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LABORATORY STUDY

Melatonin and Vitamin C Ameliorate Alcohol-Induced Oxidative Stress and eNOS Expression in Rat Kidney

Mehmet Fatih Sönmez¹, Figen Narin², Derya Akkuş¹ and Ayşegül Burçin Türkmen¹

¹Department of Histology and Embryology, Faculty of Medicine, Erciyes University, Kayseri, Turkey; ²Department of Biochemistry, Faculty of Medicine, Erciyes University, Kayseri, Turkey

Abstract

Objectives: The aim of this study was to investigate the preventive effects of melatonin and vitamin C as antioxidants on renal injury in chronic alcohol consumption. *Materials and methods*: A total of 24 adult male Wistar rats weighing 200–250 g were used in the study. Rats were divided into four equal groups. Group I (control): rats were not fed on alcohol; Group II: rats were fed on alcohol; Group III: rats were fed on alcohol; Group III: rats were fed on alcohol and 40 mg/kg vitamin C; and Group IV: rats were fed on alcohol and 4 mg/kg melatonin. *Results*: Light microscopic examination revealed atrophic renal corpuscles, dilatation and congestion of the peritubular vessels, and renal corpuscles with obscure Bowman's space and a few foamy-appearing tubules due to alcohol consumption were observed. Expression of endothelial nitric oxide synthase (eNOS) was localized to glomerulus, distal, and collector tubules. eNOS staining decreased in alcohol treatment group and melatonin and vitamin C encore increased expression pattern of eNOS. Alcohol consumption increased malondialdehyde (MDA) level and superoxide dismutase (SOD) and catalase (CAT) activities significantly in the alcohol consumption groups compared with that in the control group, while in melatonin give group just MDA level was decreased statistically significant and SOD and CAT activities were also decreased numerically compared with the alcohol consumption groups. *Conclusions*: These results indicated that chronic alcohol consumption caused renal damage by increased lipid peroxidation and melatonin and vitamin C administration produced in some degree protection against alcohol-induced damage.

Keywords: chronic alcohol consumption, eNOS, melatonin, vitamin C, kidney

INTRODUCTION

Most world populations consume alcoholic beverages. It is generally accepted that excessive alcohol consumption can induce dramatic changes in the physiological and biochemical processes of the whole organism and in the cells.¹ Three enzymatic systems are able to fulfill the ethanol oxidation: alcohol dehydrogenase, microsomal ethanol-oxidizing system, and catalase (CAT). About 80% of ingested ethanol is metabolized in the liver. Therefore, the structural and functional alterations of liver because of alcohol abuse have been extensively studied.²⁻⁵ Excessive alcohol consumption usually causes hepatic, gastrointestinal, nervous, and cardiovascular injuries. Some clinical and experimental studies have also been focused on the effects of ethanol feeding on renal function, gross and microscopic morphology of the kidney.^{6–8} While acute alcohol consumption has a nephrotoxic effect on the kidney, alternatively, chronic use in humans may result in alcohol-induced hypertension, indirectly increasing the risk of chronic kidney disease.^{9,10}

Oxidative stress is an imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to tissue damage.¹¹ Oxidative stress- and reactive oxygen species (ROS)-mediated toxicity have been considered the primary routes to alcohol-induced kidney injury.^{12,13} Thus, chronic and excessive alcohol consumption may accelerate oxidative mechanism directly or indirectly, which eventually produces cell death and tissue damage.¹⁴

Ascorbic acid (vitamin C) is a low molecular mass antioxidant that interacts directly with the oxidizing radicals and protects the cells from $ROS.^{15}$ Vitamin C scavenges the ROS by very rapid electron transfer that, thus, inhibits lipid peroxidation.¹⁶ Melatonin is

Address correspondence to Mehmet Fatih Sönmez, Department of Histology and Embryology, Faculty of Medicine, Erciyes University, 38039 Kayseri, Turkey. Tel.: +90 352 4374901/23356; Fax: +90 352 4375285; E-mail: mfsonmez@erciyes.edu.tr Received 14 September 2011; Revised 14 November 2011; Accepted 9 December 2011

synthesized by the pineal gland, retina, gastrointestinal tract, and so on and is secreted by the pineal gland. In vivo and in vitro, melatonin has been known as a radical scavenger with the ability to remove ROS and reactive nitrogen species.¹⁷ Regarding the protective effect of melatonin on renal alterations various studies have been carried out and it has been found to protect tissues against oxidative damage generated by a variety of toxic agents.¹⁸

Nitric oxide (NO) is a free radical gaseous molecule with a biological half-life of a few seconds. NO is generated from the guanidine nitrogen of L-arginine by three isoforms of nitric oxide synthase (NOS), that is, neuronal NOS (nNOS, NOS-1), endothelial NOS (eNOS, NOS-3), and inducible NOS (iNOS, NOS-2). NO acts as an intra- and intercellular messenger in different tissues.¹⁹ In the kidney, NO acts as a signaling molecule and is generated by mesangial cells and renal tubular epithelial cells.²⁰ Under physiological conditions, NO plays a prominent role in the regulation of systemic and glomerular and medullar hemodynamics, tubular function (tubular fluid and electrolyte transport), tubuloglomerular feedback, and renin release.²¹

Oxidative stress and endothelial dysfunction, which are inter-related, play a role in many diseases, including several nephropathies. Thus, in this article we report the consequences of long-term ethanol administration in rat kidney on the level of the free radical scavenger and antioxidant enzyme activities, including superoxide dismutase (SOD) and CAT. In addition, the content of lipid peroxidation product, malondialdehyde (MDA), and the expression of eNOS were examined.

MATERIALS AND METHODS

Animals

The study was conducted in Erciyes University Hakan Cetinsaya 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." A total of 24 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 humiditycontrolled room ($21 \pm 3^{\circ}$ 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: Group I served as control and received liquid diet not containing ethanol. Group II was the ethanol treatment group: rats were fed with alcohol-containing liquid diet for 28 days. Group III received alcohol-containing liquid diet and 40 mg/kg/day ascorbic acid (Redoxon, Bayer AG, Leverkusen, Germany) were injected intraperitoneally. Group IV received alcohol-containing liquid diet and 4 mg/kg/day melatonin (Merck, cat. no. 814537, Darmstadt, Germany) was injected intraperitoneally.²²

Chronic Ethanol Treatment

For chronic ethanol exposure, the rats were housed individually and ethanol was given in the modified liquid diet as previously described.²³ 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). Animals were weighted and recorded everyday and ethanol intake was also measured and expressed as g/kg. The animals were not given access to water in addition to liquid diet with or without ethanol. The daily ethanol consumption of the rats ranged from 11.3 to 15.1 g/kg.

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

Biochemical Analysis of Kidney 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 with a homogenizer (IKA Ultra-Turrax T 25 Basic, IKA, Staufen, Germany) for 2 min at 13,000 rpm. All procedures were performed at $+4^{\circ}$ C. Tissue homogenates were then centrifuged at 5000 × 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 MDA Level

The MDA level was determined with the method modified by Ohkawa et al.²⁴ 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 SOD Activity

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined based on the method of Sun et al.²⁵ 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 addition of 1 mL of ethanol– chloroform mixture (5:3, v/v) 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 CAT Activity

The CAT activity was determined based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2 . The CAT activity was expressed as nmol/min/mL.

Histological Procedures

Parts of the tissues were fixed in 10% formalin for 24 h and rinsed under running tap water for 24 h, followed by dehydration through a graded alcohol series. Tissues were made transparent in xylol and embedded in paraffin. Sections of 5 μ m thickness were stained with hematoxylin–eosin (H&E) and Masson's trichrome and photographs were taken with an Olympus BX-51 photomicroscope (Olympus, Tokyo, Japan).

Immunohistochemistry

The expression of eNOS was detected immunohistochemically in the kidney using a rabbit polyclonal antibody (sc.654; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the streptavidin-biotin peroxidase technique. The procedure was performed under identical conditions for all sections. Paraffin sections $(5 \,\mu m)$ were dewaxed in xylene. The sections were rehydrated, rinsed in deionized water, and subjected to 2N HCl solution for 20 min to antigen retrieval. Endogenous peroxidase activity was inhibited in 3% H₂O₂ in methanol for 10 min. The specimens were washed in phosphatebuffered saline (PBS) three times (5 min each time) and preincubated in a 1.5% normal goat serum (NGS; sc.2043, Santa Cruz Biotechnology) in 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 $(1 \mu g/mL \text{ diluted in PBS with } 1.5\% \text{ NGS})$ (sc.2040; Santa Cruz Biotechnology) for 30 min at room temperature in a humidified chamber, followed by streptavidin horseradish peroxidase conjugate (ready-to-use) (catalog no: 50-420Z; Zymed Laboratories Inc., South San Francisco, CA, USA) for 30 min and AEC (red) substrate kit (catalog no: 00-2007; Zymed Laboratories) for 5 min. Finally the sections were counterstained with hematoxylin, rinsed in deionized water, and mounted with clearmount solution (ready-to-use) (catalog no: 00-8010; Zymed Laboratories). The sections were examined and photographed under a light microscope (Bx-51).

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

Statistical Analysis

All statistical analyses were performed with the Statistical Package for Social Sciences software package (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). The data were expressed as mean \pm standard deviation (SD). The data were statistically analyzed using one-way analysis of variance (ANOVA). Statistical significance was set at p < 0.05.

RESULTS

No statistically significant differences were determined in body weight between groups. Light microscopic examinations exhibited normal renal corpuscles and tubules in the control group (Figure 1A). Collagen staining of the glomerulus was more pronounced (Figure 1B) in rats in Group II compared with the controls. Atrophic renal corpuscles in some of fields, dilatation and congestion of the peritubular vessels (Figure 1C), renal corpuscles with obscure Bowman's space, and a few foamy-appearing tubules (Figure 1D) due to alcohol consumption were observed. Distinctive collagen staining in the glomerulus and persistence of dilatation and congestion of the peritubular vessels could be seen even when melatonin (Figure 2A) or vitamin C (Figure 2B) was given to alcohol-administered rats. eNOS expression was seen in all groups. In control group immune staining was localized to glomerulus (+), especially more evident in distal and collector tubule (++) (Figure 2C). Decreasing eNOS staining was determined in alcohol treatment group (Figure 2D). Melatonin (Figure 3A and B) and vitamin C (Figure 3C) encore increased expression pattern of eNOS. Negative controls where incubation with the primary antisera was omitted were completely unlabelled, as seen in Figure 3D.

Biochemical analyses revealed that alcohol consumption increased MDA levels and SOD and CAT activities significantly in the alcohol consumption groups compared with the control group, while melatonin statistically significantly decreased MDA levels and SOD and CAT activities compared with alcohol consumption groups (Table 1).

DISCUSSION

Oxidative stress is defined as imbalance between production of ROS and antioxidant pathways. It has long been known that ROS are harmful for cells, because they injure lipids, proteins, and nucleic acids, which leads to structural and functional impairments.^{26,27} Oxidative stress is included in the pathophysiology of many renal diseases, involving acute renal failure and chronic renal disease. ROS cause glomerular and tubulointerstitial damage by activating and maintaining cytokine release, leukocyte infiltration, fibrosis, and



Figure 1. (A) Group I—Glomerulus, Bowman's space, and proximal and distal tubular structures can be differentiated (H&E). (B) Group II—Prominence collagen (arrow) staining compared with the control group can be observed in the glomerulus and peritubular region (Masson's Trichome). (C) Group II—Dilatation and congestion of the vessels (arrow) due to alcohol consumption can be seen (H&E). (D) Renal corpuscle with obscure Bowman's space (arrow) and foamy-looking tubules (*) can be observed in alcohol consumption rats (H&E).

Note: H&E, hematoxylin-eosin.



Figure 2. (A) Group IV—Dilatation and congestion of the peritubular vessels (*) and prominent collagen (arrow) staining can be seen (Masson's Trichome). (B) Group III—Dilatation and congestion peritubular vessels (*) and increased collagen (arrow) staining compared to control group can be seen in administrated vitamin C (Masson's Trichome). (C) Group I—The presence of eNOS in the glomerulus and tubules can be observed. (D) Group II—The eNOS immunoreactivity is lesser expressed compared with the control. Note: eNOS, endothelial nitric oxide synthase.

mesangial and endothelial cell apoptosis.²⁸ Ethanol is used to generate oxygen radicals, inhibit glutathione (GSH) synthesis, deplete GSH levels in tissues, increase MDA levels, and generally impair the antioxidative defense system in humans and experimental animals. Chronic ethanol ingestion accounts for the formation of ROS and can induce a decrease of antioxidant defense.^{29,30} The liver is the primary organ responsible



Figure 3. (A) Group IV—eNOS immunoreactivity is observed near the control in melatonin treatment group. (B) Group IV—eNOS immunoreactivity localized especially collector tubules in medulla. (C) Group III—eNOS immunoreactivity is observed near the control in administrated vitamin C. (D) Group II—Negative control. Note: eNOS, endothelial nitric oxide synthase.

Table 1. MDA and SOD levels and CAT activity in the kidney.

			Tissue MDA
	CAT	SOD (U/g	(µmol/g
	(nmol/min/mL)	protein)	protein)
Control	96.38 ± 7.47	$4.44{\pm}2.56$	2.90 ± 0.67
Alcohol	$108.88 \pm 2.10^{a,*}$	$9.86\pm4.04^{\text{a},*}$	$4.81\pm0.72^{\text{a},*}$
Vitamin C	104.20 ± 8.14	5.77 ± 4.15	3.54 ± 1.13
Melatonin	$96.23 \pm 6.34^{\mathrm{b},**}$	$4.51 \pm 1.54^{\mathrm{b},**}$	$2.76 \pm 1.74^{\mathrm{b},**}$
Р	< 0.05	< 0.05	< 0.05

Notes: SD, standard deviation.

^{a,b}Differences between means bearing different letters in each column are significant (p < 0.05). Values are expressed as mean \pm SD.

p < 0.05 compared to Group I (control), p < 0.05 compared to Group II.

for the oxidation of ingested ethanol. However, kidney may contribute to ethanol metabolism.³¹ Experimental studies on animal models have also indicated that ethanol enhanced the fatty acid oxidation by kidney microsomes and peroxisomes and affected the activities of some kidney lysosomal hydrolases.³²

It is widely accepted that alcohol consumption causes disruption in the structure and function of the kidneys. White et al.³³ demonstrated that, in Australian adults, self-categorization as a moderate or heavy drinker or an average consumption of 30 g of ethanol per day or more was associated with significantly elevated risks of developing albuminuria over 5 years of follow-up, compared with light alcohol consumption. Kronborg et al.³⁴ reported that, among males, consumption of seven or more units of alcohol per week was associated with a significant increase in estimated glomerular filtration rate (eGFR) over 7 years of follow-up. Assadi et al.³⁵ reported that ethanol treatment rats showed cytoplasmic mitochondrial atrophy and vacuolar structures of the epithelial cells of the distal tubules and collecting ducts. In another study, the dilated tubules with cloudy swelling were shown in the kidney samples of alcohol-administered rats.³⁶ In this study, light microscopic examination of the renal tissue of rats subjected to alcohol consumption revealed atrophic renal corpuscles in some of fields and renal corpuscles with obscure Bowman's space. In addition, dilatation and congestion of the peritubular vessels and a few foamy-appearing tubules could be seen. These findings clearly indicate alcoholic damage in kidney.

Enzymatic and nonenzymatic systems protect the oxidant/antioxidant status, but they are overcome during oxidative stress, a metabolic disorder due to an instability caused by excessive generation of ROS or a diminished capacity of the antioxidant defense system. Alcohol consumption results in excessive generation of free radicals, which change the biomembranes and cause damage. To scavenge ROS, cells have several antioxidant enzymes including CAT, GSH peroxidase (GPO), and SOD.³⁶ Adaramoye et al.³⁷ demonstrated that in ethanol-treated rats, serum urea, creatinine, urinary glucose, gamma-glutamyltransferase, and protein levels were increased, while creatinine clearance significantly decreased. They also found that serum and kidney MDA levels were increased, and CAT, SOD, and GSH activities were decreased in the kidney of ethanol-treated rats. Pari et al.³⁶ reported significant decreases in the enzymatic activities of some antioxidants in alcoholtreated rats compared with that in controls. In this study, biochemical analyses of renal tissue of rats subjected to alcohol consumption revealed increased MDA level and SOD and CAT activities significantly compared with that of the control group. The biochemical findings were corroborated by histopathological examination of the kidney.

In this study, melatonin and vitamin C were used as protective agents against alcohol-induced toxicity. Besides acting as a direct scavenger of free radicals, melatonin has indirect antioxidant actions. Melatonin has been shown to support several intracellular enzymatic antioxidant enzymes, including SOD, GSH reductase, CAT, glucose-6-phosphate dehydrogenase, and GSH peroxidase.³⁸⁻⁴⁰ Yurtcu et al.⁴¹ demonstrated that melatonin is a potent antioxidant agent in preventing testicular ischemia-reperfusion (I-R) injury. Ilbey et al.¹⁸ found that melatonin treatment inhibited the increase in lipid peroxidation in acetaminopheninduced nephrotoxicity. In another study, Abraham et al.⁴² reported that melatonin pretreatment reduced methotrexate-induced oxidative stress and alteration in the activity of antioxidant enzymes. Previously, we reported in our laboratory that melatonin decreased MDA and GSH levels in the kidney of smoke-inhaled rats.²² In this study, melatonin statistically significantly decreased MDA levels and SOD and CAT activities compared with alcohol consumption groups. These findings agree with the previous studies. A number of studies have shown that melatonin is better than the classic antioxidants in resisting free-radical-based molecular destruction. In this study, we also showed that melatonin was more effective than vitamin C.

NO plays important roles in the control of renal and glomerular hemodynamics, tubuloglomerular feedback response, release of renin and sympathetic transmitters, tubular ion transport, and renal water and sodium excretion.43 Alteration of renal NO production has been associated with glomerular hyperfiltration, vascular permeability, albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis.⁴⁴ All three isoforms of NOS, namely, nNOS, iNOS, and eNOS, are reported to contribute to NO synthesis in the kidney. It was determined that eNOS is strongly expressed in renal vascular endothelium and tubular epithelium.45 In this study, eNOS immune staining was localized to glomerulus and tubules. We also found decreasing eNOS staining in alcohol treatment group compared with that in control. Increased ROS activity can enhance NO inactivation and reduce bioactivity of NO. This can cause a compensatory upregulation of NOS isotype expression. Therefore, Satoh et al.⁴⁶ found that the expression of eNOS mRNA was higher in diabetic glomeruli, which is an oxidative condition. On the other hand, Majhi et al.⁴⁷ reported that arsenic exposure for an extended period can reduce eNOS expression and activity in rat kidney. It appeared that in previous findings there were contradictions. These differences may be due to experimental procedure and/or linking diverse pathogenic mechanism of oxidative stress. Being administrated to protective effect melatonin and vitamin C encore increased the expression pattern of eNOS.

The results of biochemical tests together with histological observations suggest that alcohol leads to serious changes in the histology of kidney. The increased formation of lipid peroxides and associated ROS leads to damage in membrane integrity and other pathological changes in kidney. The efficacy of any protective drug is essentially dependent on its capacity of either reducing the harmful effects or maintaining the normal physiology of cells and tissues. The membrane protective properties and antioxidant nature of melatonin might be helpful to alleviate the pathological changes caused by alcohol in kidney.

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