deletion of neurons (Dikranian et al., 2001; Ikonomidou et al., 2000; Olney et al., 2002). While the cytotoxic effects of ethanol on brain tissue are not entirely understood, three possibilities could be considered (Renis et al., 1996). Firstly, ethanol can exert its cytotoxic effects through its lipid soluble properties and hence displays its biological effects by physical action as a denaturing or disaggregating agent in cellular macromolecular organization. Secondly, the cytotoxic effects of ethanol are linked to its metabolic fate and are most probably mediated by oxygen-dependent generation of free radicals. These free radicals may directly react with vital cell constituents, or may be transformed into species that are more reactive. It is generally recognized that ethanol oxidation results in a burst of reduced pyridine nucleotides, NADH/NADPH. Increased reducing equivalents stimulate electron transport to oxygen, which predisposes the cell to increase the formation of oxygen free radicals. There is considerable evidence that free radicals may be formed during cell respiration, and when the rate of electron transport is increased, there is an increase in the formation of these molecules (Turrens, Freeman, Levitt, & Crapo, 1982). Furthermore, the cytotoxic effects of ethanol result from a combined influence of its physical, chemical and metabolic properties. During heightened metabolic activity, the cellular membranes that is more vulnerable to the oxidizing effects of free radicals. Since treatment with vitamin E significantly decreased apoptosis compared to ethanol group, the results of the current study support the latter possibility but it also imply that additional mechanism beside oxidative stress for ethanol-induced apoptosis should be contributed in the disorders. Although, a significant decrease of cerebellum SOD activities was found in ethanol group, SOD activity of the hippocampus was not changed in either ethanol or vitamin E treated groups. Under physiological conditions, SOD is an important intracellular antioxidant which catalyses the conversion of the superoxide anion radical to molecular oxygen and hydrogen peroxide (H_2O_2) and thus protects against superoxide-induced damage (Hunt, Smith, & Wolff, 1990). Catalase activities showed significant decrease in hippocampus of ethanol group compared control and vitamin E treatment normalized catalase activity compared control. Catalase activity did not change in cerebellum of different groups. However, the antioxidant mechanism fails due to overproduction of free radicals or decreased activities of scavenging enzymes, or both causing lipid peroxidation.

In conclusion, our results clearly demonstrated that oxidative stress play a crucial role in alcohol-induced brain damage, mainly by induction of apoptosis and administration of vitamin E in gestation and lactation periods alleviate oxidative stress via decreasing protein oxidation and lipid peroxidation. Consequently, it dramatically decreases ethanol-induced apoptosis in developing hippocampus and cerebellum. To our knowledge, this is the first report of an *in vivo* demonstration of such an effect. Further experimental and/or clinical studies will need to be performed to confirm such effects, including therapeutic window studies.

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