

Melatonin and Vitamin C Administration Ameliorate Alcohol-Induced Oxidative Stress and eNOS Expression in Liver of Rats

Melatonin ve C Vitamini Sıçan Karaciğer Dokusunda Alkol ile Oluşturulan Oksidatif Hasarı ve eNOS Ekspresyonunu İyileştirmektedir

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Abstract

Purpose: The aim of this study was to investigate the effects of melatonin and vitamin C on alcoholic liver disease.

Material and Methods: Twenty-four adult male Wistar rats were used in this study. Rats were divided into four equal groups. Group I (control): rats were not fed on alcohol; Group II: rats were fed on alcohol during 28 days; Group III: rats were fed on alcohol and 40 mg/kg vitamin C were injected intraperitoneally and Group IV: rats were fed on alcohol and 4 mg/kg melatonin were injected intraperitoneally. At the end of the experiment, rats were sacrificed and liver tissues were processed.

Results: Light microscopic examinations revealed steatosis in the ethanol-fed group. Expression of eNOS was distributed mainly in periportal regions of the acinus while decreased eNOS immunoreactivity was observed in fatty hepatocytes. In melatonin and vitamin C treated groups, eNOS immunoreactivity was not changed compared to alcohol groups. Chronic ethanol ingestion significantly increased MDA, SOD and catalase activity and melatonin significantly decreased MDA levels.

Conclusion: Present results indicated that alcohol consumption caused steatosis especially around central vein and mononuclear cell infiltration in some areas and melatonin and vitamin C partially ameliorated this damage.

Keywords: **Alcohol Consumption; Ascorbic Acid; Endothelial Nitric Oxide Synthase; Melatonin; Liver**

Özet

Amaç: Bu çalışmada alkolik karaciğer üzerine melatonin ve C vitamininin etkilerini araştırmayı amaçladık.

Gereç ve Yöntem: Bu çalışmada 24 adet erkek Wistar albino sıçanlar kullanıldı. Sıçanlar rasgele olarak dört gruba ayrıldı. Grup I; kontrol (n:6); Grup II; 28 gün boyunca alkol içeren sıvı diyet ile beslenen sıçanlar; Grup III; alkol ile birlikte günde 40mg/kg intraperitoneal C vitamini verilen ve Grup IV; alkol ile birlikte günde intraperitoneal 4mg/kg melatonin verilen sıçanlardan oluşturuldu. Deney sonunda sıçanlar dekapite edilerek karaciğer dokuları alındı biyokimyasal ve eNOS immunohistokimyasal incelemesi yapıldı.

Bulgular: Işık mikroskopik incelemelerde alkol alan grupta yağlı karaciğere rastlandı. İmmunohistokimyasal olarak eNOS ekspresyonu başlıca periportal alanda izlendi. Yağlı karaciğer hücrelerinde eNOS immün boyanması daha azdı. Melatonin ve C vitamini uygulanan gruplarda eNOS boyanmasında çok belirgin bir farklılık saptanmadı. Biyokimyasal olarak alkol tüketimi malondialdehit ve süperoksit dismutaz düzeyleri ve katalaz aktivitelerini artırırken melatonin malondialdehit seviyesini azalttı.

Sonuç: Bu çalışmadaki bulgular, alkol tüketiminin karaciğerde özellikle sentral ven etrafında yağlanma, bazı alanlarda mononükleer hücre infiltrasyonuna neden olduğunu ve melatonin ve C vitamininin bu hasarı kısmen düzelttiğini göstermektedir.

Anahtar Kelimeler: **Alkol tüketimi; Askorbik asit; Endotelial Nitrik Oksit Sentaz; Karaciğer; Melatonin.**

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Introduction

Excessive ethanol consumption commonly induces hepatic, gastrointestinal, nervous, and cardiovascular injuries leading to physiological dysfunctions (1). Excess ethanol leads to three pathologically distinct disorders in the liver, namely fatty liver (ethanol-associated hepatic steatosis), alcoholic hepatitis and cirrhosis. Alcohol-associated hepatic steatosis is the most common form of liver injury and is reversible with abstinence (2, 3). More serious forms of alcoholic liver disease (ALD) contain alcoholic hepatitis, characterized by persistent inflammation of the liver, and cirrhosis, characterized by progressive hepatic fibrosis. Studies suggested that chronic alcohol consumption can sensitize the liver to diverse additional pathogens and stresses such as viral hepatitis (4, 5), hemorrhagic shock (6), ischemia-reperfusion injury (7), and endotoxemic stress (8). This sensitization is thought to play a major role in the progression of ALD to nonreversible advanced stages (9).

Almost all ingested ethanol is metabolized in the liver. Two major enzyme systems, namely the oxidative and nonoxidative pathways, mediate the initial phase of ethanol metabolism (10). The oxidative pathway comprises the alcohol dehydrogenases and members of the cytochrome P450 system (11). This pathway generates acetaldehyde. Ethanol and acetaldehyde have harmful effects both direct and indirect, for example by generating reactive oxygen species (ROS) and causing damage to the intestinal mucosal barrier (10, 12).

One of the most prominent antioxidant defense systems are vitamins (A, C and E) acting as co-factors for many enzymes, protecting cells against free radical-mediated damage (13), as free radical scavengers. Vitamin C (Ascorbic acid; AA) is an important water soluble vitamin. It is an antioxidant available through dietary intake that significantly minimizes the effects of free oxygen radicals (14). The pineal hormone melatonin is involved in the circadian regulation and facilitation of sleep, the inhibition of cancer development and growth, and the enhancement of immune function. Melatonin is also known as the highly powerful endogenous antioxidant (15).

Nitric oxide (NO) is a short-living biological mediator generated from L-arginine by NO synthase (NOS). The NOS family of enzymes identified to date includes constitutively expressed endothelial NOS (eNOS or type 3 NOS) and neuronal NOS (nNOS or type 1 NOS), as

well as inducible NOS (iNOS or type 2 NOS). The NO exerts a broad spectrum of physiological functions, involving regulation of vascular reactivity, platelet and leukocyte activation, neurotransmission, regulation of cellular proliferation, and nonspecific immunity reactions (16). Inappropriate release, metabolism, or actions of NO have been associated with various vascular, ischemic, thrombotic, and inflammatory pathologies. In the liver, NO is generated by eNOS and iNOS, and this generation can mediate a number of physiological and disease reactions including this organ (16).

The aim of the present study was to determine whether AA and melatonin had a protective effect on ethanol-induced changes in lipid peroxidation and eNOS expression and antioxidant enzymes activities in the liver.

Materials and Methods

Animals. The study was conducted in Erciyes University Hakan Çetinsaya Experimental and Clinic Research Center. Ethical approval for study was obtained from Erciyes University Animal Researches Local Ethics Committee and all procedures conformed to the "Guide for the Care and Use of Laboratory Animals". Twenty-four adult male Wistar rats, 200–250 g in weight at the beginning of the experiments were used. They were housed in a quiet and temperature-and humidity-controlled room (21 ± 3 °C and $60 \pm 5\%$, respectively) in which a 12-h light: 12-h dark cycle was maintained (07:00–19:00 h light). Animals were randomly separated into four groups of six rats each. The first group served as control and received liquid diet not containing ethanol. The second group was the ethanol treatment group: rats were fed the ethanol-containing liquid diet for 28 days. The third group received ethanol-containing liquid diet and 40 mg/kg/day AA (Redoxon, Bayer) were injected intraperitoneally. The fourth group received ethanol-containing liquid diet and 4 mg/kg/day melatonin (Merck, cat. no:814537) was injected intraperitoneally (17).

Chronic ethanol treatment. For chronic ethanol exposure, the rats were housed individually and ethanol was given in the modified liquid diet as previously described by Ozbay and Kayaalp (18). At the beginning of the study, modified liquid diet without ethanol was given to rats for 7 days. Then liquid diet with 2.4% ethanol was

administered for 3 days. The ethanol concentration was increased to 4.8% and 7.2% for the following 4 and 21 days on a liquid diet, respectively. Control rats were pair fed with an isocaloric liquid diet not containing ethanol. Liquid diet was freshly prepared daily and presented at the same time of the day (10:00 h). The animals were not allowed to access the drinking water. Animals were weighted and recorded everyday and ethanol intake was also measured and expressed as grams per kilogram.

At the end of experimental period, animals were sacrificed by decapitation under intraperitoneal ketamine (75 mg/kg) + xylazine (10 mg/kg) anesthesia. After decapitation, liver tissues were quickly removed. Some of the liver tissues were used for biochemical analyses and the other tissues were used at histological procedures in the same animals.

Biochemical analysis of liver tissues. For biochemical analysis, the tissues were weighed and homogenized in four volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.50 mL/L Triton X-100 for 2 min at 13.000 rpm using a homogenizer (IKA Ultra- Turrax T 25 Basic). Tissue homogenates were then centrifuged at 5000 \times g for 60 min to remove debris. Clear supernatant fluids were separated and kept at -40°C until the enzyme activity measurements were performed.

Determination of Malondialdehyde (MDA) level.

The MDA level was determined with the method modified by Ohkawa et al (19). The MDA is the end product of fatty acid peroxidation and reacts with thiobarbituric acid to form a pink complex. The color was determined at 532 nm.

Determination of Superoxide Dismutase (SOD) activity.

Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined based on the method of Sun et al (20). The principle of the method is based on the inhibition of Nitro Blue Tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1 mL of ethanol-chloroform mixture (5:3, v/v) was added to the same volume of sample and centrifuged. Total SOD activity was measured after incubation for 20 min at 25°C spectrophotometrically at 560 nm. The SOD (1U) was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as U/g protein.

Determination of Catalase (CAT) activity

The CAT activity was determined based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H₂O₂. The CAT activity was expressed as nmol/min/ml (21).

Histological Procedures. Parts of the tissues were fixed in 10% formalin for 24 hours, rinsed under running tap water for 24 hours, followed by dehydration through a graded ethanol series. Tissues were made transparent in xylol and embedded in paraffin. Five micrometer thick sections were stained with Hematoxylin-Eosin (H&E) and Masson's trichrome and photographs were taken with an Olympus BX-51 photomicroscope.

Immunohistochemistry.

The expression of eNOS was detected immunohistochemically in the liver using a rabbit polyclonal antibody (sc.654 Santa Cruz Biotechnology, CA, USA) and the streptavidin-biotin peroxidase technique. The procedure was performed under identical conditions for all sections. Paraffin sections (5 μ m) were dewaxed in xylene. The sections were rehydrated, rinsed in de-ionized water and were subjected to 2N HCl solution for 20 min to antigen retrieval. Endogenous peroxidase activity was inhibited 3% H₂O₂ in methanol for 10 min. The specimens were washed in phosphate-buffered saline (PBS) three times (5 min each time) and preincubated in a 1.5% normal goat serum (NGS, sc.2043, Santa Cruz Biotechnology, California, USA) in phosphate-buffered saline (PBS) for 20 min at room temperature in a humidified chamber. Then, the sections were incubated overnight at 4°C with eNOS antibody (2 μ g/ml diluted in PBS with 1.5% NGS). Negative control sections were done by replacing the eNOS antibody by PBS. The sections were then incubated with biotinylated goat anti-rabbit IgG (1 μ g/ml diluted in PBS with 1.5% NGS) (sc.2040, Santa Cruz Biotechnology, California, USA) for 30 min at room temperature in a humidified chamber, followed by streptavidin horseradish peroxidase (HRP) conjugate (ready-to-use) (catalog no: 50-420Z, Zymed Laboratories Inc., South San Francisco, California, USA) for 30 min and AEC (red) substrate kit (catalog no: 00-2007, Zymed Laboratories Inc., South San Francisco, California, USA) for 5 min. Finally the sections were counterstained with haematoxylin, rinsed in de-ionized water and mounted with clearmount solution (ready-to-use) (catalog no: 00-8010, Zymed Laboratories Inc., South San Francisco, CA,

USA). The sections were examined and photographed under a light microscope (Bx-51, Olympus, Tokyo, Japan).

Immunohistochemical eNOS staining intensity in the liver tissues of all groups was evaluated semi-quantitatively by two independent histologists in a blind fashion. The intensity of eNOS expression was scored as follows: no staining (-), low (+), moderate (++), strong (+++).

Statistical Analysis. All statistical analyses were performed with the Statistical Package for Social Sciences software package (SPSS for Windows 11.5, SPSS, Chicago, Illinois, USA). The data were expressed as mean \pm standard deviation (SD), median, minimum and maximum. The data were statistically analyzed by Kruskal-Wallis. Post hoc analyses were carried out with the Student-Newman-Keuls test. Statistical significance was set at $P < 0.05$.

Results

No statistically significant body-weight changes were determined during the study period in all investigated groups. The daily ethanol consumption of the rats ranged from 11.3 to 15.1 g/kg. Light microscopic examinations revealed normal liver tissue in the control group (Figure 1A). Mixed micro- and macro-vesicular steatosis scattered in the liver lobule was seen in the ethanol-fed group. Steatosis was observed to be stronger around central vein than portal area (Fig. 1b). Mononuclear cell infiltration in some fields (Fig. 1c), activation of kupffer cells and congestion of the vessels (Fig. 1d) due to ethanol consumption were observed. Steatosis could be seen even when vitamin C (Fig. 2a) or melatonin (Fig. 2c) was less than administered to ethanol consumed rats. In these groups, mononuclear cell infiltration and congestion of the vessels were not evident (Fig. 2b, 2d).

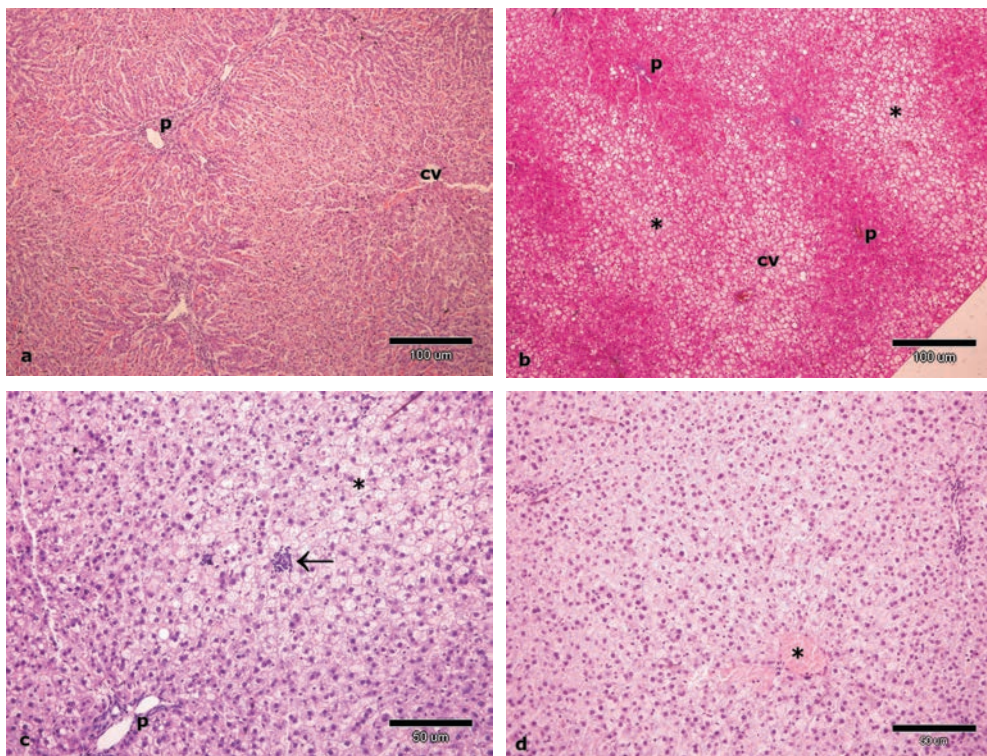


Figure 1. (a) Group I. Portal area (p), central vein (cv) and hepatocytes can be seen as normal (H&E) (Scale Bar: 100 μ m), (b) Group II. Steatosis (asterisk) is observed especially hepatocytes surrounding the central vein (Masson's Trichome) (Scale Bar: 100 μ m), (c) Group II. Mononuclear cell infiltration (arrow) and fatty hepatocytes (asterisk) can be seen in the hepatic lobule (H&E) (Scale Bar: 50 μ m), (d) Group II. Congestion of vessels (asterisk) can be observed in the central vein (H&E) (Scale Bar: 50 μ m).

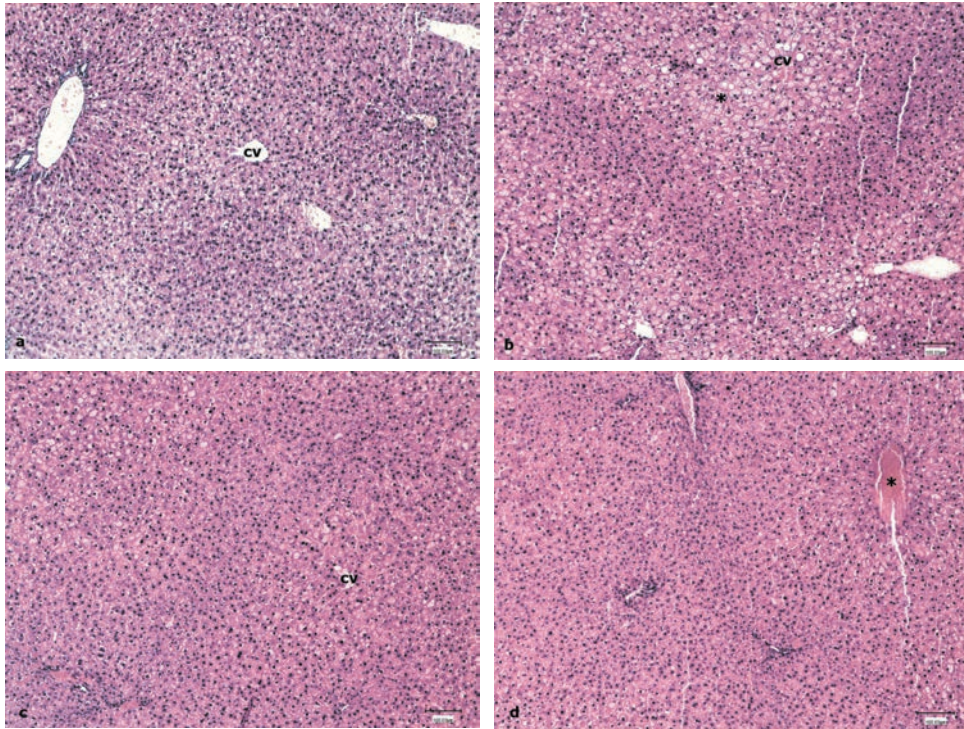


Figure 2. (a) Group III. Steatosis (asterisk) is observed especially hepatocytes surrounding the central vein (cv) (H&E) (Scale Bar: 100µm), (b) Group III. Fatty hepatocytes (asterisk) can be seen especially surrounding the central vein (cv) (H&E) (Scale Bar: 100µm), (c) Group IV. Portal area, central vein (cv) and hepatocytes can be seen as close of normal (H&E) (Scale Bar: 100µm), (d) Group IV. Congestion of vessels (asterisk) can be observed in the central vein (H&E) (Scale Bar: 100µm).

eNOS expression was seen with ethanol and melatonin and vitamin C treatment, and the immune-staining was localized to hepatocytes (+++), vascular endothelium (++) and epithelial cells of biliary duct (++) . Figure 3 shows the distribution of eNOS immunoreactivity in liver. Low-power micrograph (Fig. 3a) documents that eNOS was uniformly distributed in hepatocytes throughout all zones. Fig. 3b depicts the distribution of eNOS immunoreactivity in alcoholic liver. Expression of eNOS

was distributed mainly in periportal regions of the acinus while in ethanol-induced fatty hepatocytes was observed to decrease eNOS immunoreactivity (++) . In melatonin and vitamin C treated groups, eNOS immunoreactivity was not changed compared to ethanol groups (Fig. 3c). Negative controls, where incubation with the primary antisera was omitted, were completely unlabelled, as seen in Figure 3d.

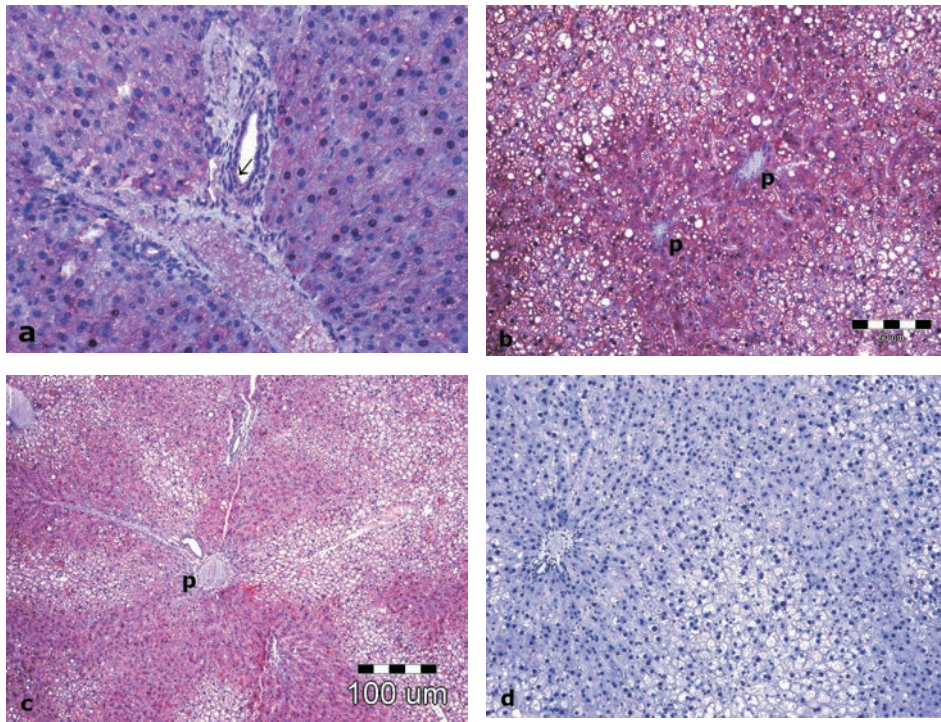


Figure 3. (a) Group I. The presence of eNOS in the hepatocytes and epithelial cells of biliary duct (arrow) (Scale Bar: 20µm)., (b) Group II. The eNOS immunoreactivity is strongly expressed in the cytoplasm of hepatocytes surrounding the portal triad (p) (Scale Bar: 50µm)., (c) Goup III. eNOS immunoreactivity are clearly seen in hepatocytes of periportal (p) area) (Scale Bar: 100µm). (d) Group II. Negative controls) (Scale Bar: 50µm).

The effect of AA and melatonin in alcoholic-liver SOD and MDA levels and CAT activity is depicted in (Table I). MDA levels were significantly found higher in chronic ethanol ingestion groups when compared control group, indicating the enhanced membrane lipid

peroxidation in the liver. In melatonin-treated group MDA levels were lower than in the ethanol groups, contrary to AA-treated group. However the SOD levels and CAT activity did not statistically different in ethanol groups from control.

Table I. Malondialdehyde (MDA) and Superoxide dismutase (SOD) levels and Catalase (CAT) activity in the liver.

	GROUPS				p
	Control (n=6)	Ethanol (n=6)	Ethanol + Vitamin C (n=6)	Ethanol + Melatonin (n=6)	
CAT	93.01±12.18 94.84 (70.25-104.53)	103.10±7.44 104.30(89.76-112.07)	108.01±5.74 110.04(96.93-113.06)	103.52±14.05 106.71(82.54-117.19)	0.112
MDA	3.23±0.88 3.03 (2.28-4.77) ^a	6.83±2.12 6.40 (4.37-9.43) ^b	5.70±1.80 5.05 (4.14-8.95) ^b	4.22±0.35 4.14 (3.87-4.66) ^c	0.003
SOD	1.25±0.81 1.33 (0.28-2.36)	1.49±0.74 1.23 (1.01-3.00)	0.57±0.27 0.53 (0.26-1.08)	0.66±0.51 0.39 (0.25-1.43)	0.095

Values are expressed as mean ± Std. Deviation, median, minimum and maximum. ^{a,b,c} : Differences between means bearing different letters in each line are significant (P<0.05). p values belong to Kruskal-Wallis.

Discussion

In this study, liver tissues of the alcohol consumption rats were observed steatosis, congestion of vessels and mononuclear cells infiltration in some fields. These findings agree with the fatty liver and hepatic inflammation.

Kupffers cells play pivotal role in the normal physiology and homeostasis of the liver as well as participating in the acute and chronic replies of the liver to toxic compounds. Activation of Kupffer cells, either directly or indirectly, results in the release of an array of inflammatory and growth control mediators as well as reactive oxygen species. This activation appears to regulate acute hepatocyte injury as well as chronic liver responses including hepatic cancer (22). In the present study, activation of Kupffer cells was determined in alcohol treated rats and this activation contributed to the potential liver damage.

Alcohol-induced oxidative stress is related to the ethanol metabolism. Three metabolic pathways of ethanol have been described in the human body so far. They involve the following enzymes: alcohol dehydrogenase, microsomal ethanol oxidation system (MEOS) and catalase. Each of these pathways could produce free radicals which affect the antioxidant system. Oxidative stress plays a pivotal role in the development of ALD (23). Yuan et al (24) demonstrated that liver MDA levels were significantly increased after 4 weeks ethanol consumption. In the current study, liver contents of MDA, a marker of lipid peroxidation, were significantly elevated in the ethanol group as compared with the control. Also, ethanol administration were not altered statistically significant the SOD levels and catalase activity in the liver. El-Sokkary et al (25) demonstrated that chronic administration of ethanol increased MDA levels in the testes, heart, lung and brain with no statistically significant change being in the liver.

In the liver, NO synthesized by the eNOS isoform regulates hepatic blood flow and vascular resistance (26). Numerous studies have shown that the production of NO is abnormal in the injured liver (27, 28). Shah and co-workers (26) showed that, in response to incremental increases in blood flow, cirrhotic animals produced significantly less NO than control animals because of reduced eNOS activity. Similarly, NO release from sinusoidal endothelial cells is reduced in liver cirrhosis; due to impaired function of endothelial cell NOS (27, 28). Wang and co-workers (29)

demonstrated that chronic alcohol intake decreases hepatic eNOS activity by increasing the expression of the inhibitory protein caveolin-1 and enhancing its binding with eNOS. Yuan and co-workers (24) showed that chronic ethanol consumption increased iNOS expression and decreased eNOS expression in the liver. We found that eNOS immunoreactivity was uniformly distributed in hepatocytes throughout all zones. Expression of eNOS was distributed mainly in periportal regions of the acinus while fatty hepatocytes were observed to decrease eNOS immunoreactivity in alcohol treated rats. These findings are in accordance with the previous reports.

Antioxidants are essential in preventing the cellular damage caused by free radical-modified lipid peroxidation. In normal metabolism, there is a balance between the generation of free radicals and antioxidant defense mechanism. Excessive ethanol use commonly leads to vitamin deficiency (30). Recent studies have shown that both vitamins C and E are reduced in alcoholics (31). Di Luzio (32) was the first to observe that pretreatment with antioxidants alleviated hepatic fat accumulation in rats treated with ethanol. The antioxidant pretreatment data clearly indicated that both vitamins C and E can prevent the generation of 1-hydroxyethyl radicals following acute ethanol treatment (33). Melatonin is highly powerful endogenous antioxidant. Recent studies have demonstrated that melatonin administration decreased liver damage (34, 35). However, Genç et al (36) demonstrated that melatonin administration did not MDA levels alcohol-induced liver damage. In the present study, melatonin and vitamin C were used as protective agents against alcohol-induced oxidative damage. Administration of vitamin C and melatonin decreased MDA and SOD levels and CAT activity compared to the alcohol consumption group but these decreases was not significant statistically apart from MDA. These findings agree with the previous studies (25). Furthermore, in melatonin and vitamin C treated groups, eNOS immunoreactivity was not changed compared to alcohol groups and was observed partially improvement as histologically.

As a conclusion, in this study the biochemical and immunohistochemical findings demonstrated increased MDA levels and decreased eNOS immunoreactivity in the fatty hepatocytes of ethanol-treated rats. Present findings suggested that melatonin and vitamin C may be beneficial in decreasing the damage caused by free oxygen

radicals and alleviation of oxidative stress by antioxidant therapy that reduces ROS-mediated NO inactivation.

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