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Neuroprotective effects of the 17β-estradiol against ethanol-induced neurotoxicity and oxidative stress in the developing male rat cerebellum: Biochemical, histological and behavioral changes

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ABSTRACT

During particular periods of central nervous system (CNS) development, exposure to ethanol can decrease regional brain growth and can result in selective loss of neurons. Unfortunately, there are few effective means of attenuating damage in the immature brain. In this study, the possible antioxidant and neuroprotective properties of 17β-estradiol against ethanol-induced neurotoxicity was investigated. 17β-estradiol (600 μg/kg) was injected subcutaneously in postnatal day (PD) 4 and 5, 30 min prior to intraperitoneal injection of ethanol (6 g/kg) in rat pups. Ninety minutes after injection of ethanol, the activities of several antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) in vermis of cerebellum were assayed. Thiobarbituric acid reactive substance (TBARS) levels were also measured as a marker of lipid peroxidation. Behavioral studies, including rotarod and locomotor activity tests were performed in PD 21–23 and histological study was performed after completion of behavioral measurements in postnatal day 23. The results of the present work demonstrated that ethanol could induce lipid peroxidation, increase TBARS levels and decrease glutathione peroxidase levels in pup cerebellum. We also observed that ethanol impaired performance on the rotarod and locomotor activities of rat pups. However, treatment with 17β-estradiol significantly attenuated motoric impairment, the lipid peroxidation process and restored the levels of antioxidants. Histological analysis also indicated that ethanol could decrease vermis Purkinje cell count and 17β-estradiol prevented this toxic effect. These results suggest that ethanol may induce lipid peroxidation in the rat pups cerebellum while treatment with 17β-estradiol improves motor deficits by protecting the cerebellum against ethanol toxicity.

1. Introduction

Ethanol exposure is a consistent and reliable producer of neuronal toxicity, particularly when the exposure occurs during periods of enhanced neuronal vulnerability (Light et al., 2002). In fact, according to some authors (Thomas et al., 1998; Tran et al., 2005; Roebuck et al., 1998) the cerebellum is one of the brain parts that are strongly influenced during the early postnatal period, a critical period of life. Because rat pups are born with a relatively undeveloped brain, especially immature cerebellum, prenatal perturbation dramatically affects cerebellar development (Hasebe et al., 2008). Therefore, the neonatal rat cerebellum would be an excellent model to investigate development and maturation disturbances.

There are critical periods of vulnerability when the developing CNS is particularly sensitive to environmental insult, including ethanol exposure (Taranukhin et al., 2010; Rice and Baron, 2000). During particular periods of CNS development, ethanol can decrease regional brain growth and can produce selective loss of neurons (Taranukhin et al., 2010). A defined period of Purkinje cell vulnerability occurs during PN4-6 in the rat during which the peak Blood ethanol concentration (BEC) determines the extent of Purkinje cell loss (Heaton et al., 2002; Light et al., 2002; Pierce et al., 2010).

A variety of mechanisms have been proposed for ethanol neurotoxicity; it is generally accepted that oxidative stress is a major one (Crews and Nixon, 2009; Haorah et al., 2008). Ethanol readily crosses the blood–brain barrier and is metabolized in the brain by enzymes, such as catalase, alcohol dehydrogenase, or ethanol-inducible cytochrome P450. This process produces reactive oxygen species (ROS) which includes superoxide free radicals, hydrogen peroxide, and hydroxyl radicals (Haorah et al., 2008). Disturbance of cellular normal redox state by excessive ROS leads to oxidative stress which causes cellular damage (Hampton and Orrenius, 1998). The

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CNS is particularly susceptible to oxidative stress due to its high oxygen consumption rate, elevated levels of polyunsaturated fatty acids, and relatively low content of antioxidative enzymes (Cohen-Kerem and Koren, 2003). Tremendous research efforts has been made to identify potential neuroprotective agents that can ameliorate ethanol-induced developmental CNS damage, so targeting ethanol-induced ROS and oxidative stress would be a logic preventative approach.

It has been well-established that estrogens influence memory and cognition (Bimonte-Nelson et al., 2006), decrease the risk and/or delay the onset of neurological diseases (e.g., AD and PD), and attenuate the extent of cell death that results from a variety of insults involving ROS-induced oxidative stress implicated among others in stroke or neurotrauma (Simpkins et al., 2004; 2005). There has been mounting evidence that estrogens enhance cell survival by suppressing the neurotoxic stimuli partly via their antioxidant activity (Niki and Nakano, 1990; Ayres et al., 1996). Free radical scavenging is believed to be a significant contributor to the rapid estrogen receptor-independent neuroprotective actions of estrogens and related compounds (Moorsmann and Behl, 1999). While these studies represent a broad range of actions for 17β-estradiol, the protective effects of 17β-estradiol against injury have not fully been explored in the developing cerebellum. Therefore, the present study investigates the neuroprotective effect of 17β-estradiol against ethanol induced toxicity in Purkinje cells using behavioral studies, as well as biochemical and histological analysis to arrive at a conclusion.

2. Materials and methods

2.1. Drugs and chemicals

17β-estradiol, sesame oil, absolute ethanol, 2-Thiobarbituric acid (TBA), 1.1.3.3-tetramethoxypropan, nitro blue tetrazolium(NBT), trichloro acetic acid (TCA), cresyl violet, xylene, formaldehyde were all purchased from Sigma-Aldrich, Germany.

2.2. Animals

The experimental protocol was approved by the Research and Ethics Committee of Damghan University. Animals were kept under standard laboratory conditions with a 12-h light/dark cycle and ad libitum food and water throughout the experiments. Evidence of pregnancy was determined by the observation of sperm in vaginal samples taken from the female rats on the morning following pairing with a male. If sperm were detected on the slide, the female was moved to individual housing until delivery. The day sperm were detected was considered gestational day (GD) 0 and delivery was expected 21 days later. The pregnant rats were monitored carefully from GD21-22; only subjects born on GD21 were used in the study. Male rat pups 4-day-old derived from timed mating of adult Wistar rats served as subjects in this study.

2.3. Drug-preparation and administration

In this study, experiments were conducted on five group (a) control, (b) ethanol (6 g/kg, i.p.), (c) 17β-estradiol (600 μg/kg, s.c.), (d) sesame oil and (e) 600 μg/kg 17β-estradiol in combination with ethanol.

Pups were separated from their mothers four days after birth and placed on a heated pad to avoid a drop in body temperature. Ethanol was mixed with saline as a 35% (w/v) solution and administered intraperitoneally at a dose of 6 g/kg. The ethanol dosage of 6 g/kg was chosen to ensure significant Purkinje cell loss, thereby revealing the protective effect of estradiol (Light et al., 2002; Lee et al., 2008). This dose of ethanol has been shown to trigger a robust cell death on postnatal day 4 in rat pups (Light et al., 2002). Control animals received intraperitoneal (i.p.) injection of saline (0.9% w/v, administered in equal volumes as ethanol-treated pups) in PD 4. 17β-estradiol was dissolved in sesame oil and administrated subcutaneously 30 min before ethanol injection. 17β-estradiol dosage was selected on the basis of earlier reports which have demonstrated its neuroprotective effects in rat pups (Asimiadou, et al., 2005). For biochemical analysis, pups were killed 90 min after the alcohol treatment. Behavioral studies, including rotarod and locomotor activity tests were performed in PD 21–23 and histological study after completion of behavioral measurements in postnatal day 23.

2.4. Blood ethanol concentration (BEC)

All blood samples were taken at 90 min following the injection of the ethanol. Twenty microliters of blood was taken from each subject by producing a small nick to the tip of the tail. The samples were immediately placed into individual glass vials containing 200 μL of a cocktail composed of 0.6 N perchloric acid and 4 mM propanol in double distilled water, tightly fastened with a septum sealed lid, and stored at room temperature until analysis by head space gas chromatography (Model 3900, Varian, Palo Alto, CA) (Lee et al., 2008).

2.5. Biochemical analysis of cerebellar homogenate

At the end of the treatment, pups were sacrificed by decapitation and cerebellums were removed. Vermis was dissected on an ice-cold surface and homogenized in cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% homogenate (w/v). The homogenates were then centrifuged at 6000×*g* for 10 min at 4 °C to remove nuclei and debris. The supernatants were separated, aliquoted, and stored at −80 °C until analysis (Enache et al., 2008).

2.5.1. Determination of TBARS levels

Thiobarbituric acid-reactive substances (TBA-RS) were measured according to Ohkawa et al. (1979). Briefly, to glass tubes were added, in order of appearance: 500 μL of sample, 50 μL of sodium dodecyl sulfate 8.1%, 1500 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5, 1500 μL of 0.8% thiobarbituric acid and 700 μL of distilled water. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. The mixture was allowed to cool on water for 5 min and was centrifuged 750 *g* for 10 min. The resulting pink stained TBA-RS were determined in a spectrophotometer at 532 nm. TBA-RS were calculated as nmol/mg protein. A calibration curve was performed using 1, 1, 3, 3-tetramethoxypropane as a standard. TBA-RS were represented as nM TBA-RS/mg protein.

2.5.2. Determination of SOD activity

Total SOD activity was assayed according to Becana et al. (1986) following the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture contained 50 mM Na-phosphate (pH 7.8), 0.1 mM EDTA, 14.3 mM methionine, 82.5 μM NBT and 2.2 μM riboflavin. The reaction was initiated by placing the test tubes under 15 W fluorescent lamps. It was terminated after 10 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. The reaction products were measured at 560 nm. One unit of SOD (U) was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under assay condition.

2.5.3. Determination of CAT activity

CAT activity was assayed by the method of Aebi (1984) using spectrophotometry. This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer pH 7.0. CAT activity is represented as absorption change in time unit (1 min) per mg protein.

2.5.4. Determination of GPx activity

GPx activity was measured according to the method of Wendel (1981) using tertbutyl hydroperoxide as substrate. NADPH disappearance was monitored at 340 nm using a spectrophotometer. The reaction medium contained 2 mM glutathione, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide and 0.1 mM NADPH. GPx activity is represented as absorption change in time unit (1 min) per mg protein.

2.5.5. Determination of protein concentration

Protein concentration was determined in cerebellar cortex homogenates using bovine serum albumin as a standard (Lowry et al., 1951).

2.6. Behavioral study

The effects of ethanol alone or in combination with 17β-estradiol on the pups' behavior were studied by rotarod test. Pups' locomotor activities were also studied by open field apparatus. All experiments started 17 days after the ethanol and 17β-estradiol injections in pups.

2.6.1. Open field test

Pups were individually placed at the center of a clean open field apparatus (40×40×15 cm, divided into nine squares). Prior to the evaluation, animals were habituated to the box for 1 min within the box. The observed parameter, number of squares crossed (locomotor activity) was recorded for 5 min by two blind observers (Burger et al., 2005).

2.6.2. Accelerating rotarod assay: a motor performance test

Rotarod test, which measures balance, coordination, and motor control, was used to evaluate motor performance. The rotarod apparatus (Hugo Sachs Electronik, Germany) consists of a suspended rod able to run at constant or at accelerating speed. Each pup was placed on a rod (8.9 cm long and 3.8 cm in diameter) covered with rubber to evaluate rotarod performance. The accelerating rotarod set to accelerate gradually from 4 to 40 rpm for each trial. The starting speed was 4 rpm, and the total time of each trial was 300 s. On a given trial, four pups were placed on the cylinder, one pup in each compartment. The cylinder was made to rotate, and a timer switch was simultaneously activated. Acceleration continued until either 40 rpm was reached or the last animal was unable to perform the running response and had fallen to the padded surface. When the rat landed on the surface, a switch was tripped and the timer for that compartment stopped. The elapsed time was recorded in tenths of a second as the measure of performance on each trial.

This test was begun in postnatal day 21 so pups received two sessions daily for 3 days for the maximum six sessions. The elapsed time of the first session was calculated separately as an initial motoric capacity before the task learning occurred in subsequent sessions. Each session consisted of four trials, with an interval of 10 min between successive trials and a minimum of 2 h between the two successive daily sessions.

2.7. Cerebellum histological analysis

After completion of behavioral measurements in postnatal day 23 the pups from control and treatment groups were sacrificed by deep anesthesia with ether. Transcardial perfusion was performed with physiological saline followed by fixing with 10% paraformaldehyde solution dissolved in 0.1% phosphate buffer solution. The animals were decapitated and the cerebellums were removed and immersed in 10% buffered paraformaldehyde for a week. Then, the block of cerebellum was immersed in 30% sucrose-buffer solution overnight. Mid-sagittal sections (10 μm thick) at the vermal level were cut using cryostat (Leica, Germany). The sections were subsequently stained with cresyl violet (0.1%). All Purkinje cells were counted in cerebellar lobules of I–III, using light microscopy at 40× magnification and digital image analysis (McGoey et al., 2003).

For each pup, the density of Purkinje cells was determined for each of the 3 cerebellar lobules (I–III) of each section, and then the average value for the 3 lobules was calculated for each section. The analyst was unaware of the experimental treatment. Purkinje cell density was expressed as the average value of the cell number per millimeter of tissue length.

2.8. Data analysis

The data of behavioral, biochemical and histological studies were expressed as Mean ± SEM (standard error of mean) and analyzed using one-way analysis of variance (ANOVA). When analysis of variance showed a significant difference, post hoc Tukey HSD test were applied to demonstrate the difference in behavioral and histological experiments. In biochemical study, post hoc LSD test was used to demonstrate the difference. P-value less than 0.05 ($P<0.05$) was considered to be statistically significant.

3. Results

3.1. Blood ethanol concentration

Student's *t*-test (ethanol vs ethanol + estradiol groups) performed on BEC data showed no significant difference in BEC between two groups receiving ethanol treatment. The mean BECs for the ethanol and estradiol + ethanol groups were 322 (±29) and 351 (±25) mg/dL, respectively.

3.2. Effects of estradiol on lipid peroxidation levels and antioxidant enzyme activities

Administration of ethanol significantly increased TBARS levels in the cerebellum compared to control pups (Fig. 1A, $P<0.01$). Estradiol treatment caused a significant decrease in the cerebellum TBARS levels when compared to the ethanol-treated group ($P<0.05$). Ethanol-treated group demonstrated significant reduction in the cerebellum GPx activity (Fig. 1B, $P<0.01$), as compared to the control group. Estradiol + ethanol treated pup showed a significant increase in the cerebellum GPx and CAT activities as compared to the ethanol-treated group (Fig. 1B and C, $P<0.05$).

3.3. Behavioral studies

3.3.1. Effect of estradiol on initial motoric capacity in ethanol-treated pups

Ethanol-treated group had a significantly shorter latency on rotarod than that observed for the control group ($P<0.01$). Estradiol treatment caused a significant increase in initial motoric capacity when compared to the ethanol-treated group ($P<0.001$) but it was not significantly different from the latency observed for the control group (Fig. 2).

3.3.2. Effect of estradiol on subsequent latencies to fall from the rotarod in ethanol-treated pups

The latency to fall from the rotarod gradually increased in all four groups as a function of test sessions (Fig. 3). However, the overall latency of the ethanol group was shorter than that of the control group ($P<0.01$ in sessions 1, 2, 3, 4, 6 and $P<0.001$ in session 5).

Treatment with estradiol significantly increased the latency of the estradiol + ethanol treated group at sessions 1, 2, 3 and 4 compared to findings for the ethanol group ($P<0.001$ in sessions 1, 2 and $P<0.01$ in session 3 and $P<0.05$ in session 4). Although estradiol increased the

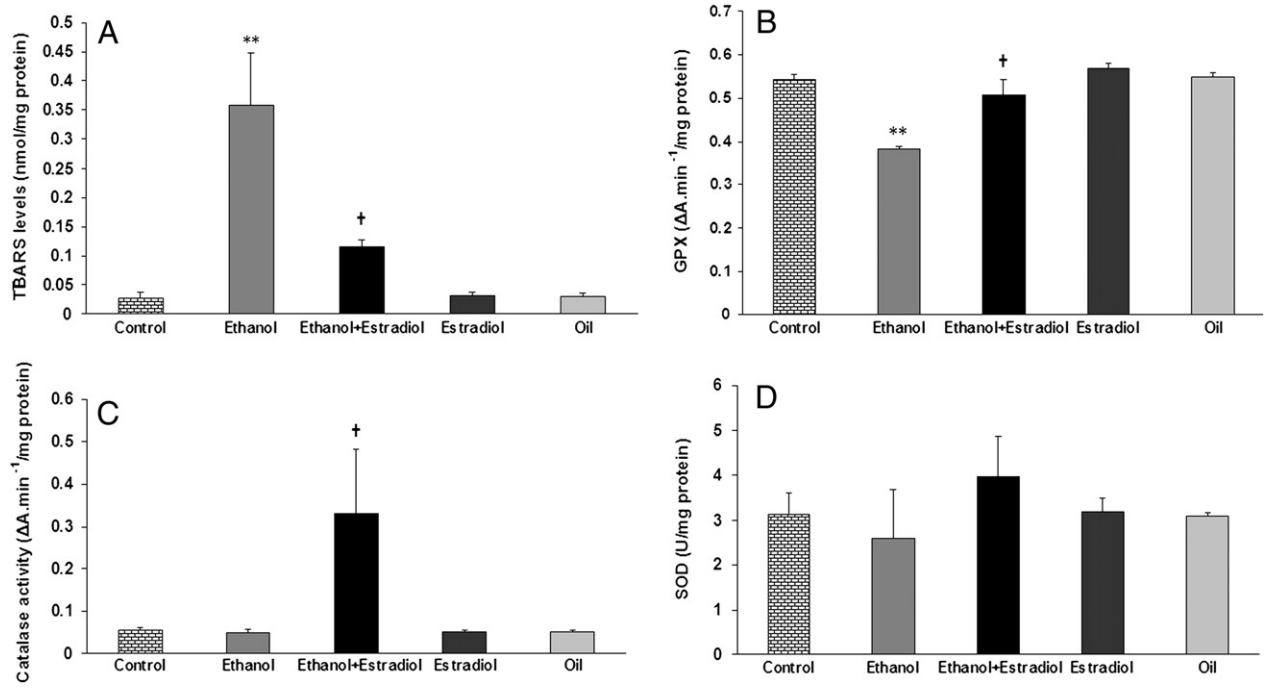

Fig. 1. Effects of estradiol and/or ethanol treatment on activities of CAT, GPx, SOD, and TBARS levels in cerebellar vermis. Administration of ethanol significantly increased TBARS levels as compared to control pups (A, $P<0.01$), whereas estradiol treatment improved it. Pups treated with ethanol exhibited a marked decrease in the activity of GPx (B, $P<0.01$) but estradiol could increase GPx activity (B, $P<0.05$). Activities CAT and SOD showed reduction in ethanol group as compared with control but were not significant (C and D). There were no significant differences in the activities of CAT, GPx, SOD, and TBARS levels between control, estradiol and sesame oil groups. SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase ΔA: Absorption change. Values are shown as means ± SEM of 6–7 animals per group. ** $P \leq 0.01$ compared with control group. † $P \leq 0.05$ compared with ethanol group.

latency of the estradiol + ethanol treated group at sessions 5 and 6 but the difference wasn't statistically significant.

On the other hand, there was significant difference in the rotarod performance between control and ethanol-treated pups (Fig. 3). Ethanol-treated pups stayed on for a shorter time than control pups on rotarod. When two trials were repeated for each day, ethanol-treated pups stayed on for a shorter time than control pups after learning whereas estradiol + ethanol treated group stayed on for a higher time than ethanol-treated pups after learning (Fig. 3).

3.3.3. Effect of estradiol on open field test in ethanol-treated pups

In the open field test ethanol demonstrated statistically significant reduction in the number of crossings in ethanol group (39.05 ± 4.46) compared to control group (61.38 ± 4.41, $P<0.001$) which implies a decrease in locomotor spontaneous activity. Moreover, administration of estradiol along with ethanol improved the performance of the estradiol + ethanol treated pups (52.13 ± 5.44, $P<0.05$, Fig. 4).

3.4. Histological evaluation

3.4.1. Effects of estradiol in ethanol-induced neuronal density

Light microscopy of the lobules I, II and III of cerebellar cortex for control and estradiol + ethanol groups showed a similar pattern in Purkinje cell shape and distribution. Purkinje cells in those groups were round or pear-shaped, and the cells were regularly distributed between the molecular layer (light stain) and the granular layer (dark stain). In contrast, some of the Purkinje cells of the ethanol group were sparse, shrunken, and irregularly shaped. Their Purkinje cell

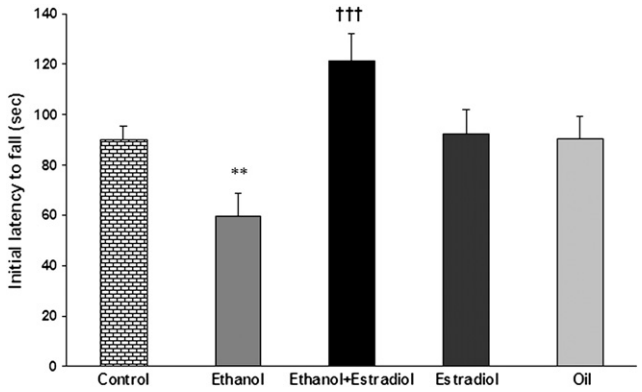

Fig. 2. Effects of estradiol on initial motoric capacity in ethanol-treated pups. 17 days after termination of administration of ethanol, pups were tested for initial latency to fall from the rotarod. Ethanol significantly reduced latency to fall of ethanol treated pups in comparison to control group ($P<0.01$). Post hoc Tukey comparison indicated a longer latency in the estradiol + ethanol group than observed for the ethanol-treated group ($P<0.001$).

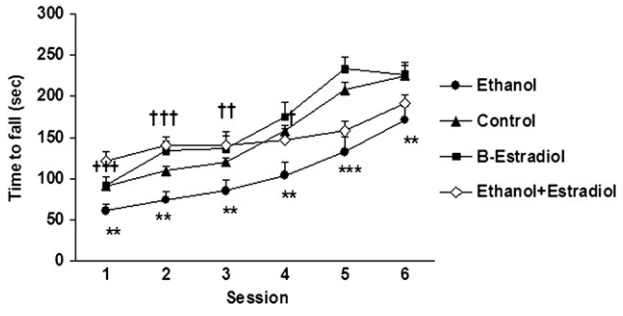

Fig. 3. Effects of estradiol on subsequent latencies to fall from the rotarod in ethanol-treated pups. Motor dysfunction was tested by the rotarod test which measures balance, coordination, and motor control in PD 21–23. Comparison of the rotarod performances between control and ethanol treated pups showed a significant motor impairment in ethanol treated group. In vivo co-treatment with estradiol and ethanol improved motor performance on the rotarod as reflected in a longer time spent walking on rotating drum before falling. Values are mean ± SEM of ten pups. ** $P \leq 0.01$, *** $P \leq 0.001$ compared with control group. †† $P \leq 0.01$, ††† $P \leq 0.001$ compared with ethanol group.

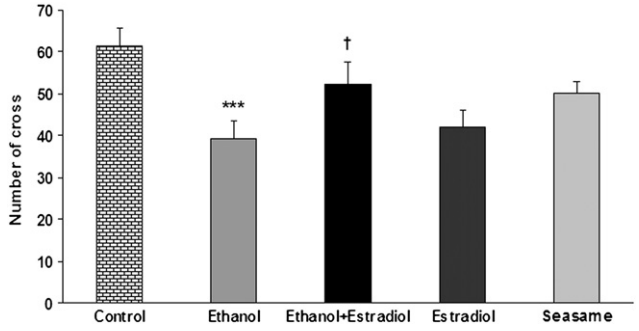

Fig. 4. Effects of estradiol treatment on the locomotor activity in ethanol-treated pups. Ethanol significantly decreased the number of squares crossed whereas estradiol treatment could increase it in estradiol treated pups. There were no significant differences in the number of squares crossed between control, estradiol and sesame oil groups. Values are mean ± SEM of ten pups. *** $P \leq 0.001$ compared with control group. † $P \leq 0.01$ compared with ethanol group.

layers showed a disconnected pattern, with a wide gap between the cells (Fig. 5).

Purkinje cell counted in lobules I, II and III of vermis in several cerebellar mid-sagittal slices from 23-day-old pup. There were 6.55 ± 0.17, 4.2 ± 0.21 and 6.61 ± 0.14 Purkinje cell in 250 μm length of cerebellar lobule I of control, ethanol and estradiol + ethanol groups (Fig. 5D, $P \leq 0.001$, $n = 8$), respectively. Purkinje cell numbers of cerebellar lobule II of control, ethanol and estradiol + ethanol groups were 7.9 ± 0.16, 5.36 ± 0.27 and 7.6 ± 0.14, respectively. Also, Purkinje cell numbers of cerebellar lobule III of control, ethanol and estradiol + ethanol groups were 8.3 ± 0.16, 6.33 ± 0.23 and 7.91 ± 0.13, respectively (Fig. 5E and F, $P \leq 0.001$, $n = 8$). There was no significant difference in the PCs number between control and estradiol + ethanol groups Purkinje cell numbers in cerebellar lobules I, II and III for the pups in estradiol + ethanol groups were significantly higher than those from pups in the ethanol group (Fig. 5D, E and F, $P \leq 0.001$, $n = 8$).

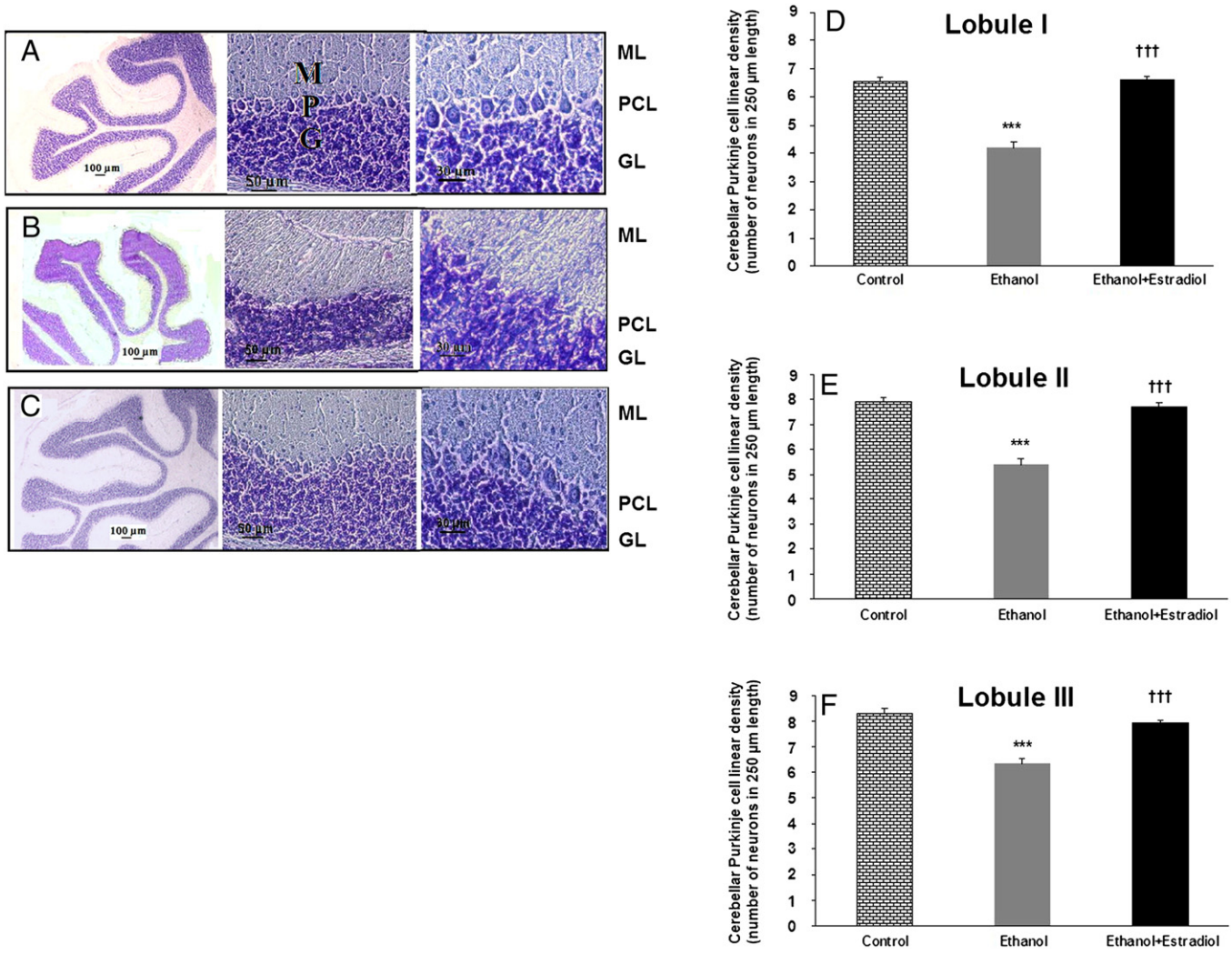

Fig. 5. Neuroprotective effect of estradiol on the cerebellar Purkinje cells following application of ethanol. (A), the spherical cell bodies of Purkinje cells in control pups are aligned nicely between the granular and molecular layers. In ethanol treated pups (B), Purkinje cells were darkly stained and scattered within the Purkinje cell layer and shrinkage of the cell somata is seen. (C) Unlike ethanol treated pups, Purkinje cells from estradiol + ethanol groups treated pups was almost normal, displaying an aligned Purkinje cell layer that was indistinguishable from control and the shape of Purkinje cell somata remained more or less normal and were rescued by neuroprotective action of estradiol. In comparison to controls, the cerebellar Purkinje cell linear density in ethanol-treated pups was significantly low (D, E and F, $P < 0.001$); however, no significant differences were found between the control and estradiol + ethanol treated groups (D, E and F). The Purkinje cell linear density in estradiol + ethanol treated group significantly higher than those obtained for ethanol pups (D, E and F, $P < 0.001$). Data were collected from several histological sections (lobes I–III per section) per pup and five or six pups in each group. ML: molecular layer; PCL: Purkinje cell layer; GL: granular cell layer. *** $P \leq 0.001$ compared with control group. ††† $P \leq 0.001$ compared with ethanol group.

4. Discussion

The idea that ethanol affects the developing brain was apparent from the first modern clinical report describing the offspring of alcohol-abusing women, where a number of brain and behavioral deficits were noted (Jones and Smith, 1973). Experimental evidence demonstrates that alcohol interferes with many molecular, neurochemical and cellular events occurring during the normal development of the brain. Some brain areas are more affected than others and, even within a given region, some cell populations are more vulnerable than others. The neocortex, hippocampus and cerebellum are especially susceptible to alcohol and have been associated with behavioral deficits (Guerri, 2002). Since ethanol produces a wide array of effects, including alterations of coordination, speech, and cognitive function, the cerebellum is an important target of investigation. Purkinje neurons, which are GABAergic, constitute the sole output of the cerebellar cortex, are involved in motor function (Dow and Moruzzi, 1958) and genetic deletions of Purkinje neurons (for example, the Lurcher mouse) can produce motor impairments (Vogel et al., 2007).

The aim of this study was to investigate and clarify the neuroprotective effects of estradiol, an antioxidant agent, against ethanol neurotoxicity in developing cerebellum. Previously Gonenc et al. (2005) demonstrated that melatonin decreased ethanol-induced lipid peroxidation and increased glutathione peroxidase activity in the rat hippocampus. They reported that ROS seem to play an important role in ethanol-induced neurotoxicity and melatonin protects neurons from acute exposure to ethanol-induced oxidative stress in the rat hippocampus. Light et al. (2002) also showed that a single dose of ethanol (a dose of 6.0 g/kg delivered on PN4) resulted in a significant and extensive lobular dependent loss of Purkinje cells within 24 h after administration. Extensive loss of Purkinje cells was observed in the early developing lobules (I–III, VIII–X) while the later developing lobules (IV–VII) showed little or even no Purkinje cells loss.

Biochemical results of the present study indicated that ethanol significantly increased TBARS level (lipid peroxidation) and decreased GPx activities in the pup's cerebellum (Fig. 1A, B). These results are in accord with those of earlier reports that also demonstrated the oxidative stress induced after acute ethanol in the rat brain (Gonenc et al., 2005). Prenatal ethanol exposure has been shown to cause an increase in oxidative stress in developing organs, including the brain (Chu et al., 2007; Heaton et al., 2002). Indeed, even a brief exposure to ethanol during gestation can produce an imbalance in the brain's intracellular redox state (Dong et al., 2010) that can be correlated with behavioral deficits (Vink et al., 2005).

Both human and animal research provide evidence that the CNS is vulnerable to the damaging effects of ethanol during development, and one particular form of damage is neuronal loss. Alcohol exposure during the period of extensive connectivity among Purkinje and other neurons, either from postnatal days (PD) 4–6 (Light et al., 2002; Pierce et al., 1999) or only on PD4 (Goodlett et al., 1990), results in a significant and permanent loss of Purkinje cells. In the present study, the loss of cerebellar Purkinje cells by ethanol was observed. McCray et al. (1976) indicated that glutathione peroxidase activity inhibited lipid peroxidation in membrane, as glutathione peroxidase activity exerted its effect on this system by preventing free radical attack on the polyunsaturated membrane lipids in the first place. Therefore, it seems that ethanol could result in lipid peroxidation and Purkijne cell loss through decrease GPx activity. The consequence of Purkinje cell loss were thought to be involved in the deficiencies of motor coordination and gait exhibited by children diagnosed with fetal alcohol syndrome (Marcus, 1987).

Breton et al. (1998) and Ogura et al. (1980) have assessed abnormal motor function in ethanol treatment group to monitor behavioral manifestations that are known to be associated with cerebellar neuronal damage. Using a rotarod assay, Ogura et al. (1980) were able to demonstrate that rats with defective cerebellum development showed poor rotarod performance. Massive degeneration of cerebellar Purkinje cells induced by neurotoxin of penitrem A was correlated with motor behavior impairment in rats (Breton et al., 1998).

In the present study, motor dysfunction was tested by the rotarod test. We measured initial motor capacity as well as learning capacity of a cerebellar-dependent task by recording latencies of the first session and subsequent sessions, respectively. The initial latency data reflect the unlearned potential motoric capacity, because pups learn training in the rotarod task in the initial session. As expected, poor rotarod performance was observed in ethanol-treated pups. Comparison of the rotarod performances between control and ethanol treated pups showed a significant motor impairment in ethanol treated group in all of sessions. The ethanol group had significantly lower initial latency time than control group, indicating that ethanol could diminish motor coordination before and after learning in pups. The effects of ethanol on the motor coordination were investigated in some of the earlier studies. Valenzuela et al. (2010) reported that ethanol impaired cerebellar functions including balance, posture, motor coordination, and cognition in part, by impairing synaptic plasticity mechanisms at cerebellar Purkinje neurons. It was found that long-term depression at both parallel fiber- and climbing fiber-Purkinje cell synapses were inhibited by acute ethanol exposure. In earlier studies, chronic ethanol treatment resulted in a persistent deficit in the acquisition of a variety of tasks, including water maze learning (Gonenc et al., 2005) and motor coordination tasks (Fehr et al., 1976).

Behavioral tests, such as the open-field task, permit the evaluation of primary motor activity. Previous studies have shown that ethanol exposure could decrease activity levels in animals (Lyvia et al., 2005). The present study established decreased locomotors activities in ethanol-treated pups. The neocortex, hippocampus and cerebellum were especially susceptible to alcohol and have been associated with behavioral deficits (Guerri, 2002).

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 et al., 2004). The 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). Therefore, due to Prooxidant action of ethanol and an antioxidant action of estradiol, we also studied effects of estradiol on antioxidant levels and lipid peroxidation in cerebellum. Our results indicated that estradiol treatment inhibited lipid peroxidation significantly and increased GPx and CAT levels in the ethanol treated pups (Fig. 1B and C). Nonetheless, estradiol also improved ethanol mediated motor coordination deficits and increased locomotors activity in open field tests (Figs. 2–4). Also, histological analysis showed that ethanol induced Purkinje cell loss, were significantly improved by treatment with estradiol (Fig. 5).

Jung and Metzger (2010) showed that 17β-estradiol interferes with the ethanol withdrawal-induced alteration of oxidative signaling pathways and thereby protects neurons, mitochondria, and behaviors. On the other hand, there has been mounting evidence that estrogens enhance cell survival by suppressing the neurotoxic stimuli partly via their antioxidant activity (Niki and Nakano, 1990; Ayres et al., 1996). Free radical scavenging is believed to be a significant contributor to the rapid ER-independent neuroprotective actions of estrogens and related compounds (Moorsmann and Behl, 1999). The antioxidant property of these compounds is due to the free phenolic hydroxyl group on the A-ring of the steroid (Badeau et al., 2005). Estrogens are potent lipid antioxidants, and thus, block membrane oxidation (Gridley et al., 1998). 17β-estradiol treatment reduces the by-

products of lipid peroxidation (Green and Simpkins, 2000) and reduces the oxidation of low-density lipoproteins (Sacks et al., 1994).

GPx is the most important antioxidative enzymes in the brain that metabolizes peroxides such as H_2O_2 protects cell membranes from lipid peroxidation. In our data, estradiol improved the GPx activity that was decreased by ethanol, and therefore, it could inhibit lipid peroxidation in membrane. Also, estradiol significantly increased the CAT activity that in turn could detoxifies hydrogen peroxide (H2O2) by reducing it to water.

Heaton et al. (2000) demonstrated that neonatal alcohol exposure on postnatal days (PDs) 4 and 5 significantly reduced the Purkinje cell numbers in lobule I of the cerebellum and that such deficits were attenuated by pretreatment and cotreatment with vitamin E. Also, Shirpoor et al. (2009) demonstrated that oxidative stress plays 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. Ozaras et al. (2003) investigated free-radical scavenger effect of n-acetylcysteine in rats intragastrically fed with ethanol. This study demonstrated that ethanol-induced liver damage was associated with oxidative stress, and co-administration of n-acetylcysteine attenuated this damage effectively in rat model.

The present study provides neurobehavioral evidence that ethanol administration is associated with a loss of cerebellar Purkinje cells and a related behavioral deficit in a manner that is prevented by estradiol. Presumably, antioxidant action of estradiol may counteract the neurotoxic prooxidants produced during administration of ethanol, and thus, may prevent cerebellar neuronal death.

In conclusion, at the very least, the present study findings provide evidence that estradiol treatment prevents cerebellar Purkinje cell death and related motor deficit in ethanol-treated pups, in part through its antioxidant action and inhibiting the peroxidation of lipids and improving the activity of antioxidant enzymes. However, we know that the protective effects of estradiol against Purkinje cell death observed during ethanol toxicity may be mediated by multiple factors in addition to oxidative stress. Therefore, the accurate mechanism is not clear so far to propose the potential therapeutic use of estradiol in preventing the cerebellum from ethanol-induced oxidative damage and further studies are needed.

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References

Aebi H. Catalase in vitro. Methods Enzymol 1984;105:121–6.
Asimiadou S, Manthey D, Kaindl AM. Protection with estradiol in developmental models of apoptotic neurodegeneration. Ann Neurol 2005;58:266–76.
Ayres S, Tang M, Subbiah MT. 17ß-estradiol as an antioxidant: some distinct features when compared with common fat-soluble antioxidants. J Lab Clin Med 1996;128:367–75.
Badeau M, Adlercreutz H, Kaihovaara P, Tikkanen MJ. Estrogen A-ring structure and antioxidative effect on lipoproteins. J Steroid Biochem Mol Biol 2005;96:271–8.
Becana M, Aparicio-Tejo P, Irigoyen JJ, Sanchez-Diaz M. Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*. Plant Physiol 1986;82:1169–71.
Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, reactive oxygen species and tissue damage. Curr Pharm Des 2004;10:1611–26.
Bimonte-Nelson HA, Francis KR, Umphlet CD, Granholm AC. Progesterone reverses the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in middle-aged ovariectomized female rats. Eur J Neurosci 2006;24:229–42.
Breton P, Bizot JC, Bueeand J, De La Manche I. Brain neurotoxicity of Penitrem A: electrophysiological, behavioral and histopathological study. Toxicon 1998;36:645–55.
Burger ME, Fachinetto R, Zeni G, Rocha JB. Ebselen attenuates haloperidol-induced orofacial dyskinesia and oxidative stress in rat brain. Pharmacol Biochem Behav 2005;81:608–15.
Chu J, Tong M, de la Monte SM. Chronic ethanol exposure causes mitochondrial dysfunction and oxidative stress in immature central nervous system neurons. Acta Neuropathol 2007;113:659–73.
Cohen-Kerem R, Koren G. Antioxidants and fetal protection against ethanol teratogenicity. I. Review of the experimental data and implications to humans. Neurotoxicol Teratol 2003;25:1–9.
Crews FT, Nixon K. Mechanisms of neurodegeneration and regeneration in alcoholism. Alcohol 2009;44:115–27.
Dong J, Sulik KK, Chen SY. The role of NOX enzymes in ethanol-induced oxidative stress and apoptosis in mouse embryos. Toxicol Lett 2010;193:94–100.
Dow RS, Moruzzi G. The Physiology and Pathology of the Cerebellum. Minneapolis: University of Minnesota Press; 1958.
Enache M, Waes VV, Vinner E, Lhermitte M, Maccari S, Darnaudéry M. Impact of an acute exposure to ethanol on the oxidative stress status in the hippocampus of prenatal restraint stress adolescent male rats. Brain Res 2008;1191:55–62.
Fehr KA, Kalant H, LeBlanc AE. Residual learning deficit after heavy exposure to cannabis or alcohol in rats. Science 1976;192:1249–51.
Gonenc S, Uysal N, Acikgoz O, Kayatekin BM, Sonmez A, Kiray M, et al. Effects of melatonin on oxidative stress and spatial memory impairment induced by acute ethanol treatment in rats. Physiol Res 2005;54:341–8.
Goodlett CR, Marcussen BL, West JR. A single day of alcohol exposure during the brain growth spurt induces brain weight restriction and cerebellar Purkinje cell loss. Alcohol 1990;7:107–14.
Green PS, Simpkins JW. Neuroprotective effects of estrogens: potential mechanisms of action. Int J Dev Neurosci 2000;18:347–58.
Gridley KE, Green PS, Simpkins JW. A novel, synergistic interaction between 17beta-estradiol and glutathione in the protection of neurons against beta-amyloid 25–35-induced toxicity in vitro. Mol Pharmacol 1998;54:874–80.
Guerri C. Mechanisms involved in central nervous system dysfunctions induced by prenatal ethanol exposure. Neurotox Res 2002;4:327–35.
Hampton MB, Orrenius S. Redox regulation of apoptotic cell death. Biofactors 1998;8:1–5.
Haorah J, Ramirez SH, Floreani N, Gorantla S, Morsey B, Persidsky Y. Mechanism of alcohol-induced oxidative stress and neuronal injury. Free Radic Biol Med 2008;45:1542–50.
Hasebe M, Matsumoto I, Imagawa T, Uehara M. Effects of an anti-thyroid drug, methimazole, administration to rat dams on the cerebellar cortex development in their pups. Int J Dev Neurosci 2008;26:409–14.
Heaton MB, Mitchell JJ, Paiva M. Amelioration of ethanol-induced neurotoxicity in the neonatal rat central nervous system by antioxidant therapy. Alcohol Clin Exp Res 2000;24:512–18.
Heaton MB, Paiva M, Mayer J, Miller R. Ethanol-mediated generation of reactive oxygen species in developing rat cerebellum. Neurosci Lett 2002;334:83–6.
Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. Lancet 1973;2:999–1001.
Jung ME, Metzger DB. Alcohol withdrawal and brain injuries: beyond classical mechanisms. Molecules 2010;15:4984–5011.
Lee Y, Rowe J, Eskue K, West JR, Maier SE. Alcohol exposure on postnatal day 5 induces Purkinje cell loss and evidence of Purkinje cell degradation in lobule I of rat cerebellum. Alcohol 2008;42:295–302.
Light KE, Belcher SM, Pierce DP. Time course and manner of Purkinje neuron death following a single ethanol exposure on postnatal day 4 in the developing rat. Neuroscience 2002;114:327–37.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
Lyvia MVC, Joao PLD, Silvania MM, Vasconcelos GF, Aragao EC, Noronha PBG, et al. Behavioral and neurochemical effects on rat offspring after prenatal exposure to ethanol. Neurotoxicol Teratol 2005;27:585–92.
Marcus JC. Neurological finding in fetal alcohol syndrome. Neuropediatrics 1987;18:158–60.
McCray PB, Gibson DD, Fong KL, Hornbrook KR. Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. Biochim Biophys Acta 1976;431(3):459–68.
McGoey TN, Reynolds JN, Brien JF. Chronic prenatal ethanol exposure-induced decrease of guinea pig hippocampal CA1 pyramidal cell and cerebellar Purkinje cell density. Can J Physiol Pharmacol 2003;81:476–84.
Moorsmann B, Behl C. The antioxidant neuroprotective effects of estrogens and phenolic compounds are independent from their estrogenic properties. Proc Natl Acad Sci USA 1999;96:8867–72.
Niki E, Nakano M. Estrogens as antioxidants. Methods Enzymol 1990;186:330–3.
Ogura H, Mikami T, Takamura N, Suzuki Y, Chiba T. Development of behavioral function of cerebellar hypoplasia rats as induced by cytosine arabinoside (ara-C). Nippon Yakurigaku Zasshi 1980;76:33–44.
Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.
Ozaras R, Tahan V, Aydin S, Uzun H, Kaya S, Senturk H. N-acetylcysteine attenuates alcohol-induced oxidative stress in the rat. World J Gastroenterol 2003;9(1):125–8.
Pierce DR, Williams DK, Light KA. Purkinje cell vulnerability to developmental ethanol exposure in the rat cerebellum. Alcohol Clin Exp Res 1999;23:1650–9.
Pierce DR, Hayar A, Williams DK, Light KE. Developmental alterations in olivary climbing fiber distribution following postnatal ethanol exposure in the rat. Neuroscience 2010;169:1438–48.
Rice D, Baron S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 2000;108(Suppl. 3):511–33.
Roebuck TM, Mattson SN, Riley EP. A review of the neuroanatomical findings in children with fetal alcohol syndrome or prenatal exposure to alcohol. Alcohol Clin Exp Res 1998;22:339–44.
Sacks FM, McPherson R, Walsh BW. Effect of postmenopausal estrogen replacement on plasma Lp(a) lipoprotein concentrations. Arch Intern Med 1994;154:1106–10.

Shirpoor A, Minassian S, Salami S, Khadem-Ansari MH, Ghaderi-Pakdel F, Yeghiazaryan M. Vitamin E protects developing rat hippocampus and cerebellum against ethanol-induced oxidative stress and apoptosis. Food Chem 2009;113:115–20.
Simpkins JW, Yang SH, Liu R, Perez E, Cai ZY, Covey DF, et al. Estrogen-like compounds for ischemic neuroprotection. Stroke 2004;35:2648–51.
Simpkins JW, Wang J, Wang X, Perez E, Prokai L, Dykens JA. Mitochondria play a central role in estrogen-induced neuroprotection. CNS Neurol Disord Drug Targets 2005;4: 69–83.
Taranukhin AG, Taranukhina EY, Saransaari P, Podkletnova IM, Pelto-Huikko M, Oja SS. Neuroprotection by taurine in ethanol-induced apoptosis in the developing cerebellum. J Biomed Sci 2010;17(Suppl. 1):S12.
Thomas JD, Goodlett CR, West JR. Alcohol-induced Purkinje cell loss depends on developmental timing of alcohol exposure and correlates with motor performance. Brain Res Dev Brain Res 1998;10:159–66.
Tran TD, Jackson HD, Horn KH, Goodlett CR. Vitamin E does not protect against neonatal ethanol-induced cerebellar damage or deficits in eyeblink classical conditioning in rats. Alcohol Clin Exp Res 2005;29:117–29.
Valenzuela CF, Lindquist B, Zamudio-Bulcock PA. A review of synaptic plasticity at Purkinje neurons with a focus on ethanol-induced cerebellar dysfunction. Int Rev Neurobiol 2010;91:339–72.
Vink J, Auth J, Abebe DT, Brenneman DE, Spong CY. Novel peptides prevent alcohol induced spatial learning deficits and proinflammatory cytokine release in a mouse model of fetal alcohol syndrome. Am J Obstet Gynecol 2005;193:825–9.
Vogel MW, Caston J, Yuzaki M, Mariani J. The Lurcher mouse: fresh insights from an old mutant. Brain Res 2007;1140:4–18.
Wendel A. Glutathione peroxidase. Methods Enzymol 1981;77:325–33.