Comparative Effects of Antioxidants on Chronic Ethanol-Induced Oxidative Stress in Rat Hippocampus

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Summary

Objective: Long-term ethanol exposure can cause serious damages on central nervous system functions. The aim of the study was to investigate the effects of several antioxidant agents on chronic ethanol-induced oxidative stress in rat brain.

Material and Methods: Oxidative stress was evaluated by measuring malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), levels in the brain tissue. Ethanol was given to adult male Wistar rats by a liquid diet for 28 days. Control rats were fed by no ethanol contained isocaloric liquid diet. Melatonin (4 mg), ebselen (20 mg), proantocyanidin (PAC) (100 mg), vitamin E (100 mg) and vitamin C (100 mg) were applied to rats by oral route with gavages for 7 days from 22nd day of ethanol administration. Blood alcohol levels were measured by gas chromatography technique. MDA, SOD and GSH-Px levels were measured in hippocampal tissue.

Results: While chronic exposure to ethanol caused a significant increase in MDA level, it significantly decreased both SOD and GSH-Px levels in hippocampal tissue. All antioxidants used in the study significantly reversed both the decrease in SOD and the increase in MDA levels. Vitamin E and vitamin C were ineffective on decreased GSH-Px level in ethanol administered rats.

Conclusions: Our results suggest that chronic ethanol exposition causes oxidative stress responses that can be reversed by melatonin, ebselen, PAC, vitamin E and vitamin C treatments in hippocampal area of rat brain. In addition, melatonin, ebselen and PAC were more effective than vitamin E and vitamin C.

Key words: Ethanol, oxidative stress, hippocampus, antioxidants, rat(s)

Siçan Hipokampusunda Kronik Etanolün Oluşturduğu Oksidatif Stres Üzerine Antioksidanların Karşılaştırılmış Etkileri

Özet

Giriş: Uzun süreli alkole maruz kalma santral sinir sisteminde ciddi hasarlara neden olabilir. Bu çalışmanın amacı siçan beyinde kronik etanolun neden olduğu oksidatif stress üzerine çeşitli antioksidanların etkilerini incelемektir.

Materyal-Metod: Oksidatif stres beyin dokusunda malondialdehid (MDA), superoksidit dismutat (SOD) ve glutatyon peroksidaz (GSH-Px) düzeylerinin ölçülmesi vasıtasıyla değerlendirildi. Etanol erişkin erkek Wistar siçanlarına sıvı bir diyet ile 28 gün süre ile verildi. Kontrol siçanlar etanol içermeyen izokalorik sıvı dieta aldı. Siçanlara melatonin (4 mg), ebselen (20 mg), proantosiyanidin (PAC) (100 mg), vitamin E (100 mg) ve vitamin C (100 mg) etanole maruziyetin 22. gündünden itibaren gavaj ile oral yoldan 7 gün süre ile verildi.
Kan etanol düzeyleri gaz kromatografisi tekniği ile ölçülü. MDA, SOD ve GSH-Px düzeyleri hipokampal dokuda ölçüldü.

**Bulgular:** Kronik alkol maruz kalma hipokampal dokuda MDA düzeyinde anlamlı bir artış yaparken SOD ve GSH-Px düzeylerini anlamlı ölçüde düştü. Çalışmada kullanılan tüm antioksidanlar hem SOD düzeylerindeki azalmayı hem de MDA düzeylerindeki artışa tersine çevirecek düzeltti. Vitamin E ve vitamin C etanol verilen çıkanlardaki hipokampal GSH-Px düzeyindeki düşüş üzerine etkisizdi.

**Sonuç:** Bulgularımız kronik alkol maruz kalmanın çıkan hipokampusunda melatonin, ebselen, PAC, vitamin E ve vitamin C tedavisi ile düzeltilebilen oksidatif stress yanıtlarına neden olduğunu işaret etmektedir. Ayrıca, sonuçlarımız melatonin, ebselen ve PAC’ın hipokampal oksidatif stress üzerine vitamin E ve vitamin C’den daha etkili olduğunu göstermektedir.

**Anahtar Kelimeler:** Etanol, oksidatif stres, hipokampus, antioksidanlar, çıkan

**INTRODUCTION**

Ethyl alcohol (ethanol) is one the most widely consumed psychoactive substance all over the world. Ethanol abuse and dependence remain among the greatest substance abuse problems worldwide and abuse of ethanol has also become one of the largest public health problems. Ethanol withdrawal syndrome precipitated by discontinuing chronic ethanol intake is the most important evidence indicating the presence of physical ethanol dependence\(^{(22)}\). The brain, like most body organs, is susceptible to damage from the toxic effect of ethanol ingestion. The risk of brain injury and related neurobehavioral deficits are important results of chronic ethanol abuse and/or consumption\(^{(24)}\).

Many toxic compounds, which generate a cellular oxidative stress, induce morphological alterations of cells, and in turn lead to cell death\(^{(3)}\). Alcohol could represent a prime example of a substance that alters the redox state of the brain. It has been suggested that ethanol consumption can induce proinflammatory factors, result in a generation of additional reactive oxygen species (ROS) and increase cellular oxidative stress and consequent lipid peroxidation in the rat brain\(^{(6,8)}\). The mechanism underlying the cellular toxicity of ethanol has been widely investigated, but remains poorly understood. One possible mechanism that appears to contribute to this pathology is ethanol-related induction of free radicals/oxidative stress processes, and/or the down-regulation of protective antioxidants. The relationship between chronic ethanol administration and mentioned events has been demonstrated in many previous studies in a variety of subjects\(^{(1-3,16)}\). Findings from these studies implied that oxidative stress could be a potential mechanism for ethanol-induced injury in brain. According to this aspect, supplementation with antioxidants can ameliorate ethanol-induced damages in the brain. Thus, the results of many studies are in line with this hypothesis\(^{(3,13-16,28)}\).

Hippocampal formation is known to be the most important region of the brain for memory function\(^{(40)}\). It is well demonstrated that ethanol administration results in structural, neurochemical and functional changes in hippocampus\(^{(7,19,26,29)}\). These changes are also directly related to impairments in spatial learning and memory\(^{(29)}\). Although effects of antioxidants such as melatonin, vitamin E and vitamin C on ethanol-induced injuries in brain have been subjected in a number of studies, comparative experimental studies with relatively newer ones, such as ebselen and proantocyanidin (PAC) are very limited. Although there are many studies indicating beneficial effects of antioxidants in ethanol-induced brain injury\(^{(3,13-16,28)}\), some authors showed inability of antioxidants on ethanol-
induced neurotoxicity\(^{(9,10)}\). In addition, effects of PAC, an antioxidant in grape seed extract\(^{(42)}\), on ethanol-induced oxidative stress or brain injury has not been subjected in any study yet. On the other hand, studies focused involved in the effects of antioxidants on ethanol-induced hippocampal injury are inadequate. Thus, clarification of benefit of antioxidants on ethanol-related impairments in brain has still been required additional studies.

The main objective of the present study was to further demonstrate the ability of melatonin, ebselen, PAC, vitamin E and vitamin C to prevent oxidative stress-induced biochemical alterations such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and malondialdehyde (MDA) in the hippocampus of chronic ethanol administered adult rats. We also compared the effectiveness of these antioxidants on the biochemical changes after ethanol-induced oxidative stress.

**MATERIAL AND METHODS**

**Animals and laboratory**

The experiments performed in this study have been carried out according to the rules in the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA) and the Declaration of Helsinki. All efforts were made to minimize animal suffering to reduce the number of animals used. Local ethical committee approval was also obtained (Date: February 2\(^{nd}\), 2009; No: 2009-09/20K).

Adult male Wistar rats (180–250 g) were subjects. They were placed in a quiet and temperature and humidity controlled room (22 ± 2 °C and 60 ± 5%, respectively) in which a 12/12-hour light/dark cycle was maintained (07:00 am – 07:00 pm light).

Exposure to ethanol and other experiments were carried out in separate and isolated laboratories, which have the same environmental conditions with the colony room. The experiments were performed at the same time-period of the day, and during the light period of the light-dark cycle.

**Chronic exposure to ethanol**

For chronic exposure to ethanol, the rats were housed individually and ethanol was given in the form of a modified liquid diet as previously described\(^{(32,38,39)}\). The rats received a modified liquid diet with or without ethanol ad libitum. No extra chow or water was supplied. The composition of the modified liquid diet with ethanol is: cow milk 925 ml (Balkan Süt, Turkey), 25-75 ml ethanol (96.5% ethyl alcohol; Tekel, Turkish State Monopoly), vitamin A 5000 IU (Akpa İlaç Sanayi, Turkey) and sucrose 17 g\(^{(32)}\). This mixture supplies 1000.7 kcal/L.

The deficiency in vitamin A content is the most significant disadvantage of cow's milk when it is used as a vehicle for chronic ethanol administration. Vitamin A deficiency is associated with growth retardation and liver injury during chronic ethanol exposure\(^{(32)}\). For this reason, we added vitamin A supplement to the liquid diet. Because cow's milk has sufficient vitamin B and other mineral ingredients, we did not add these ingredients to the liquid diet.

At the beginning of the study, the rats received the liquid diet without ethanol for 7 days. Then liquid diet with 2.4% (v/v) ethanol was administered for three days. The ethanol concentration was increased to 7.2% (v/v) for the following 21 days. Control rats were pair fed with an isocaloric liquid diet containing sucrose as a caloric substitute for ethanol for 28 days. The liquid diet was freshly prepared daily and presented at the same time (10:00 h). The weight of the rats was recorded every day, and daily ethanol intake was measured and expressed as g per kg per day.

**Antioxidants used**

Melatonin, ebselen and vitamin C were purchased from Sigma Chemical (USA). Pure grape seed PAC extract was
purchased from GNC, Preventive Nutrition (USA). Vitamin E was purchased from Aksu Farma (Turkey).

**Antioxidant treatments**

On the 22nd day of the ethanol-containing liquid diet administration, the subjects were assigned into individual groups randomly. Each group had eight animals in the present study. All antioxidant administrations to separately grouped rats were done according to the protocols in Table 1. All compounds except vitamin E were dissolved in saline and applied to rats by oral route with gavages for 7 days from 22nd day of ethanol administration. Vitamin E was dissolved in sunflower oil and applied to animals as mentioned above. Oral gavages technique was also applied to control animals with solvents (saline or sunflower oil) without antioxidants.

<table>
<thead>
<tr>
<th>Group No</th>
<th>Administration protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No EtOH administered rats</td>
</tr>
<tr>
<td>2</td>
<td>Chronic EtOH administered rats</td>
</tr>
<tr>
<td>3</td>
<td>BEL measuring group*</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin (4 mg/kg/day)</td>
</tr>
<tr>
<td>5</td>
<td>Ebselen (20 mg/kg/day)</td>
</tr>
<tr>
<td>6</td>
<td>Proanthocyanidin (100 mg/kg/day)</td>
</tr>
<tr>
<td>7</td>
<td>Vitamin E (100 mg/kg/day)</td>
</tr>
<tr>
<td>8</td>
<td>Vitamin C (100 mg/kg/day)</td>
</tr>
</tbody>
</table>

n= 8 for each group; EtOH= Ethanol; BEL= Blood ethanol level; * BEL measured 28th day of ethanol administration 2 hrs before presenting the new liquid diet.

**Biochemical analysis**

Biochemical analyses were performed in individual groups of ethanol-treated and control rats (n= 8 for each group). Ethanol receiving rats in individual groups were decapitated at 21st, and 28th days of ethanol ingestion. Brains were removed and hippocampus was dissected within 5 min on a block of ice. The samples were homogenized in phosphate buffer (pH= 7.4) on an ice cube by a blade homogenizer and they were kept at -70°C until the extraction procedure. The protein content of tissue homogenates was measured as previously described with bovine serum albumin as the standard(17). Lipid peroxidation levels were measured by the thiobarbituric acid (TBA) reaction, as described by Ohkawa et al.(23). This method was used to perform a
spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 535 nm. The MDA levels were detected as nmol/g protein. SOD activity was assayed using by the nitroblue tetrazolium (NBT) method as described by Sun et al. (31), in which NBT was reduced to blue formazan by O₂, which has a strong absorbance at 560 nm. One unit (U) of SOD was defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The calculated SOD activity was expressed as U/g protein. GSH-Px activity was measured according the method described by Paglia & Valentine (25), in which GSH-Px activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically followed up at 340 nm at 37 °C. The absorbance at 340 nm was recorded for 5 min. The activity was the slope of the lines as mmol of NADPH oxidized per minute. GSH-Px activity was expressed as U/g protein.

**Determination of blood ethanol levels**

Blood ethanol levels (BELs) were determined in an individual group of the ethanol-receiving rats (n= 8) at 28th day of ethanol consumption following 2 hour before presenting the new liquid diet. Blood samples were taken by lateral tail vein. For determination of BEL, headspace equipped gas chromatography-flame ionization detector (GC-FID) was used. GC column was DB-1 fused silica wide-bore capillary column (30 m x 0.53 mm i.d., film thickness 5 µm, (J&W Scientific, Folsom, CA, USA) and GC conditions were as follow: Column (oven) temperature: 40 °C (isothermal); injection port and detector temperature: 170 °C; carrier gas: helium; its flow rate: 20 mL/min. injection port and detector temperature: 170 °C; carrier gas: helium; its flow rate: 5 mL/min.

**Statistical analysis**

Data were expressed as mean ± SEM. Changes in GSH-Px, SOD and MDA levels of ethanol-dependent rats as compared with ethanol non-dependent control rats were analyzed by unpaired (between groups) Student's t-test. The level of significance was set at p< 0.05 level.

**RESULTS**

Daily ethanol consumption of the rats was over 11 g/kg during the exposure to ethanol (7.2%). No significant differences between the ethanol-ingesting groups were observed. Body weights of the ethanol-treated rats did not change significantly at the end of chronic exposure to ethanol as compared to beginning of the study.

BELs were found to be in a range from 80 to 100 mg/dL (95.0±1.74 mg/dL) in ethanol administered rats.

GSH-Px, SOD and MDA levels in hippocampal formation of control and ethanol-dependent rats, and the effects of melatonin, ebselen, PAC, vitamin E and vitamin C on changes in GSH-Px, SOD and MDA levels have been shown in figures 1-3.

Chronic ethanol administration significantly increased MDA level in rat hippocampus. All antioxidants used in the present study significantly prevented the ethanol-induced increase of MDA level. Melatonin, ebselen and PAC were significantly more effective than vitamin E and vitamin C on increased MDA level (p< 0.05, Student's t test) (Figure 1).

Chronic ethanol administration significantly decreased both SOD and GSH-Px level in rat hippocampus (p< 0.05, Student's t test) (Figure 2 and 3). All antioxidants used in the present study prevented significantly the ethanol-induced decrease in SOD level. Melatonin, ebselen and PAC were significantly more effective than vitamin E and vitamin C on decreased MDA level (p< 0.05, Student's t test) (Figure 2).

Melatonin, ebselen and PAC prevented significantly the ethanol-induced decrease in GSH-Px level (p< 0.05, Student's t
test). Vitamin E and vitamin C was found to be ineffective on the ethanol-induced decrease in GSH-Px level (ps> 0.05, Student’s t test) (Figure 3).

**Figure 1:** Effects of antioxidant treatments on chronic ethanol-induced change in MDA level of rat hippocampus [n= 8 for each group; MDA: malondialdehyde; control (-): no ethanol administered control group; control (+): chronic ethanol administered control group; PAC: proanthocyanidin; vit E: vitamin E; vit C: vitamin C; #: significantly different from control (-), *: significantly different from control (+) and $: significantly different from vitamin E and vitamin C, p< 0.05, Student’s t test].

**Figure 2:** Effects of antioxidant treatments on chronic ethanol-induced change in SOD level of rat hippocampus [n= 8 for each group; SOD: superoxide dismutase; control (-): no ethanol administered control group; control (+): chronic ethanol administered control group; PAC: proanthocyanidin; vit E: vitamin E; vit C: vitamin C; #: significantly different from control (-), *: significantly different from control (+) and $: significantly different from vitamin E and vitamin C, p< 0.05, Student’s t test].
DISCUSSION

The main finding of the present study is that chronic administrations of ethanol caused marked oxidative stress responses i.e. increased MDA and decreased SOD and GSH-Px levels in rat hippocampus and antioxidants such as melatonin, ebselen, PAC, vitamin E and vitamin C had an ability to reverse these changes. According to our findings, melatonin, ebselen and PAC were more effective than vitamin E and C on ethanol-induced oxidative stress responses. Thus, we found to be ineffective of vitamin E and C on decreased level of GSH-Px after ethanol-induced oxidative stress in hippocampal formation of the rats. Although vitamin E and C had some significant effects on elevated MDA and reduced SOD levels, their effects were significantly lower than those by melatonin, ebselen and PAC. As important, we showed for the first time that the beneficial effects of PAC on ethanol-induced oxidative stress in brain. In addition, our findings involved in the beneficial effects of vitamin C on MDA and SOD levels in rat hippocampus are the first data in literature.

Chronic ethanol consumption in humans and animals is associated with the development of significant neurological abnormalities in cortical and subcortical structures of brain. In mammals, ethanol is metabolized in two steps. First, alcohol dehydrogenase converts ethanol to acetaldehyde, a toxic and reactive molecule. Second, aldehyde dehydrogenase converts acetaldehyde to acetate. Each of these reactions leads to the formation of one molecule of nicotinamide adenine dinucleotide, reduced form (NADH) which enhances the activity of the respiratory chain, including increased O$_2$ consumption and ROS formation.$^{(41)}$
Besides to promote the formation of ROS, further chronic ethanol exposure inhibits the neuronal growth factors and alters function of many neurotransmitter receptors and ion channels in brain.(27). Composed ROS depending on the oxidative stress are related to several damages which affect cognitive processes. Hippocampus is one of the important brain areas related to cognitive processes. Thus, abnormal processes in the hippocampus result in memory disorders in mammalian brain. Chronic ethanol consumption markedly impairs important hippocampal function and causes problems such as disruption of long-term potentiation and progressive learning and memory deficits.(12). Therefore, antioxidant administration may be helpful for preventing and/or treatment of hippocampal damages in alcoholics. Results from a number of previous studies have been indicated that melatonin,(11,30) ebselen(12,13) and vitamin E(20,21,28) had protective and curative effects on ethanol-induced oxidative stress responses in rat hippocampus are in line with our findings. We confirmed those findings with melatonin, ebselen and vitamin E and we expanded them with some additional data regarding PAC and vitamin C.

Results of the present study indicate that both vitamin E and vitamin C were ineffective on reduced GSH-Px level in hippocampus. Furthermore, these antioxidants had significantly lower action on elevated MDA and reduced SOD levels than melatonin, ebselen and PCA. Our data has a limitation to explain what can be responsible for lower activity of the vitamins. Absorption and/or distribution problems by gastric route may be related to their lower activity. Testing of higher doses of these compounds could be thought. However, used dose (100 mg/kg) in our study seems to be adequate because of observing some significant effects on MDA and SOD levels by this dose. On the other hand, these observations implied that biochemical changes, especially reduced GSH-Px levels, in hippocampal formation after chronic ethanol consumption could be more resistant to the effects of vitamin E and C. Melatonin, ebselen and PAC may be better choice for preventing chronic ethanol-induced cognitive impairments.

Previous studies indicated that chronic ethanol consumption above 9 g/kg/day had some neurotoxic behavioral impairment such as reduced locomotor activity and motor coordination in rats(4,18,33). In our study, we detected higher ethanol consumption (over 11 g/kg/day). In addition, high blood alcohol concentration (80-100 mg/dl) was detected in rats at 28th day of the chronic ethanol consumption. According to the results of our previous studies daily ethanol consumption over 11 g/kg/day more than two weeks also produced physical dependence in rats(32,34,36). Some studies from our laboratory also indicated that chronic alcohol administration by liquid diet to rats caused some marked neurochemical and metabolic changes in rat brain.(35,37,39) In the present study, it could be expected that the heavy ethanol consumption may cause some marked changes in biochemical markers of oxidative stress in rats. Overall these data imply that chronic heavy ethanol administration to rats by a liquid diet technique for almost four weeks might be used as a brain damage model in rats. Thus, we observed some marked changes in biochemical components due to oxidative stress in rat hippocampus following chronic ethanol exposure.

Doses of the antioxidants used in our study were selected as to the results of our preliminary experiments and previous studies. These doses of antioxidants did not cause any abnormal behaviors or any prominent changes in locomotor activities of the naïve rats.

CONCLUSION

Our results suggest that chronic ethanol exposition causes oxidative stress responses that can be reversed by melatonin, ebselen, PAC, vitamin E and
vitamin C treatments in hippocampal area of the rat brain. Melatonin, ebselen and PAC are more effective than vitamin E and vitamin C on ethanol-induced oxidative stress and these antioxidants may be helpful for preventing oxidative-stress induced damages in hippocampal formation in alcoholics.

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Contributors

Dr. Uzbay was the study coordinator. He also wrote the manuscript. Dr. Uzbay, Dr. Macit, Dr. Ulusoy and Dr. Celik were involved in the protocol design. Dr. Macit and Dr. Kayir were involved in the collection and analysis of data. All authors were involved in writing of the preliminary paper; reviewed and revised the final manuscript.

Conflict of interest

The authors have no conflicts of interest to disclose in relation to this manuscript.

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