Betaine prevents ethanol-induced oxidative stress and reduces total homocysteine in the rat cerebellum

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Abstract Oxidative stress is a hypothesis for the association of reactive oxygen species with cerebrovascular and neurodegenerative diseases. Thus, we examined whether oral betaine can act as a preventive agent in ethanol-induced oxidative stress on the cerebellum of rats. Thirty-two adult male Sprague–Dawley rats were divided into four equal groups (control, ethanol, betaine, and betaine plus ethanol) with different dietary regimens and were followed up for 1 month. Total homocysteine (tHcy) of plasma and cerebellum homogenate was determined by an Axis® homocysteine EIA kit, and antioxidant enzyme (glutathione peroxidase (GPx), SOD, and CAT) activities of cerebellum homogenate were measured chemically by a spectrophotometer. Lipid peroxidation of cerebellum was shown by the measurement of thiobarbituric reactive substances (TBARS) via a spectrophotometer. Ethanol-induced hyperhomocysteinemia was manifested by an increase in the concentrations of tHcy in the plasma and cerebellum homogenates of the ethanol group, while ethanol-induced oxidative stress was indicated via an increase in lipid peroxidation marker (TBARS) in cerebellum homogenates of ethanol-treated rats. In contrast, betaine prevented hyperhomocysteinemia and oxidative stress in the betaine plus ethanol group as well as the betaine group. The results of the present investigation indicated that the protective effect of betaine is probably related to its ability to strengthen the cerebellum membrane cells by enhancement of antioxidant enzyme activity principally GPx, while the methyl donor effect of betaine to reduce hyperhomocysteinemia has been explained previously and confirmed in the present study.

Keywords Betaine · Cerebellum · Antioxidant enzymes · Ethanol · Rat

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Introduction

In recent years, there has been increasingly more evidence to support the hypothesis that elevated total homocysteine (tHcy) is an independent risk factor for coronary vascular and neurodegenerative diseases [1, 2, 12, 14, 38, 47]. High homocysteine (Hcy) has been suggested as a mediating factor in alcohol-related brain atrophy [41]. The high prevalence of hyperhomocysteinemia in the population and its easy treatability make

Hcy an interesting amino acid for studies in the prevention of degenerative brain disorders [41]. Homocysteine plays a role in a shared biochemical cascade involving overstimulation of N-methyl-D-aspartate receptors, oxidative stress, activation of caspases, DNA damage, and endoplasmic reticulum and mitochondrial dysfunction [13]. The formation of methionine from homocysteine can occur either via betaine or via 5-methyltetrahydrofolate [21]. Animal studies have shown that both pathways are equally important and that betaine is a vital methylating agent [6, 26, 27]. Betaine (trimethylglycine) transfers a methyl group via the enzyme betaine homocysteine methyltransferase (BHMT) to become dimethylglycine. Ethanol feeding can affect several hepatic enzymes in animals, including decreasing methionine synthetase activity [7]. This leads to increased BHMT activity to maintain hepatic S-adenosyl methionine (SAM) at normal concentrations [21] (supplementary file).

During the past decades, several studies have examined the role of oxidative stress on developmental alcohol-mediated neurotoxicity, possibly via the formation of free radicals [18, 31, 48, 52]. Reactive oxygen species (ROS) are generated during oxidative metabolism and can inflict damage on all classes of cellular macromolecules, eventually leading to cell death [11, 48]. Oxidative stress is believed to contribute to neurodegeneration and cognitive and behavioral deficits after ischemia, anoxia, carbon monoxide poisoning, and alcoholism [9, 53, 54]. During the metabolism of alcohol, acetaldehyde is formed as the principal metabolite. When acetaldehyde is oxidized, it produces superoxide free radicals that are able to react with hydrogen peroxide to form other types of free radicals, such as hydroxyl radicals [48]. Chronic and excessive ethanol consumption is associated with various biochemical and physiological changes in the central nervous system (CNS). The most affected brain regions seem to be superior frontal association complex, hypothalamus, and cerebellum [35].

Although the brain has defenses against ROS including dietary free radical scavengers (ascorbate, α-tocopherol), the endogenous tripeptide glutathione, and enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), there is considerable evidence that oxidative damage directly or indirectly, due to free radical production and ROS, can lead to brain injury [23]. Indeed, brain tissues have unique characteristics that make them especially susceptible to damage due to low levels of antioxidant defenses [10]. Lipid peroxidation is one of the consequences of oxidative stress induced by metabolism of ethanol [35]. In this regard, a previous study demonstrated that chronic gestational exposure to alcohol caused a decrease in the membrane integrity while increasing oxidative stress in cerebellar neurons [48]. Malondialdehyde (MDA) is one of the major aldehyde derivatives of lipid peroxidation, and it is a by-product of the lipid peroxidation process [48]. In this content, oxidative stress and lipid peroxidation due to ethanol consumption were evaluated by MDA concentration in brain tissue via the measurement of thiobarbituric acid reactive substances (TBARS) [35].

It has been shown methyl tetrahydrofolate reductase (MTHFR) deficiency in mice delayed brain development and caused severe cerebellar abnormalities, with effects on granule cell development and neuronal organization [45, 46]. In this regard, previous betaine supplementation showed a beneficial effect on cerebellar development of the pups. It reduced the severity of lamination disruption in the cerebellum and limited the defects to only one or two anterior lobules [19]. Very recently, we proved the beneficial properties of betaine in ethanol-induced hyperhomocysteinemia in a rabbit animal model [1, 2].

Taking the above into consideration, we hypothesized that the oral administration of betaine prior to ethanol can act as an antioxidant agent to increase the activity of GPx, SOD, and CAT in ethanol-induced oxidative stress and decrease TBARS concentration (as a lipid peroxidation marker) in the rat cerebellum. We also investigated how tHcy of plasma and cerebellum varied with betaine therapy in rats.

Materials and methods

Materials

Alcohol (ethanol 95%) and TBARS were purchased from Merck Chemical Company (KGaA, Darmstadt, Germany), and betaine (Betafin® 96%) was obtained from Biochem Company (Brinkstrasse 55, D-49393 Lohne, Germany). GPx and SOD kit were obtained via Randox ® Company (Randox, Germany). The homocysteine kit was prepared by Axis® Homocysteine EIA (Axis-Shield AS, Germany). All chemicals used were of analytical grade.

Animals

Thirty-two adult male Sprague–Dawley rats (weighing 220–250 g, purchased from Shiraz University of Medical Sciences, Animal House Center, Iran) were housed in temperature-controlled conditions under a 12:12-h light/dark photocycle with food and tape water supplied ad libitum. All rats were treated humanely and in compliance with the recommendations of Animal Care Committee for the Shiraz University of Medical Sciences (Shiraz, Iran). All the experimental procedures were carried out between 8.00-11.00 hours, and all treatments were applied orally by gavage.

Experimental design

The rats were divided into four equal groups, and weight gain and food consumption were determined at weekly intervals and treated daily for 1 month in the following order: the control group received 1 ml of normal saline by gavage orally, the ethanol group received ethanol (4 g/kg by gavage), the betaine group received betaine soluble in water (1.5% w/w of the total diet, approximately 180 mg/mouse soluble in water daily by gavage), and the betaine plus ethanol group received betaine (similar to the betaine group) and after 120 min, fed with ethanol solution (4 g/kg by gavage). Doses of ethanol and betaine were determined according to previous studies [1, 33, 49]. One day after the last gavage, the rats were killed using diethyl ether anesthesia (Dagenham, UK) by decapitation. Immediately after rat killing, blood samples were collected via cardiac puncture, whole blood containing EDTA was centrifuged at $3,000 \times g$ for 5 min, and plasma was prepared in microtubes. The brain was removed and carefully cleaned of adhering, and then the cerebellum in all groups was separated. Cerebellum and plasma samples were stored at $-70°C$ until analysis.

Tissue preparation for protein measurement, tHcy and TBARS detection, and enzyme assay

Rat cerebellum was thawed and manually homogenized in cold phosphate buffer (pH 7.4) containing 5 mM EDTA, and debris were removed by centrifugation at $2000 \times g$ for 5 min (Centrifuge 5415 R; Rotofix 32A, Germany). Supernatants were recovered and used for antioxidant enzyme activities, TBARS and tHcy concentrations, and protein measurement. Protein content of tissue homogenates was determined using a colorimetric method of Lowry with bovine serum albumin as a standard [39].

Measurement of tHcy concentration

Total homocysteine of cerebellum homogenates and plasma, which refers to the sum of protein-bound, free-oxidized, and reduced species of homocysteine, was determined by the Axis® Homocysteine EIA kit [29, 36]. The sample volume used was 25 µl. Absorbance was measured at a wavelength of 450 nm using an ELISA reader (STAT FAX 2100, USA). All estimations were performed in duplicate and the intra assay coefficient of variation was <10%.

Measurement of GPx activity

The activity of GPx was evaluated with Randox GPx detection kit according to the manufacturer's instructions. GPx catalyze the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH), the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured spectrophotometrically (S2000 UV model; WPA, Cambridge, UK) against blank at 340 nm. One unit of GPx was defined as 1 µmol of oxidized NADPH per minute per milligram of tissue protein. The GPx activity was expressed as unit per milligram of tissue protein.

Measurement of SOD activity

The activity of SOD was evaluated with Randox SOD detection kit according to the manufacturer's instructions. The role of SOD is to accelerate the dismutation of the toxic superoxide produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity is then measured by degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of INT under the

conditions of the assay. SOD levels were recorded at 505 nm and through a standard curve and expressed as unit per milligram of tissue protein.

Measurement of CAT activity

Tissue catalase activity was assayed using the method described by Claiborne [20]. The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 19 mM H_2O_2, and a 20–50 µl sample. The reaction was initiated by the addition of H_2O_2, and absorbance changes were measured at 240 nm (25°C) for 30 s. The molar extinction coefficient for H_2O_2 is 43.6/M cm^{-1}. The CAT activity was expressed as the unit that is defined as 1 µmol of H_2O_2 consumed per minute. The catalase activity was expressed as unit per milligram of tissue protein.

Measurement of lipid peroxidation

The level of lipid peroxidation was indicated by the content of TBARS in the cerebellum. Tissue TBARS was determined by following the production of thiobarbituric acid reactive substances as described by Subbarao et al. [50]. In short, 40 µl of homogenate was added to 40 µl of 0.9% NaCl and 40 µl of deionized H_2O, resulting in a total reaction volume of 120 µl. The reaction was incubated at 37°C for 20 min and stopped by the addition of 600 µl of cold 0.8 M hydrochloride acid, containing 12.5% trichloro-acetic acid. Following the addition of 780 µl of 1% TBA, the reaction was boiled for 20 min and then cooled at 4°C for 1 h. In order to measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 1,500×g in a microcentrifuge for 20 min, and the absorbance of the supernatant was spectrophotometrically read at 532 nm, using an extinction coefficient of 1.56×10^5. The blanks for all of the TBARS assays contained an additional 40 µl of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nano-mole per milligram of tissue protein.

Statistical analysis

All results are presented as mean±SEM. Data were compared by one-way analysis of variance (ANOVA) with Tukey's post hoc analysis. A calculated P value of less than 0.05 was considered to be statistically

significant. Statistical analysis was performed using the statistical package SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA).

Results

The mean values (±SEM) of the GPx, SOD, and CAT activity of the four groups of rat cerebellum are presented in Fig. 1. GPx activity is significantly higher in the betaine group compared to the control, ethanol, and betaine plus ethanol group ($P < 0.05$). Interestingly, GPx activity is insignificantly higher in the ethanol group compared to the control group ($P > 0.05$). SOD activity is significantly higher in the betaine group compared to the control and ethanol group ($P < 0.05$). However, CAT activity is significantly higher in the betaine group compared to the ethanol group ($P < 0.05$). Although the activities of SOD and CAT in the betaine plus ethanol group were higher compared to the ethanol group, these enhancements were not statistically significant ($P > 0.05$). Indeed, when betaine was administered prior to ethanol, it could increase the level of these parameters near to the betaine group.

TBARS concentration (mean±SEM) in the ethanol group (33.60±7.3) was increased significantly compared to the control (11.21±2.15), betaine (13.52±1.85), and betaine plus ethanol group (14.49±1.98; $P < 0.05$). Although the betaine plus ethanol group showed slightly increased TBARS concentration compared to the control group, this was not significant ($P > 0.05$; Fig. 2).

Treatment of rats with ethanol significantly increased tHcy in cerebellum of the ethanol group
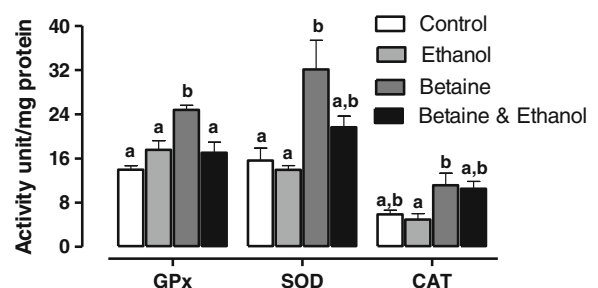
Fig. 1 Comparison of antioxidant enzyme activities: glutathione peroxidase (*GPx*), superoxide dismutase (*SOD*), and catalase (*CAT*) among the control and treatment groups of rats. Values represent mean±SEM of enzyme activity (unit/mg protein of cerebellum tissue). *Means* for each enzyme with *different superscripts* differ statistically (one-way ANOVA followed by Tukey's post hoc test; $P < 0.05$)
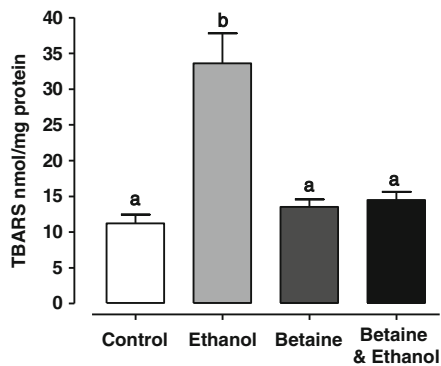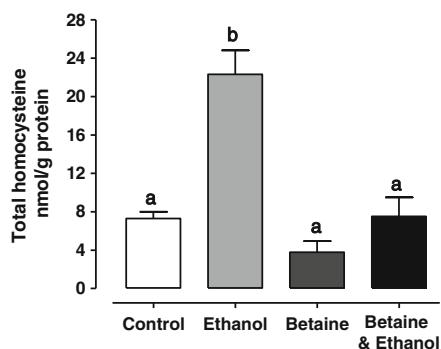
Fig. 2 .Comparison of thiobarbituric acid reactive substances (TBARS) concentration among the control and treatment groups of rats. Values represent mean±SEM of TBARS (nanomoles per milligram protein of cerebellum tissue. *Means with different superscripts* differ statistically (one-way ANOVA followed Tukey's post hoc test; $P<0.05$)

Fig. 4 Comparison of plasma total homocysteine (tHcy) concentration among the control and treatment groups of rats. Values represent mean±SEM of tHcy (micromoles per liter of plasma). *Means with different superscripts* differ statistically (one-way ANOVA followed by Tukey's post hoc test; $P<0.05$)
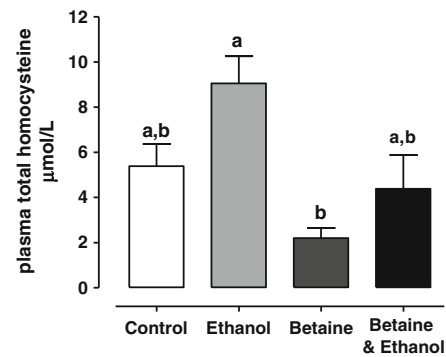
compared to the other groups, while administration of betaine to the betaine and betaine plus ethanol group could prevent increase of tHcy concentration ($P<0.05$; Fig. 3). In contrast, the plasma concentration of tHcy in the betaine group was remarkably lower compared to ethanol-ingested rats ($P<0.05$). Although plasma homocysteine for ethanol-treated rats was not significant compared to the control group, it tended to approach significance ($P=0.058$; Fig. 4).

Discussion

Our data support the hypothesis that betaine can reduce ethanol-induced oxidative stress and suggests it does by

Fig. 3 Comparison of total homocysteine (tHcy) concentration among the control and treatment groups of rats. Values represent mean±SEM of tHcy (nanomoles per gram protein of cerebellum tissue). *Means with different superscripts* differ statistically (one-way ANOVA followed by Tukey's post hoc test; $P<0.05$)

promoting the antioxidant enzyme activity including GPx, SOD, and CAT. The observation for antioxidant enzymes, TBARS concentration, and tHcy in the treatment groups supports the idea that betaine is associated with antioxidant and methyl donor properties through its involvement in cell membrane stabilization and homocysteine remethylation [1].

The results of the present investigation indicated that the protective effect of betaine is probably related to its ability to strengthen the cerebellar cell membrane by its membrane-stabilizing action or to a counteraction of free radicals by its antioxidant property [28]. The present data indicate that ethanol consumption increases tHcy and TBARS content in the rat cerebellar tissue [14, 15, 35]. Interestingly, our results indicate that ethanol induces oxidative damage and enhances GPx activity. In this context, a previous report showed that ethanol can enhance glutathione content in the rat pups brain [48].

Chronic alcohol consumption results in brain injury, leading to a number of neuropsychiatric symptoms including alcoholic cerebellum degeneration and dementia [17, 35]. Because brain consumes approximately 29% of oxygen received by organism, ethanol-induced ROS production and consecutive oxidative stress play an important role among the mechanisms of ethanol neurotoxicity [4, 48, 51]. Previous studies showed that betaine exerted cellular and subcellular membrane stabilization in the liver and myocardium cells by restoring both non-enzymatic and enzymatic antioxidants [24, 28]. We have already reported the protective effect of betaine on ethanol-induced hyperhomocystei-

nemia in our recent studies [1, 2]. However, the protective effect of betaine on cerebellar antioxidant defense system has not yet been previously explored. In the present study, we found significantly elevated antioxidant enzyme activity in cerebellum of rats exposed to betaine, and this is consistent with a previous report [28].

During the past decade, administration of betaine has been shown to exert a significant role within tissue as a methyl donor, which in turn may be used for the synthesis of methionine, carnitine, phosphatidylcholine, creatine, and these substances play a key role in protein and energy metabolism in the cells [21]. Betaine is believed to play a significant role in maintaining the structural and functional integrity of cell membranes. A previous study has been demonstrated that betaine, through its participation in sequential methylation within the cellular membranes, maintains a proper balance between phosphotidyl ethanolamine and phosphotidyl choline, thus sustaining proper membranes [28, 37]. In the present study, betaine, a methyl donor that continuously generates SAM, is shown to lead to long-term lowering of plasma homocysteine during supplementation in the dietary intake range of 1.5% (w/w) of total diet. Furthermore, since humans produce little betaine from choline due to lack of choline oxidase [30], betaine is practical for investigations regarding the treatment of hyperhomocysteinemia in humans.

Although betaine can across the blood–brain barrier, homocysteine remethylation to methionine catalyzed by BHMT occurs mainly in liver [42, 43]. SAM is released from liver and can also across the blood–brain barrier, with partial restoration of the decreased methyl donor pool in brain caused by MTHFR deficiency [18]. This phenomenon may explain why there is still a high level of homocysteine in the brain of betaine plus ethanol group despite the increase in homocysteine remethylation to methionine in liver following betaine administration. Indeed, plasma total homocysteine decreased in the betaine group after betaine treatment, while tHcy in the cerebellum of ethanol-ingested rats due to lack of SAM elevated significantly. In this regard, previously, Broch et al. have shown a much higher concentration of Hcy (about 6 nmol/g tissue) in the cerebellum of the rats, while the concentration in the liver was about 4 nmol/g of tissue in rats [16].

Homocysteine inhibits the expression of antioxidant enzymes which might potentiate the toxic effects

of ROS [13, 32]. In addition, autooxidation of homocysteine is known to generate ROS, whereby the prevention of homocysteine-induced toxicity by catalase suggests that hydrogen peroxide acted as a mediator of oxidative injury, leading to oxidative stress [5, 22, 40]. In this regard, only catalase activity was significantly higher in the betaine group compared to the control group. Indeed, catalase prevented H_2O_2 accumulation and elevated its activity in the betaine plus ethanol group compared to the control group, although it was not significant.

The brain is more vulnerable to oxidative stress than other organs due to its low antioxidant protection system and increased exposure of target molecules to ROS [23]. The nervous tissue has a high content of polyunsaturated fatty acids, which are easy targets to oxidative damage by free radicals due to the unsaturated bonds they contain [48]. In our study, ethanol consumption caused significantly increased TBARS concentration in the ethanol group, and betaine treatment restored this elevated TBARS concentration in the betaine plus ethanol group near to the control group. On the other hand, there were significant differences among the ethanol and other groups for the TBARS concentration, indicating oxidative stress in ethanol-treated rats. These results of ethanol-exposed rats were consistent with previous literature [35].

Animal models have shown that a number of antioxidants prevent oxidative brain injury through a variety of cellular mechanisms which have described oxidative damage on the CNS [10, 23, 53, 54]. Glutathione antioxidant system plays a fundamental role in cellular defense against reactive oxygen species. The cellular tripeptide, GSH (γ-glutamyl cysteinyl glycine), thwarts peroxidative damage by neutralizing the free radicals [28]. In the present study, significant elevation of TBARS concentration in the cerebellum tissue of the ethanol group suggests an enhanced oxidative stress in the experimental animal model.

It is well known that SOD and CAT, which are responsible for the destruction of peroxides, have a specific role in protecting tissues against oxidative damage [3, 28, 34]. In the present study, the unpaired electron present in the hydroxyl free radical might have been trapped and subsequently dismuted by betaine [8, 44]. However, the protective effect of betaine against ethanol-induced oxidative stress observed in this study may also be associated with the restoration of SAM, which contributes to an increase

in the supply of substrate needed for the synthesis of glutathione that protects the cell from reactive metabolites and reactive oxygen species [28].

To the best of our knowledge, this is the first in vivo study to show that betaine treatment results in an overall increase in the antioxidant enzyme activities in rat cerebellum. Betaine is a methylating agent like SAM, and it also stabilizes SAM levels via BHMT pathway [1, 2, 14]. Therefore, betaine may have an antioxidant effect against oxidative damage in brain. In addition, betaine may have some advantages than endogenous SAM application because SAM application enhances the levels of homocysteine, which is undesirable due to the toxicity of this amino acid, whereas betaine treatment decreases homocysteine levels by directly inducing the remethylating process, which transforms homocysteine into methionine [38]. With regard to BHMT, which is abundant in primates, the beneficial properties of betaine are promising and reduce the elevated plasma homocysteine concentrations via the BHMT pathway [12, 25, 26]. Although, betaine demonstrated as a potential neuroprotective agent for prevention of ethanol-induced oxidative damage in the cerebellum. However, further studies including extra biochemical parameters and histochemical techniques should be performed to validate this assumption.

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