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ALTERATIONS IN ERYTHROCYTE ANTIOXIDANT SYSTEM AFTER CHRONIC ADMINISTRATIONS OF HIGH DOSE OF ETHANOL TO MICE

Ebru BEYTUT* Aysel GÜVEN** Nadide Nabil KAMİLOĞLU*

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Summary: The aim of this work was to determine the levels of thiobarbituric acid reactive substances (TBARS) as indicator of lipid peroxidation and the activity of glutathione peroxidase (GSH-Px) and the levels of reduced glutathione (GSH) in red blood cell (RBC) of high dose ethanol-treated mice. Sixteen mice (3 weeks of age) were used and all mice were randomly divided into two groups. The first group was used as a control and a placebo was given to the group by oral route twice per week. The second group were given for 12 weeks with ethanol by oral route twice per week, 5ml/kg bw.

TBARS levels in the plasma of the ethanol group were significantly increased ($p<0.001$) compared with that of the control group. The GSH-Px activity of RBC was significantly ($p<0.001$) lower in ethanol group than in control group. In addition, GSH levels of mice RBC significantly ($p<0.01$) decreased in ethanol-treated group compared to control group. These results indicated that ethanol-induced increase in plasma TBARS levels was associated with reductions in GSH-Px activity and in GSH levels of the mice RBC.

Key Words: Ethanol, Lipid Peroxidation, Glutathione, Glutathione Peroxidase, Erythrocyte, Mice.

Kronik Olarak Yüksek Dozda Etanol Verilen Farelerin Eritrosit Antioksidan Sistemindeki Değişiklikler

Özet: Bu çalışmanın amacı, yüksek dozda etanol verilen farelerin eritrositlerindeki redükte glutatyon düzeyleri (GSH) ve glutatyon peroksidaz aktivitesi (GSH-Px) ile lipid peroksidasyonunun bir indikatörü olan tiyobarbiturik asit substratlarının (TBARS) düzeylerini belirlemekti. Bu amaç için çalışmada toplam 3 haftalık 16 fare kullanıldı.Tüm fareler 2 gruba ayrıldı. İlk grup kontrol grubuydu ve bu gruba serum fizyolojik (plasebo) 12 hafta süre ile haftada iki kez ağız yoluyla verildi. İkinci gruba ise, yine 12 hafta süresince haftada iki kez 5 ml/kg c.a. etanol ağız yolu ile verildi.

Etanol grubunun plazma TBARS düzeyleri kontrol grubuna kıyasla önemli düzeyde yüksek ($p<0.001$) bulundu. Ancak, etanol grubunun eritrosit GSH-Px aktivitesi ile GSH düzeyleri kontrol grubuna göre daha düşük düzeylerdeydi (sırasıyla $p<0.001$, $p<0.01$). Bu sonuçlar gösterdi ki; farelere yüksek dozda etanol'ün kronik olarak verilmesine bağlı olarak meydana gelen plazma TBARS düzeylerindeki artış, eritrositlerindeki GSH düzeyleri ve GSH-Px aktivitelerinin azalışına neden olmuştur.

Anahtar Sözcükler: Etanol, Lipid Peroksidasyon, Glutatyon, Glutatyon Peroksidaz, Eritrosit, Fare.

INTRODUCTION

Experimental evidence indicates that a number of toxic and carcinogenic processes, induced by physical and chemical agents in liver and other organs, involve the formation of reactive radical species[1,2] which can induce autoxidative changes in biomembranes and other cellular components, resulting eventually in cell death[3].

Free radical-mediated peroxidation phenomena play an important role in the mechanism of cellular damage caused by ethanol[4]. In addition, hepatotoxins-induced oxidative damage in erythrocytes causes loss of membrane function by enhancing lipid peroxidation and alterning the erythrocyte antioxidant system[5]. Antioxidant defences, present in all aerobic organisms, include antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and free radical scavengers (Vitamin C and E, carotenoids, glutathione) whose function is to remove oxygen reactive species (ROS), thus protecting organisms from oxidative stress. Hydrogen peroxide (H_2O_2) and organic hydroperoxides will also be destroyed in the cytosolic and mitocondrial compartments by glutathione peroxidase (GSH-Px, EC 1.11.1.9) in the presence of reduced glutathione (GSH). GSH, which has been previously oxidised, will be regenerated by glutathione reductase (GR, EC 1.6.4.2), participating in the turnover of reduced GSH pools[6,7].

Various toxic agents are known to have a high affinity for thiol groups. Despite an accumulating body of data concerning the effcets of erythrocytes GSH levels and GSH-Px activities and subsequent cell function, the relationship of altered GSH level and GSH-Px activity of erythrocytes to toxicity of hepatic agents are rather limited and contradictory. Moreover, although increased lipid peroxidation (LPO) has been

* Department of Physiology, Faculty of Veterinary Medicine, University of Kafkas, Kars-TURKEY
** Department of Biology, Faculty of Science, University of Kafkas, Kars-TURKEY

reported in the liver after ethanol consumption, little information is available as to LPO caused by ethanol on the plasma. Therefore, the aim of the present study was to investigate the effect of chronic administration of high dose of ethanol on plasma LPO levels and GSH-Px activities and reduced GSH levels in erythrocytes of mice.

MATERIALS and METHODS

Animals and Collection of Samples: Three old weeks clinically healthy female Swiss Albino mice (n=16) weighing 25-30 g were used in the this study. They were housed, 4 mice to a cage, in stainless-steel wire-mesh cages in a temperature-controlled room at 24 C with 55% relative humidity and 12 h light-dark cycle. The animals were given ad libitum a diet including the ingredients shown in Table 1 and tap water.

The animals were allowed to acclimatize for 15 days and were then divided randomly into two groups of 8 animals each. The first group was used as a control and a placebo was given by oral route to the group twice per week. The second group were given for 12 weeks with a high dose of ethanol by oral route twice per week, 5ml/kg bw.

At the end of the 12 weeks, mice were killed by decapitation and blood was collected using heparin (nevparin 5000 U/ml) as anticoagulant. The plasma and the red blood cells (RBC) were separated by centrifugation (2500 g, for 15 min at 4 °C). Plasma was frozen (-20 °C) until further analysis. Packed cells were washed 3 times with 0.9 % sodium chloride and then hemolysed by exposure to 9 parts redistilled water followed by freezing (-20 °C for 18 h) and thawing before analysis. Plasma specimens were used for the determination of malondialdehyde (MDA).

Analysis of Thiobarbituric Acid Reactive Substances in the Plasma: Lipid peroxidation (MDA) was estimated by the method of Placer et al.[8] based on thiobarbituric acid-reactive substance (TBARS) reactivity. MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex that has maximum absorbance at 532 nm. Values were expressed as MDA equivalents in nmol/ml plasma.

GSH-Px and Reduced Glutathione Assay: Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was measured in RBC by the direct method as described by of Lawrence and Burk[9]; oxidation of GSH was determined with Ellman's reagent[10]. In the reaction, cumene-hydroperoxide was used as oxidizing agent. The GSH-Px activity was expressed as units (1 unit the enzyme quantity that oxidizes 1 mol GSH/ min in the above system at 25 °C). The GSH level of the hemolysate was measured spectrophotometrically using Ellman's reagent[10]. The hemoglobin concentration in lysed erythrocytes was determined by cyanmethemoglobin method[11].

Statistical Analysis: The results were expressed as mean values ± SE. The significances between mean values were determined according to Mann-Whitney U-test evaluated by the SPSS (version 6.0) computer program. The 0.05 level was chosen as the minumum statistical significance throughout.

RESULTS

A statistically significant increase in TBARS levels was found in plasma of the animals treated with ethanol when compared to controls ($p<0.001$, Table II). In addition, GSH-Px activity in RBC was also significantly reduced in mice exposed to ethanol when compared to controls ($p<0.001$) (Table II). Also, decreased levels of GSH were found in RBC after treatment with ethanol ($p<0.01$) (Table II).

Table I: Diet composition
Tablo I: Diyet bileşimi

Ingredients	%
Wheat	10
Corn	23
Barley	15
Wheat bran	8
Soybean	26
Fish flour	8
Meat-bone flour	4
Pelled	5
Salt	0.8
Vitamin mineral mix*	0.2

* Vit. A, D_3, E, K_3, B_1, B_2, B_6, and B_{12}, nicotinamid, folic acid, biotin, Mn, Fe, Zn, Cu, I, Co, Se, antioxidants (butylhydroxytoluol) and Ca

Table II: Levels of investigated paremeters in plasma and RBC of mice in the control, and in group given high dose of ethanol.
Tablo II: Yüksek dozda etanol verilen grup ile kontrol grubundaki farelerin plazma ve eritrositlerinde araştırılan bazı parametrelerin düzeyleri

	Groups	
	Control	Group with Ethanol[1]
Plasma TBARS (nmol/ml)	1.34±0.54	3.12±0.12a
RBC, GSH-Px (U/g Hb)	43.25±3.25	21.28±0.62a
RBC, GSH (nmol/ml)	1.98±0.09	0.87±0.05b

Note: Results are expressed as mean ± SE.
a: $p<0.001$, b: $p<0.01$
[1] Mice were given by oral route for 12 weeks 5 ml/kg bw ethanol twice per week.

DISCUSSION

Previous studies have demonstrated that a marked elevation of LPO (determined as malondialdehyde) was observed in the plasma of mice after chronic CCl_4 intoxication[13]. Ethanol-induced oxidative stress in plasma was mediated via LPO, reflected by significant reductions in GSH-Px activity and in reduced GSH levels in erythrocytes (Table II). We also found an increase in LPO due to ethanol in the plasma of mice after 12 weeks. Similarly, Topinka et al.[14] reported an increase in peroxyl radicals in the plasma of chronic alcoholics. However, there are few reports studying ethanol-induced LPO in plasma.

It has been suggested that the primary event in the development of the ethanol-induced fatty liver, as well as in liver injuries induced by other chemicals, is the formation of lipoperoxides at selective subcellular sites because of the alteration in antioxidant activity of the hepatic cell[15-17]. As lipid peroxide could be initiated by the enhanced production of oxygen free radicals and the impaired enzymatic and non-enzymatic defences of the liver, it is conceivable that the liver of mice treated with high dose of ethanol easily tends to the production of the reactive oxygens and lipid peroxides.

In living cells, there is a continuous production of free radicals and many pathologies are belived to result from damage to tissue initiated by them due to the production of the radicals overwhelming the defense mechanisms. Antioxidative enzymes play important roles in eliminating free radicals and preventing LPO in tissues and plasma[18] and reduction of activities associated with increased LPO[19]. GSH participates in a number of important cellular processes, including protection of cells against the toxic effects of oxygen, radiation, and other compounds[20-22]. The literature on GSH levels in RBC after ethanol toxication are rather limited and contradictory. Likewise, Goebel and Schneider[23] reported a two fold reduction in GSH levels in the erythrocytes of the chronic alcoholic. Similarly, as shown in Table II, we also found a decrease in the levels of GSH in the erythrocytes after chronic administration of ethanol. This data indicate that the antioxidant defense system against oxidative stress was impaired after chronic administration of ethanol. In addition, intracellular GSH gives protection from ethanol-dependent LPO[24], suggesting that GSH may represent an important protection mechanism against chronic ethanol toxicity.

The antioxidant enzymes and other antioxidants provide the cells with protection against oxidative stress[5]. For inctance, GSH-Px uses GSH to reduce various hydroperoxides, and is involved in the removal of H_2O_2 and in the protection of unsaturated lipids of biomembranes from LPO[25,26]. We observed a decrease in the GSH-Px activity due to chronic ethanol administration. Similarly, Luty-Frackiewicz et al.,[27] reported a reduction in GSH-Px activity in liver of alcholics. These results indicated that the increased LPO caused by ethanol administration depended on the reduction in the activity of GSH-Px.

In conclusion, adverse effects of ethanol produced decrease of the antioxidant defence system, the significant elevation of oxidative activity and lipid peroxides in the plasma, possibly leading to the appearance of some adverse effects. Further examination of the changes in the metabolic pattern in plasma should help to clarify the mechanism of oxidative stress produced by chronic administration ethanol.

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Yazışma adresi (corresponcende address

Yrd.Doç.Dr. Ebru BEYTUT
Department of Physiology, Faculty of Veterinary Medicine,
University of Kafkas, Kars-TURKEY
Tlf: +90 474 2426800