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The New Perspective Neuroprotective Effect Of Boric Acid Against Ethanol-Induced Oxidative Damage On Synaptosome

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Abstract

In this study, the effects of ethanol toxicity (200 mM) in vitro and the application of boric acid (BA) (5, 10 and 25 mM) at different doses were investigated for potential protective and antioxidant roles on rat brain synaptosomes. Synaptosomes were used as a sample of five groups (control, ethanol, ethanol+5 mM BA, ethanol+10 mM BA, ethanol+25 mM BA), which include six samples. Malondialdehyde (MDA), Nitric Oxide (NO) levels and Catalase (CAT) activity were measured as oxidative stress markers in ethanol-induced oxidative stress in rat brain synaptosomes. The levels of MDA significantly increased in the ethanol-treated synaptosomal samples, as compared those in the control samples. However, the levels of MDA significantly decreased in the BA-treated groups having a greater effect with the highest concentration (25 mM) of BA used ($P<0.05$). The levels of MDA in the ethanol-treated + boric acid (25 mM) group were found to be significantly lower than in the ethanol-treated group ($P<0.05$). The CAT activities of the ethanol-treated group were higher than in control group, and the CAT activities of the BA (5 mM, 25 mM) groups were found to be close to that of the control groups ($P<0.01$). NO levels in ethanol-treated groups were slightly decreased as compared to control groups but not statistically significant. Nevertheless, NO levels in ethanol-treated +boric acid (25 mM) groups were increased ($P<0.05$). In conclusion, our data showed that BA treatment might be neuroprotective effective against ethanol-induced neurotoxicity as antioxidant properties.

Keywords: Boric Acid, Ethanol, Synaptosome, Oxidative Stress, Neuroprotective Effect.

INTRODUCTION

In aerobic life, it is almost inevitable that organisms are exposed to free radicals and reactive oxygen species (ROS). Because molecular oxygen production requires ATP in the $O_2 \rightarrow O_2^-$ pathway. ROS consists of molecular species with the ability to break electrons from radicals (H_2O_2, HOCl) or other molecules containing unpaired electrons (OH, O_2^-). These species can directly damage biomolecules or initiate radical chain reactions [1]. Besides, it can change membranes and proteins in cell structure. Many studies have shown that free radicals are related with many diseases, such as cardiovascular disorders, neurodegenerative diseases, and cancer, which are known to have high morbidity and mortality rates, but antioxidant molecules delay or prevent these undesirable oxidation reactions [1].

Alcohol-induced oxidative stress is known to cause neuronal damage, such as alcoholic neuropathy, cerebellum degeneration, fetal alcohol syndrome, Wernicke's Korsakoff syndrome, cognitive dysfunction, alcohol withdrawal syndrome, and dementia [2].

Synaptosomes have many different functions. Therefore, their use in synaptic studies are fairly common and conveniently [3]. Many studies have shown that synaptosomal fractions have all the functions of a synapse. Similarities of synapses and synaptosomes were shown in experiments [4]. Purity is very important in the sense of synaptosomes. Because of various investigations such as glycolysis, oxidative phosphorylation, ion transport, neurotoxicity, and neurotransmitter are shown on synaptosomes [5]. Because of these similarities, synaptosomes are preferred for in vitro studies, without exposure to the difficulties and confusions associated with in situ use of synapses [6]. Since the brain is composed of a large number of polyunsaturated fatty acids and consumes about 29% of the total oxygen in the whole body, it is highly sensitive to oxidative stress. Earlier studies have shown that ethanol affects the synaptic structure of the brain through several mechanisms such as changes in protein and lipid compositions and deterioration of membrane structures [7]. Lipid peroxidation has been observed ethanol-induced oxidative stress in the brain. Mechanisms that result from oxidative damage include peroxidation of membrane lipids and proteins, which cause degradation in membrane structure and cellular functions [8,9]. Ethanol can affect the brain tissue directly or indirectly. It is believed that the direct effect of ethanol on the biological membrane is due to the alteration of the functions of the transmembrane protein and ion channels by increasing the fluidity membranes. The results of oxidative and non-oxidative mechanisms of ethanol toxicity, acetaldehyde is formed by three different mechanisms such as alcohol dehydrogenase, cyt-p450-dependent microsomal ethanol oxidation system and the catalase-H_2O_2 system are responsible for indirect effects [10]. Acetaldehyde produces protein adducts which damage the role of restorative enzymes and the mitochondria. As free radical generators, catalase and microsomal ethanol oxidation systems are also responsible for these damages [11]. Acetaldehyde has been reported to sensitize the organism to oxidative stress. This is accomplished by reducing glutathione groups, neutralizing free glutathione and reducing hydrophobic regions in the organism [12,13]. It is known that the catalase-peroxide system affects the formation of acetaldehyde from ethanol. In many neuronal tissue preparations, it has been shown that the formation of acetaldehyde from ethanol is also significantly reduced when this system is interrupted by pharmacological agents or genetic effects [14]. Therefore, it is believed that the catalase-peroxide mechanism is the key enzymatic pathway for the oxidation of ethanol to acetaldehyde in the brain. Through the nonoxidative system, ethanol makes fatty acid ethyl esters, which play a role in the degradation of myelin metabolism and the prevention of esterification of neuronal membrane cholesterol together with mitochondrial function [15]. Ethanol-derived reactive oxygen species (ROS), free radicals production and sequential oxidative stress play a critical role in neurotoxicity caused by ethanol [16]. However, despite all this information, the

relationship between alcoholic-induced oxidative stress and membrane features is not totally understood. It is known that all the features of a cell may change with the deterioration of membrane fluidity.

Boric acid (BA) is an important trace element that supports metabolic events for plants, humans, animals even for some technological equipment. Monobases are known to be proton donors, but BA is not. Instead, it accepts a hydroxyl ion such as Lewis acid from water and releases a proton [17]. It assumes membrane integrity, function and redox metabolism due to high attraction to some metabolites in the organism, for example, S-adenosyl methionine (SAM), Flavin adenine dinucleotide (FAD), oxidized nicotinamide adenine dinucleotide (NAD^+), glycolipids, glycoproteins, and phosphonoazides. It is known that some foods and drugs contain boron and when they are digested orally, it enters the bloodstream rapidly and completely, and about 100% is excreted through the urine [17,18]. Boron Neutron Capture Therapy (BNCT) is known to be used for some types of cancer [19]. Some studies have shown that it reduced the adversity and incidence of inflammatory diseases [20]. The effects of BA on the body are in particular on some minerals, enzymes, hormones and metabolic pathway [21.]In addition to all these effects, some studies have presented and discussed that BA has antioxidant properties and protective roles [23].

Recent studies in our laboratory, ethanol has been shown to cause inflammatory and neurodegenerative diseases in synaptic vesicles obtained from a fraction of rat neurons [22]. Moreover, in earlier studies, we showed that the most suitable dose of ethanol toxicity on rat brain synaptosomes were 200 mM ethanol-concentration. Besides, we evaluated the potential neuroprotective effect of different doses of boric acid (BA) (5, 10 and 25mM) on synaptosomes in our current study.

MATERIALS AND METHODS

Procedure for Obtaining Experimental Animals and Synaptosomes. Sprague Dawley male albino rats (250 ± 50 g) supplied the Laboratory of Eskisehir Osmangazi University (Eskisehir, Turkey) and were used in experiments. The rats nourished feed and tap water ad libitum. Moreover, they were maintained at laboratory conditions at a temperature of 22◦C ± 3◦C and 55% ± 5% relative humidity in a 12-h dark/light cycle. This study was approved with as number 97/530 by the Ethical Committee of Eskisehir Osmangazi University.

Preparation of Synaptosomes in Experimental Rats. All necessary equipment and materials are prepared for experiments. The crude synaptosomal fractions for this study were composed by changing the method of Whittaker et al. used in many studies [5]. After 5 healthy *Sprague Dawley* male albino rats (250 ± 50 g) were sacrificed by cervical dislocation for their brain without other tissue, their forebrains were removed and separation from other regions of the nervous system was performed. After they were cleaned in physiological saline solution to remove residues and then the weights were recorded. Each piece obtained was divided into about four equal cuts and a pool was set up for use in the measurements. 5 (rats) x 4 = 20 cuts were obtained. 5 groups were prepared with randomly chosen cuts. After, the all cuts were weighted and then divided into about 30 equal weights for use in each group (n=6). Each group contained six samples of the forebrain (n=6). The homogenization process was carried out after determining the about equal weights in per group. They were used as a sample of five groups (control, ethanol, ethanol+5 mM BA, ethanol+10 mM BA, ethanol+25 mM BA), which include six samples. We used the same method in our group's previous studies [22].

The following solutions were freshly prepared. Rat's forebrain fragments were homogenized in an ice-cold solution of HEPES (10 mmol/L) and sucrose (0.32 mmol/L). After, the homogenate was centrifuged at 3,000 × g for 10 min; the supernatant was removed and again centrifuged at 15,000 × g for 20 min. After artificial cerebrospinal fluid was composed (aCSF; 116 mM NaCl, 5.4 mM KCl, 0.9 mM $MgCl_2$, 0.9 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 10 mM glucose, pH 7.2) , the synaptosomal rich pellet was resuspended (5). Depending on the content (Table 1), the control group and the experimental groups were treated with ethanol (200 mM), and different dose of boric acid (5, 10 and 25 mM) and were incubated at 37°C for 30 min. After the incubation, the parameter of MDA, NO levels, and CAT activities that we specified in our study method was calculated. The quantity of protein in the samples was concluded with the Bradford method [24].

Table 1. The distribution of ethanol (200 mM), boric acid (5, 10 and 25 mM) concentrations on synaptosomal fractions, according to the experimental groups; each group has six samples (n= 6).

Group 1	**Control**
Group 2	**200 mM ethanol**
Group 3	**200 mM ethanol + 5 mM boric acid**
Group 4	**200 mM ethanol + 10 mM boric acid**
Group 5	**200 mM ethanol + 25 mM boric acid**

Measurement of Nitric Oxide Levels in Synaptosomes. It is hard to measure NO levels directly. Determination of NO levels in various body fluids and tissue homogenates is based on the measurement of nitrite and nitrate, the end products of nitric oxide oxidation. The amount of nitrite (NO^{-2}) and nitrate (NO^{-3}) in the samples is determined in two steps. Firstly nitrate should be reduced by enzymatic transformation or by cadmium garnels. In the acidic environment, the nitrite reacts with diazotization with sulfanilamide, which is the aromatic amine, and forms a purple azo compound with N- (1-naphthyl) ethylenediamine. Deproteinization is performed for proteins that may have adverse effects on measurement sensitivity. In our study, the nitrite level in rat brain synaptosomes was determined with the method of Cortas and Wakid [25]. NO levels were stated in nanomoles per milligram of protein.

Measurement of MDA Levels in Synaptosomes. The quantitative determination of lipid peroxidation in biological samples are based on the color response between MDA, the end product of lipid peroxidation, and thiobarbituric acid (TBA). Lipid peroxidation in body fluids or cells is assessed as a marker of oxidative stress. MDA level in tissue homogenate was determined by Ohkawa et al. measured according to the method reported [26]. 0.1 g portions were weighed out from the tissues and 1/10 KCl solution was added as 0.1 g to 1 mL and homogenized. Homogenates were centrifuged at + 4 ° C at 1500xg for 15 min. Measurements were made from the supernatant. The results were stated in nmol/mg protein

Measurement of CAT Levels in Synaptosomes. The determination of CAT activities was measured and calculated according to the method of Beutler [27]. It is known that catalase catalyzes the breakdown of H_2O_2 to H_2O. The rate of H_2O_2 degradation by the catalase enzyme is measured and calculated at 230 nm because H_2O_2 absorbs light at this wavelength. Briefly, after addition of 50 ml of tris, 900 ml of H_2O_2 and 30 ml of distilled water, the incubation is carried out at 37 ° C before the hemolysate is added. After the he-

molysate has been added, the reduction in optical densities against the blank at 412 nm is monitored for the next 10 minutes.

Protein Estimation in Synaptosome in Experimental Rats. It is extremely important to determine the protein content so that all measurement results can be interpreted. The protein contents were determined according to the method of Bradford [28] using Bowin serum albumin (BSA) was prepared at a range of 10 to 100 mg protein to form a standard chart. Briefly, 20 mL of biological sample and 3 mL of Bradford reagent were mixed for the analysis; then 1.5 mL of ethanol (95%) and 3 mL of o-phthalaldehyde OPA (85%) were added to each tube, thoroughly mixed and incubated at room temperature for at least 5 minutes. Standard chart created. The absorbance of each tube was measured at 595 nm.

Statistical Analysis. In the interpretation of this study, a commercial SPSS 21.0 Windows program was used for the statistical analysis of the data obtained from the experimental and control groups. The results were given as ± standard deviation and values of P <0.05 were considered significant. One-way ANOVA was applied for data showing normal distribution.

RESULT

In this study, ethanol-induced oxidative stress markers in synaptosomes of rats, malondialdehyde (MDA), nitric oxide (NO) and catalase (CAT) levels were measured.

Effect of Boric Acid (5, 10 and 25 mM) and 200 mM Ethanol on MDA Levels in Synaptosomes. The change in MDA levels with administration of ethanol-treated (200 mM) and BA (5, 10 and 25 mM) are shown in Figure 1.

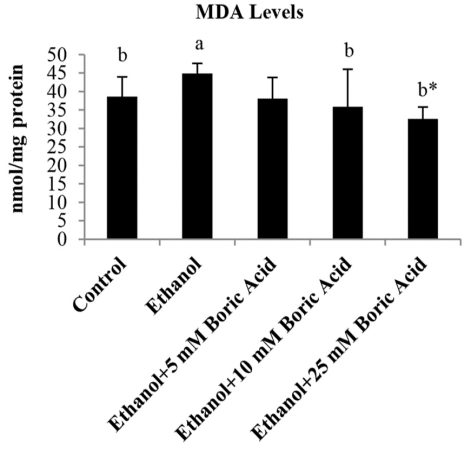

Figure 1. Comparing MDA (nmol/mg protein) levels among the control group, 200 mM Ethanol Group and Ethanol + 5, 10, 25 mM Boric Acid Groups.
Data shown are mean ± standard deviation.
a: Statistically significant differences were found in the control group (P<0,05)
b: Statistically significant groups differing from ethanol group (P<0,05)
*: Significantly advanced (P<0,01).

The levels of MDA (44.87 ± 2.73) significantly increased in the ethanol-treated group when compared to those in the control group (36.07 ± 3.47), however, it decreased significantly after BA was added to the samples. MDA level in the ethanol-treated (200 mM) + BA (25 mM) group was shown to be significantly decreased compared with that in the ethanol group (P<0.05). While MDA level of ethanol-treated (200 mM) + BA (5 mM) group were (40.77 ± 1.16), ethanol-treated (200 mM) + BA (10 mM) group were (36.19 ± 9.85) and ethanol-treated + BA (25 mM) were (33.37 ± 2.58).

Effect of Boric Acid (5, 10 and 25 mM) and 200 mM Ethanol on NO Levels in Synaptosomes. The change in NO levels with administration of ethanol-treated (200 mM) and BA (5, 10 and 25 mM) are shown in Figure 2.

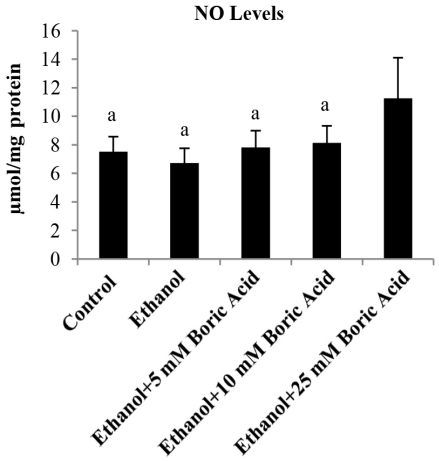

Figure 2. Comparing NO (µmol/mg protein) levels among the control group, 200 mM Ethanol Group and Ethanol + 5, 10, 25 mM Boric Acid groups.
Data shown are mean ± standard deviation.
a: Statistically different from the group treated with 25 mM boric acid (P<0.05)

NO levels (7.24 ± 0.36) in ethanol-treated group were decreased but unchanged compared with control groups (7.63 ± 0.47) as statistically. Neverthless, NO levels in ethanol-treated + BA (25 mM) groups were increased (P<0.05). While NO level of ethanol-treated (200 mM) + BA (5 mM) group were (7.61 ± 0.21), ethanol-treated (200 mM) + BA (10 mM) group were (7.93 ± 0.64) and ethanol-treated + BA (25 mM) were (11.47 ± 0.74).

Effect of Boric Acid (5, 10 and 25 mM) and 200 mM Ethanol on CAT Activities in Synaptosomes. The change in CAT activities with the administration of ethanol-treated (200 mM) and BA (5, 10 and 25 mM) groups are shown in Figure 3.

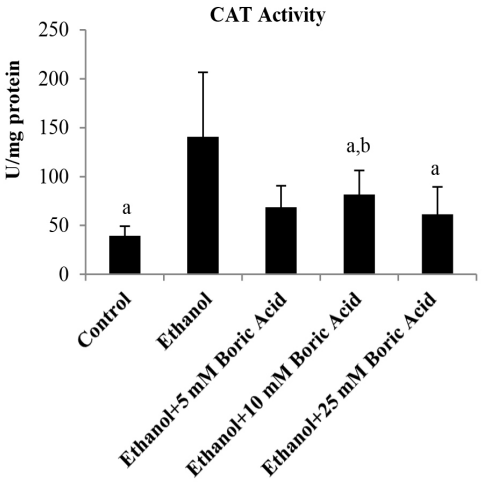

Figure 3. Comparing CAT (U/mg protein) levels among the control group, 200 mM Ethanol Group and Ethanol + 5, 10, 25 mM Boric Acid Groups.
Data shown are mean ± standard deviation.
a: Statistically significant differences from the ethanol group (P<0,001).
b: Statistically significant differences from control group (P<0,05).

The CAT activities (182.13 ± 10.23) of the ethanol-treated group were significantly higher than that in the control group (39.50 ± 9.77) and CAT activities of the BA (5 mM, 25 mM) groups were close compared with control groups (P<0.01). While CAT activities of ethanol-treated (200 mM) + BA (5 mM) group were (61.41 ± 25.88), ethanol-treated (200 mM) + BA (10 mM) group were (81.66 ± 24.61) and ethanol-treated + BA (25 mM) were (61.30 ± 28.22).

DISCUSSION

In order to determination neurotoxic effect of ethanol on rat brain synaptosomal fractions and also the evolution of neuroprotective effects of three different concentration of BA, we measured MDA, NO levels, and CAT activities.

Oxidative stress is associated with neurotoxicity of branched long chain fatty acids occurring in mitochondrial dysfunction and neurodegenerative diseases [29]. Synapses that play an important role in neuronal signal transduction are highly correlated with oxidative stress-induced neurotoxicity. Synaptosomes are highly vulnerable to lipid and protein oxidation due to their high mitochondrial content and energy consumption in presynaptic neuronal axons, and thus synapses may be the starting point for progressive neurodegenerative cascades with the maintenance of energy balance between neurons and mitochondrial function abnormalities [30-32]

Membranes are the main target of ethanol-induced damage due to phospholipids having saturated and unsaturated fatty acids content. This increased ROS production causes membrane lipid peroxidation leading to loss of cellular integrity. Studies have shown that ethanol promotes free radical production and causes peroxidation of lipids in the membrane structure, which leads to deterioration and alteration of the membrane lipid composition [33]. In this study, it was demonstrated that in vitro application of 200 mM ethanol increases lipid peroxidation by the effect on rat brain synaptosomes. Parallel with our results, according to Sogut et. al [34], there was a statistically significant increase (P<0.05) in the MDA levels when the 200 mM ethanol-treated group was compared with other ethanol and control groups. In another previous study, high-dose ethanol consumption has been shown to increase MDA levels in synaptosomes [22]. Montoliu et al. [35] have reported that ethanol increases ROS in synaptosomes and causes deterioration of membrane integrity. In addition, studies on rats have also shown that ethanol exposure increases MDA levels in synaptosomes associated with lipid peroxidation. It has also been shown in previous studies that both acute and chronic ethanol administration reduced antioxidant activity [36]. The results of our study were found to be consistent with all previous studies [37].

It is known that when ethanol is metabolized in synaptosomal fractions of rat brain, the catalase enzyme found in peroxisomes in the oxidative pathway and the enzyme of fatty acid synthase in the non-oxidative pathway are involved. There is also a direct effect on the membrane of the ethanol [45]. Ethanol is converted to acetaldehyde, a metabolite of the reaction carried out with the alcohol dehydrogenase and catalase enzyme involved in the microsomal cytochrome P450 system [46]. Enzymatic and non-enzymatic antioxidants (such as GR, GPx, GST, SOD, CAT and thiol, tocopherol, ascorbic acid, GSH, respectively) have an important role in the prevention of cellular damage caused by ROS that results from alcohol exposure [47]. SOD and CAT antioxidant enzymes, which have cooperative effects with each other, are two of the most important defense mechanisms against the toxic effects of ROS, especially those involved in oxygen metabolism. The H_2O_2 formed by the SOD reaction is converted to water together with the CAT enzyme. In the study on fetal alcohol syndrome rats previously performed in our laboratory, ethanol + BA test group CAT activities were found to be higher than the alcohol group (P<0.05). However, there was no statistically significant difference in CAT activities between the control group and the alcohol group. In another study investigating the effects of phytanic acid exposure on CAT activities, a marked reduction in the CAT activities of the group of phytanic acid was observed compared with the control group. In contrast, melatonin administration significantly improved CAT activities (P<0.05) when compared to the group with phytanic acid exposure [14]. We have already mentioned that catalase is the main enzymatic pathway that metabolizes ethanol to acetaldehyde in the brain. Thus, if the activity of the catalase enzyme is controlled by genetic or pharmacological effects, the formation of the resulting acetaldehyde in ethanol metabolism in many different nerve tissue preparations can be significantly reduced. Aragon and colleagues have conducted in vivo studies demonstrating the link between catalase-peroxide system and ethanol. If all blood has been drawn from the rat brains, they have shown that acetaldehyde can be formed from ethanol [48]. Thus, according to our hypothesis, when in vitro ethanol-treated on rat brain synaptosomes, the catalase activity was increased due to alcohol content. According to a study by Turkez et al. [49], 15 mg/L of boron exposure increased CAT activities of erythrocytes; however, exposure of 500 mg/L boric acid was found to cause a decrease in CAT activities. In a study to investigate the toxic effects of carbon tetrachloride on rats, exposure of BA to doses of 50, 100, and 200 mg/kg was reported to increase liver CAT activities [21]. In our study, the induction of reduced CAT activities by BA (5 and 25 mM) may be due to superoxide radical flux. Treatment of BA on rat brain synaptosomes have significantly improved antioxidant enzyme levels due to the effect of reducing free radical accumulation during ethanol-treated (200 mM) exposure.

In this study, the in vitro exposure of BA to synaptosomes was found to significantly increase the NO levels. Our results consistent with the literature show that neurotoxicity in synaptosomes has not caused solely by ROS, but also induced RNS production have shown to cause oxidative stress [38,39]. It is believed that the underlying mechanism of reduced NO levels following administration of high dose acute ethanol in vitro to brain synaptosomes is due to ethanol having the inhibitory effect on N-methyl-D-aspartate (NMDA) receptors. The primary pathway for NO production is glutamate-dependent ionotropic NMDA receptor activation. Activation of these receptors causes the sodium and calcium to enter the cell as the potassium moves out of the cell through the ion channels, causing the membrane potential to change [40]. In parallel with our studies, ethanol and its metabolites inhibit NMDA receptors in the brain, thereby decreasing nitric oxide synthase (NOS) activity and causing NO levels to decrease [50]. We found that the NO level of the group exposed to ethanol-treated was not statistically significant when compared to the control group. However, NO levels in ethanol-treated + BA (25 mM) groups were increased (P<0.05).

The studies investigating the integration of BA with antioxidant mechanisms, which have an important role in protecting the cellular integrity, are increasing day by day but still not fully understood [41,42]. According to İnce and et al. [43], daily 100 mg/kg boron administration has been shown to reduce oxidative stress by reducing ROS production and increase antioxidant capacity [43]. In another study, BA administered at the same dosage for a short time (≤4 weeks) was shown not to be toxic to rodents [44]. An in vitro study, Genotoxicity was established with paclitaxel in the

cell culture medium and the addition of Boric acid (2,5 or 5 mg/L) significantly reduced genotoxicity [51]. In another in vitro study, application of a boric acid (3%) solution has been shown to significantly improve wound healing [52]. According to the proposed hypothesis, BA administration at increasing doses may be a possible mechanism for reducing oxidative stress induced by ethanol. BA, which acts as a Lewis acid, can complex with many biological compounds via hydroxyl groups. Also, in this study, dose studies related to BA were performed for the first time. Appropriate dose ranges were determined with reference to cell culture studies [53] and the effects on different doses of synaptosomes were examined. There are two important potential functions of BA on CAT enzyme activity. Primarily, BA reduces ROS production by linking hydrogen peroxide produced with alcohol metabolism. Second, it protects the CAT enzyme by reducing the amount of free hydrogen peroxide by BA.

CONCLUSION

Administration of ethanol (200 mM) on rat brain synaptosomes have neurotoxic effects. High doses of alcohol may cause functional changes in some metabolic pathways, such as NO and CAT enzyme activities while damaging synaptosomal membranes, as demonstrated by MDA parameters. Exposure to ethanol (200 mM) on synaptosomes increased MDA levels, decreased NO levels, and increased CAT activities. BA (25 mM) reduced the MDA levels, increased the NO levels, and decreased CAT activities more than other BA (5, 10 mM) concentrations. These results demonstrate that exposure to ethanol (200 mM) causes damage in rat brain synaptosomes and that BA acts with antioxidant mechanisms against ethanol-induced oxidative stress. This fundamental study of the possible neuroprotective effect of boric acid against alcohol-induced neurotoxicity will provide new perspectives for both researchers and clinicians to work towards the therapeutic use of boric acid. In the future, along with developing engineering and medical applications, especially boron-based compounds will be used in biomedical research in the treatment of many deadly diseases such as neurodegeneration and cancer.

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