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The effects of chronic exposure to ethanol and cigarette smoke on the formation of peroxynitrite, level of nitric oxide, xanthine oxidase and myeloperoxidase activities in rat kidney

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Abstract

The aim of this study was to investigate the effects of chronic ethanol intake and cigarette smoke exposure on rat kidney. The animals were divided into four experimental groups: (1) the control group (C), (2) the ethanol group (E), (3) the cigarette smoke group (CS), and (4) the cigarette smoke plus ethanol group (CS+E). Rats in E, CS and CS+E groups were treated with ethanol and/or cigarette smoke for 6 months. The animals were killed and the kidneys were removed to determine the activity of xanthine oxidase (XO), myeloperoxidase (MPO) and the levels of nitric oxide (NO). Histopathological and immunohistochemical analysis were performed in kidney tissues. The activity of XO/g protein were 2.8 ± 0.3, 5.2 ± 0.3, 3.2 ± 0.1, and 7.4 ± 0.7 U for C, E, CS and CS+E groups, respectively. In groups E, and CS+E, the XO values were significantly higher than in group C ($P < 0.05$). The increase in XO activity of CS was not significantly different from group C ($P > 0.05$). There was a significant increase in XO activity of group CS+E as compared to CS and E groups ($P < 0.05$), and also a significant difference in XO activity between E and CS was observed ($P < 0.05$). The activity of MPO/g protein were 13.5 ± 0.6, 16.2 ± 1.1, 14.7 ± 1.1, 23.8 ± 0.9 U for C, E, CS, and CS+E groups, respectively. While MPO activity of kidneys from group CS+E were significantly higher as compared to C, CS, and E groups ($P < 0.05$), there was no significant difference among the groups of C, CS, E ($P > 0.05$). The levels of NO/g wet tissue were 347.7 ± 8.5, 261.1 ± 4.8, 329.8 ± 5.6, and 254.2 ± 3.8 nmol for C, E, CS, and CS+E groups, respectively. In groups of E and CS+E, the NO values were significantly lower than that of group C animals ($P < 0.05$). Although we detected lower NO levels in the E and CS+E groups than in CS group ($P < 0.05$), a significant difference in NO levels between CS+E and E groups was not observed. In the histopathological analysis of the kidney slices, severe degenerations in kidney tissues of group CS, E, CS+E were observed. Generally, the histological changes in kidney of

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CS+E and E groups were more severe than those observed in CS alone. While we observed a strong immunoreactivity for anti-nitrotyrosine antibody in kidneys of group CS+E, examination of sections from rat kidneys in group E revealed moderate staining. On the other hand, group CS had very little immunostaining. There was no immunostaining in group C. We concluded that chronic ethanol administration and cigarette smoke exposure may cause oxidative and nitrosative stress which lead to rat kidney damage. (Mol Cell Biochem **291:** 127–138, 2006)

keywords: cigarette smoke, ethanol, MPO, nitric oxide, nitrotyrosine, kidney, XO

Introduction

Cigarette smoking and alcohol consumption could result in a number of acute and chronic harmful effects in several organs including kidneys. It has been reported that smoking is a main risk factor in the pathogenesis of renal disorders [1]. Data on the occurrence of renal cell carcinoma showed that 30% of men and 24% of women were suffering from renal cell carcinoma that is developed by cigarette smoking [2]. Chronic use of ethanol could affect the kidneys and liver adversely. The occurrence of glomerulonephritis has been highly correlated with alcoholism [3]. Alcohol consumption has been also reported to cause acute tubular necrosis [4] and renal tubular dysfunction [5]. Increasing evidence also suggests that ethanol and smoking-induced reactive oxygen species (ROS) are the major source of many disorders including the kidney toxicity. A great deal of experimental evidence demonstrated that pathophysiology of renal diseases and renal tissue damage could be associated with the alterations induced by ROS [6]. Oxygen free radicals could be generated by a variety of reasons at the cellular levels. An important source of oxygen free radical is known to be xanthine oxidase (XO) that could be formed from xanthine dehydrogenase (XD) either reversibly (via oxidation or blockage of its thiol groups) or irreversibly (via limited proteolysis) under pathological conditions [7]. The aforementioned conversion reaction leading to the elevated tissue XO levels is thought to be responsible for mechanism of several pathological conditions including alcoholism and smoking [8, 9]. ROS generated through XO could be an important factor leading to an injury during early and late stages in the epithelial cells of proximal and distal renal tubules [10]. Polymorphonuclear (PMN) leukocytes themselves are an important source of ROS causing cellular injury [11]. It was reported that free radicals leading to pathological lesions in the kidneys could be generated via infiltration of neutrophils [12, 13]. Detection of PMN leukocytes infiltration in an inflamed tissue has been based on the measurement of the myeloperoxidase (MPO) activity. Several studies have shown that cigarette smoke and ethanol consumption increased MPO activity in various tissues [14, 15]. Nitric oxide (NO) is a free radical gas molecule that is produced from L-arginine by the catalytic action of enzyme, nitric oxide synthase (NOS) [16]. NO participates in a variety of physiological processes consisting of neurotransmission [17] and regulation of blood vessel wall [18]. Modulatory effect of NO also exists on glomerular hemodynamics through the regulation of renin release and tubuloglomerular feedback system [19, 20]. In addition to its physiological effects in the kidney, it was reported that altered NO concentration could result in several renal diseases. The inactivation of NO by superoxide radical ($O_2^{\bullet -}$) could also lead to a decrease in the glomerular filtration rate [21]. Involvements of NO in the pathogenesis of inflammation and some degenerative disorders have been linked to the generation of peroxynitrite ($ONOO^-$). NO can interact with $O_2^{\bullet -}$ to form $ONOO^-$ [22], which is also a powerful oxidant, causing a number of potentially dangerous reactions including thiols or thioethers, degrading carbohydrates as well as initiating lipid peroxidation and DNA cleavage. Studies have shown that NO metabolism can be altered under pathological conditions [9, 23].

The purpose of the present study was to determine individual and combined effects of chronic ethanol consumption and chronic cigarette smoke exposure by measuring the activity of XO, MPO and the level NO in rat kidney. In addition, histological examination and immunohistochemical staining for nitrotyrosine, a nitrosative stress marker, were also performed to further determine the effects of ethanol intake and cigarette smoke.

Materials and methods

Animals

Male Wistar rats initially weighing between 180 and 220 g at 6 weeks of age were obtained from the Inonu University Animal Research Laboratory. The rats were housed in individual cages in a room at a certain temperature (20–22 °C), humidity (60–70%) and 12:12 h light:dark cycle.

Experimental protocol

Twenty eight rats were randomly divided into four experimental groups each containing seven rats: (1) the control group (C), (2) the ethanol group (E), (3) the cigarette smoke

group (CS), and (4) the cigarette smoke plus ethanol group (CS+E). Ethanol and cigarette smoke treatment continued during 6-month period of the experiment. Throughout the study, each rat in all groups was supplied with 100 ml liquid diet, and liquid consumption of each rat was recorded daily. Animals in group E were orally given ethanol in a modified liquid diet (MLD; cow's milk: 925 ml, vitamin A: 5000 IU and sucrose: 17 g) as described by Uzbay and Kayaalp [24]. Briefly, the rats were fed with ethanol free MLD for 7 days and then, 2.4% ethanol (v/v) (ethanol 95.6%, Tekel, Turkish State Monopoly) containing MLD was given for 3 days. Then, the ethanol concentration was increased to 4.8% for another 3 days. Finally, ethanol concentration was increased to 7.2% for the rest of the 6-month period. In order to keep total calorie at a constant value, sucrose concentration of the diet was reduced accordingly in the diet. Rats in group C received only MLD with sucrose as a caloric substitute for ethanol for 6 months. The rats in CS group received the same modified diet as in control group. The application of cigarette smoke was carried out according to the method of Zhu *et al.* [25]. Briefly, the rats were placed in a closed plexiglass cage (dimensions: $0.75 \times 1.00 \times 0.85$ m., volume: $0.64\,m^3$). Smoke was provided from a burning cigarette through a cigarette machine and directly applied to the cage in about 7 min at a frequency of 20 cigarettes per day. Fresh air was also provided to the cage by means of a pump. The last group received a combination of ethanol and cigarette smoke (group CS+E).

Following 6 months of experimental period, the rats were killed and samples of kidney tissues from each rat were collected. For histopathological and immunohistochemical examinations, a portion of kidney tissue was fixed in 10% formalin. Another portion of kidney from the same animal was stored at –70 °C until XO, MPO and NO analyses were performed. To provide uniformity, all analyses were done under the same conditions at the same time.

Biochemical analysis

Xanthine oxidase activity (XO)
XO activity was determined spectrophotometrically according to the method of Prajda and Weber [26], based on the formation of uric acid from xanthine, which increases absorbance at 292 nm ($\varepsilon_M = 9.2 \times 10^3$). One unit of activity was defined as 1 μmol of uric acid formed per minute and data are presented as U/g protein.

Myeloperoxidase activity (MPO)
Determination of MPO activity was carried out spectrophotometrically using 4-aminoantipyrine/phenol that is a substrate for MPO-mediated oxidation by H_2O_2. Absorbances were read at 510 nm, and the data were given as U/g protein [27].

Total nitrite measurement
Total nitrite was measured by a slight modification of cadmium-reduction method [28]. Results were expressed as nanomoles per gram of wet tissue (nmol/gwt).

Immunohistochemical staining
Immunohistochemical analysis was performed according to Yildirim *et al.* [29], with some modifications. Briefly, five-micron sections of kidney were deparaffinized in xylene and rehydrated in ethanol series. Then, they were treated with 0.3% hydrogen peroxide in phosphate buffered saline (PBS, 0.1 M, pH 7.4) to block endogenous peroxidase activity for 30 min. Following once washing with PBS, the sections were exposed to 1.5% normal goat serum for 30 min to block nonspecific antibody binding. The sections were incubated with a mouse anti-nitrotyrosine antibody (Calbiochem, Darmstadt, Germany) diluted 1:1000 in PBS for 1 h at RT. After they were washed three times in PBS, the sections were incubated in a goat biotinylated anti-mouse IgG, secondary antibody (1:200 dilution; Calbiochem, Darmstadt, Germany) for 30 min. Then, they were finally incubated in a avidin-biotin peroxidase complex (ABC kit, Calbiochem, Darmstadt, Germany) for 30 min. Peroxidase was developed with diaminobenzidine-hydrogen peroxide solution (0.005% 3,3-diaminobenzidine and 0.1% hydrogen peroxide in 0.1 M PBS). Finally, the sections were counterstained with hematoxylin and examined under a light microscope.

Protein determination

Lowry method was utilized to determine protein concentration using bovine serum albumin as a standard [30].

Kidney histology

Kidney samples were fixed in 10% formaldehyde, buffered with PBS (0.01 M, pH 7.4), and embedded in paraffin. They were sectioned at 5 μm thickness and stained in hematoxylin-eosin (H-E). The stained sections were analyzed using a light microscope.

Statistical analysis

Results are expressed as means $\pm$ SEM. Analysis of variance was used to test the differences between multiple groups and the differences were considered significant if the P value was less than 0.05 by Tukey's multiple comparison test.

Table 1. The effects of chronic ethanol consumption and cigarette smoke exposure on the levels of NO and activity of MPO and XO in rat kidney[a]

Groups	NO (nmol/gwt)	MPO (U/g protein)	XO (U/g protein)
C	347.7 ± 8.5	13.5 ± 0.6	2.8 ± 0.3
E	261.1 ± 4.8	16.2 ± 1.1	5.2 ± 0.3
CS	329.8 ± 5.6	14.7 ± 1.1	3.2 ± 0.1
CS+E	254.2 ± 3.8	23.8 ± 0.9	7.4 ± 0.7
Stastistical comparison (*P*)			
C vs E	0.000	0.234	0.014
C vs CS	0.156	0.789	0.913
C vs CS+E	0.000	0.000	0.000
E vs CS	0.000	0.686	0.047
E vs CS+E	0.829	0.000	0.026
CS vs CS+E	0.000	0.000	0.000

[a]Groups of Control (C), Ethanol (E), Cigarette Smoke (CS) and Cigarette Smoke plus Ethanol (CS+E). Each group represents the mean ± SEM for seven rats.

Results

Biochemical parameters

Daily mean liquid diet (containing 7.2% alcohol) consumption was 59.1 ± 0.9 ml in group E and 53.6 ± 1.5 ml in CS+E for the 6 months. Table 1 shows XO, MPO activities and NO levels in rat kidney tissues. XO activities in group E (5.2 ± 0.3 U/g protein) and group CS+E (7.4 ± 0.7 U/g protein) were significantly higher than in group C (2.8 ± 0.3 U/g protein) ($P < 0.05$). However, no difference in XO activity was found between group C and group CS (3.2 ± 0.1) ($P > 0.05$). Furthermore, XO activity of renal tissue from group CS+E was higher than that of group CS and E ($P < 0.05$). XO activity of kidney from rats receiving ethanol treatment was found to be increased compared to that of group CS ($P < 0.05$). While MPO activity of kidneys from group CS+E (23.8 ± 0.9 U/g protein) was significantly higher than groups of C (13.5 ± 0.6 U/g protein), CS (14.7 ± 1.1 U/g protein), and E (16.2 ± 1.1 U/g protein) ($P < 0.05$), there was no significant differences among groups of C, CS and E ($P > 0.05$). Kidney NO levels in group E (261.1 ± 4.8 nmol/gwt), and CS+E (254.2 ± 3.8 nmol/gwt) were significantly lower than in groups of C (347.7 ± 8.5 nmol/gwt) and CS (329.8 ± 5.6 nmol/gwt) ($P < 0.05$). Rats treated with cigarette smoke showed decreased levels of NO when compared to the control group, but the decrease in NO level was not statistically significant ($P > 0.05$). However, no difference was observed in NO levels between groups of E and CS+E ($P > 0.05$).

Histopathology

Histopathological evaluation of the tissues in group C (Fig. 1) did not reveal any pathological abnormalities; however, in group CS (Fig. 2), severe hydropic degeneration of the kidney tubules was clearly visible. In group E (Fig. 3), glomerular hypercellularity and small widening in glomerular space

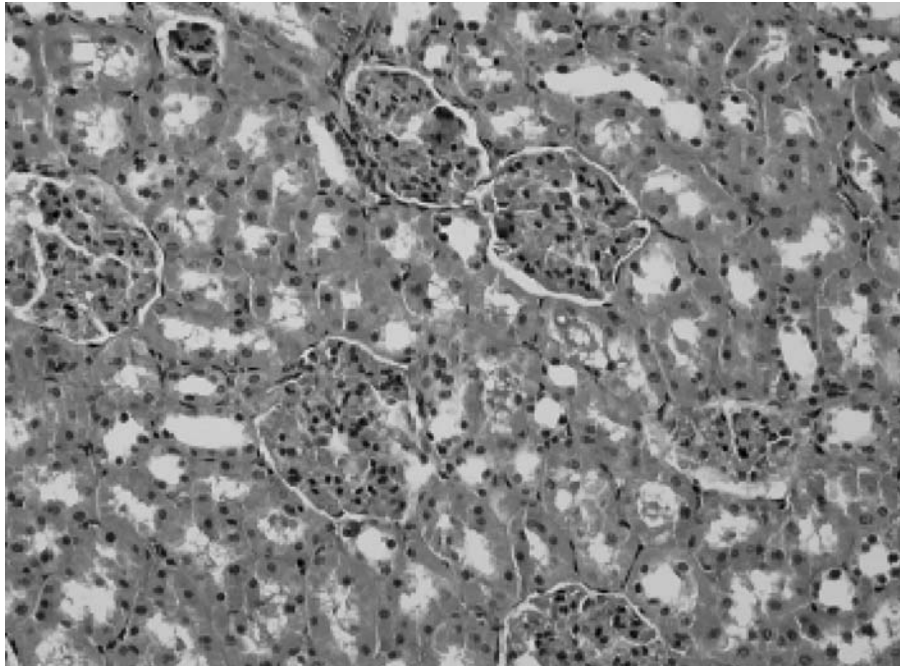

Fig. 1. Kidney showing normal architecture in group C (H-E ×185).

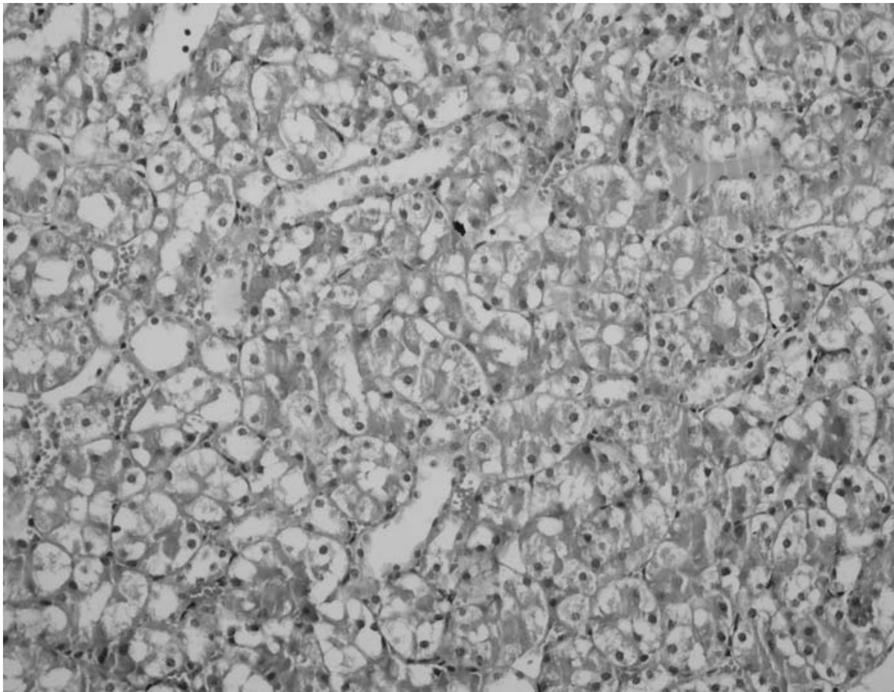

Fig. 2. Kidney showing severe hydropic degeneration in group CS (H-E ×185).

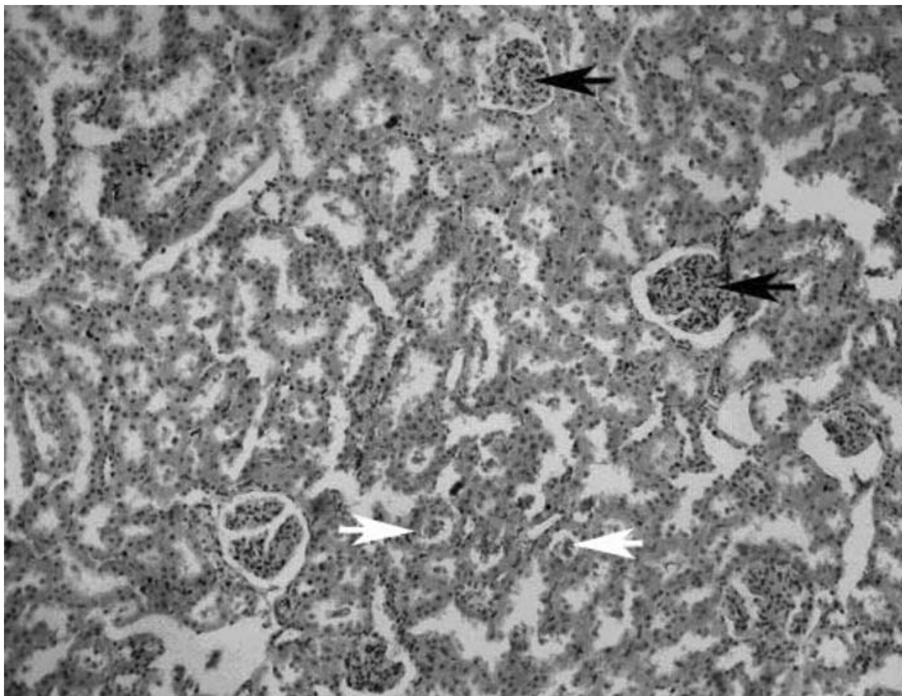

Fig. 3. Kidney showing glomerular hypercellularity (black arrows) and desquamated epithelial cells in tubule lumens (white arrows) in group E (H-E × 92).

was observed. Moderate hydropic degeneration in the proximal tubule epithelia and occasional interstitial mononuclear cell infiltration was also observed. In group CS+E (Figs. 4, 5), there were increased widening in glomerular space and hypercellularity in glomeruli. Widespread vacuolar degeneration in tubule epithelia and desquamation in some tubule epithelia were observed. Few erythrocytes in some tubule lumens were also seen. Some renal tubules were dilated and

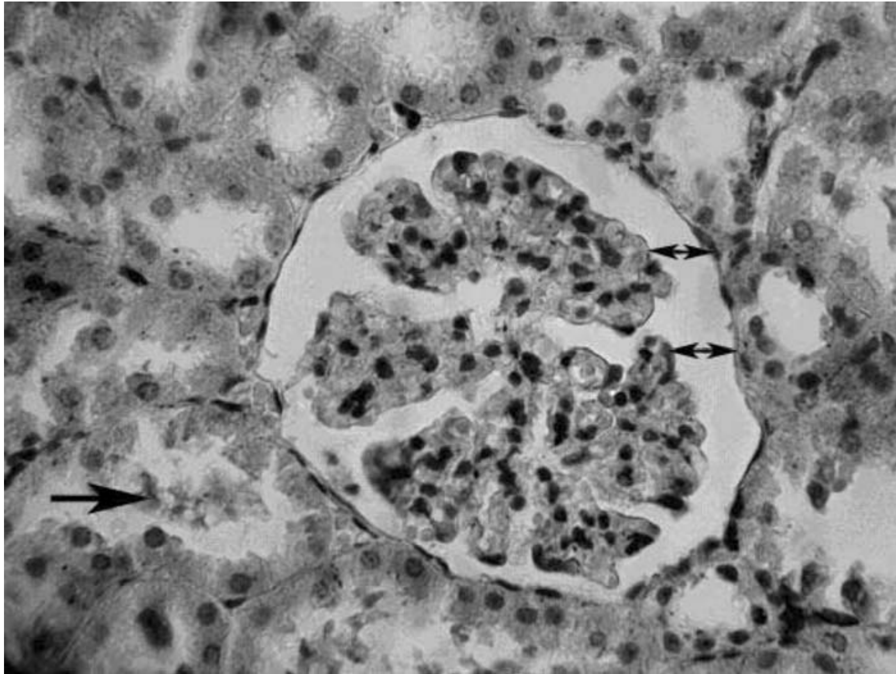

Fig. 4. Kidney showing tubular degeneration and desquamated epithelial cells (black arrow) and expansion of glomerular cavity (double headed arrow) in group CS+E (H-E × 925).

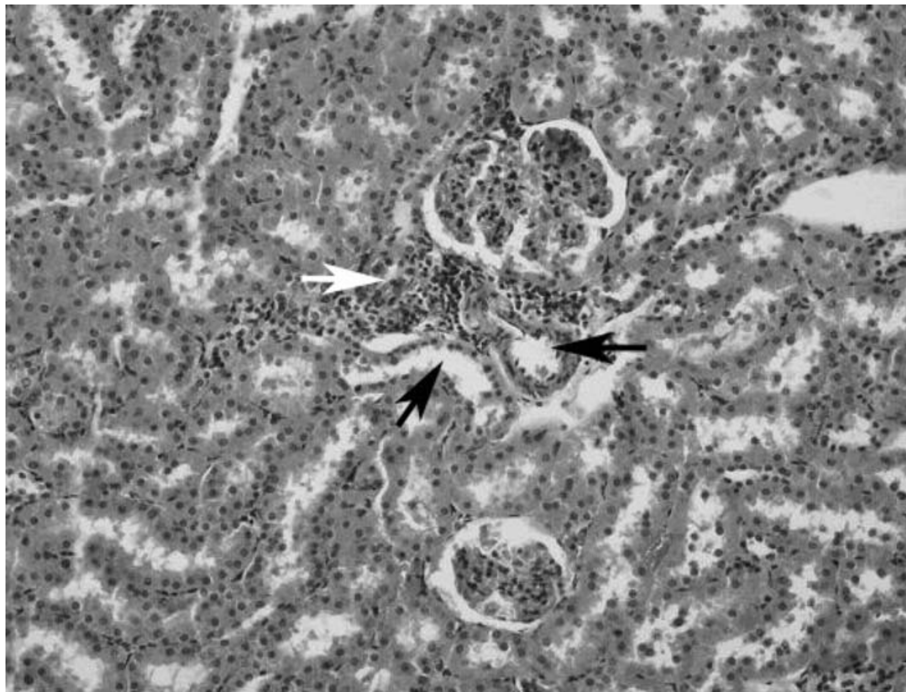

Fig. 5. Kidney showing mononuclear cell infiltration in the interstitium (white arrow) and marked tubular dilatation (black arrows) in group CS+E (H-E ×185).

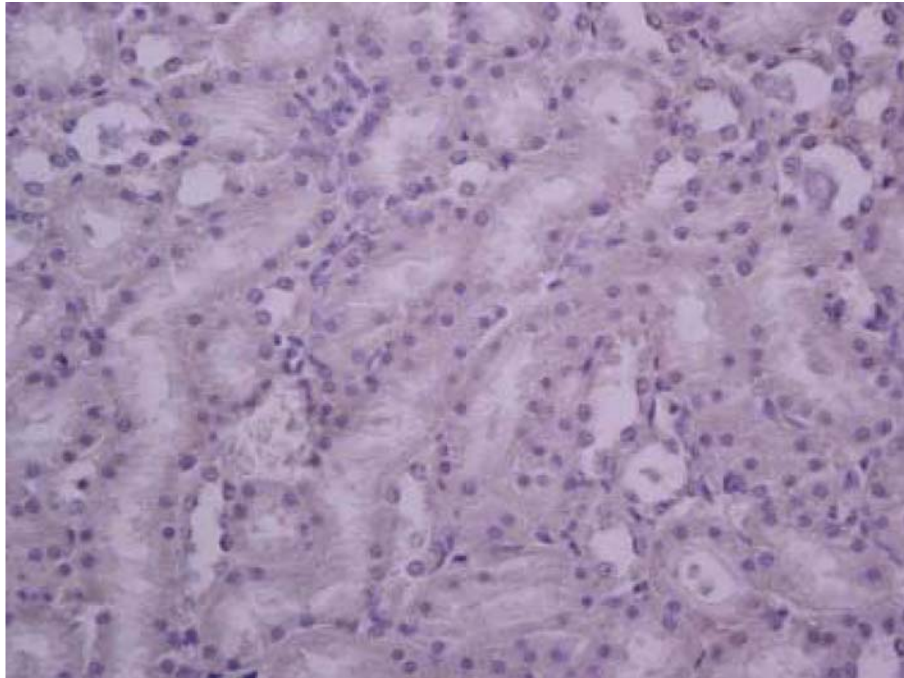

Fig. 6. No immunostaining was found in the proximal tubular epithelial cells in group C (×370).

the tubular epithelia were observed as necrotic. Interstitial mononuclear cell infiltration was prominent in most sections.

Immunohistochemistry

There was a strong immunoreactivity for anti-nitrotyrosine antibody especially in proximal tubules of kidney in group CS+E (Fig. 9). The kidney sections from rats in group E (Fig. 8) showed moderate staining while group CS (Fig. 7) had very little immunostaining. There was no detectable immunostaining in kidney sections from group C (Fig. 6).

Discussion

The role of smoking and chronic alcohol intake on human health has not been very well described. However, the number of studies on the effects of smoking and alcohol are increasing, and these studies proved that these two habits are able to induce harmful effects on various organs in human body. Much of the attention has been focused on the organs that appear to be directly affected by smoking and alcohol use. Such organs included the lungs, liver and heart, but the risk on the kidneys posed by smoking was underestimated and ignored [31]. There is little information available on the effects of ethanol and cigarette smoke especially on kidney, therefore we examined the individual and combined effects of ethanol and cigarette smoke on this organ in this study. It has been proposed that damage in several organs can be attributed to the oxidative and nitrosative stress. To test this hypothesis, several researchers have studied the mechanisms of cellular antioxidant defense systems and demonstrated that chronic ethanol consumption and cigarette smoking increase oxidative stress in various tissues [32–34].

In the present study, histopathological investigation of kidneys of group CS animals revealed that the only observed pathological change was severe hydropic degeneration (Fig. 2). This seems to be an indication of early degenerative changes in the kidney. In an experimental study, Odoni *et al.* [35] reported glomerulosclerosis and tubulointerstitial fibrosis in the subtotally nephrectomized rats treated with cigarette smoke extract in solvent acetone. However, such an association between glomerulosclerosis and chronic cigarette smoking in human was not observed [36]. On the other hand, severity of pathological changes increased in kidneys of ethanol treated rat (Fig. 3). Hypercellularity in glomeruli and widening in glomerular space were observed in these rats. Also, occasional mononuclear cell infiltration, degeneration in proximal tubule epithelia and desquamation in tubule epithelia were noted. These findings were partially similar to those reported in the literature [37]. In our current study, the most prominent pathological abnormalities were observed in CS+E treated animals (Figs. 4 and 5). Similar findings seen in ethanol group were also observed in this group though the pathological changes were more severe. Additionally, there

were few erythrocytes in the proximal tubule lumens. It seems to be that cigarette smoking increases the harmful effects of ethanol use. The histopathological changes in kidney tissues of groups E and CS+E were more severe than those observed in the tissues of group CS alone. The histopathological alterations observed in the present study could be associated with excessive production of ROS due to ethanol and cigarette smoke exposure.

The reason for the overproduction of ROS could be originated from a variety of sources under the pathological conditions. For example, ethanol itself is able to induce formation of ROS in kidney tissue. Ethanol is mainly metabolized to acetaldehyde by alcohol dehydrogenase (ADH), catalase, and a microsomal ethanol-oxidizing system in the liver [38]. Rat kidney also contains ADH that appears to have similar or identical mechanism that is present in the liver [39]. Ethanol is metabolized to acetaldehyde by ADH, and then further oxidized by acetaldehyde dehydrogenase to final product acetate that contributes to the ROS. It was shown that, rats receiving ethanol for 10 weeks exhibited increased ADH activity resulting in increased acetaldehyde production [40]. These results suggested that chronic ethanol treatment could cause enhanced ethanol oxidation and ROS production in rat kidney leading to kidney damage.

Polymorphonuclear leukocytes might also play a role in the initiation of ROS production. Free radicals leading to pathological lesions in the kidneys could be generated via infiltration of neutrophils [13]. In this study, we found that MPO activity, an indicator of PMN leukocyte infiltration, in the kidney tissues of CS+E treated rats was highest among all groups ($P < 0.05$). In addition, we also observed an increased MPO activity in groups of CS and E as compared to group C, which was not statistically significant ($P > 0.05$). Similar alterations in MPO activity have also been reported in other studies such as ethanol and cigarette smoke induced pancreatic injury [15], and gastric mucosa damage [14]. Additionally, it was reported that administration of ethanol for 6 months resulted in increased MPO activity in the parenchyma of brain tissue [41]. In contrary, Chow *et al.* [42], and Nguyen *et al.* [43], found that ethanol or cigarette smoke alone did not increase MPO activity in the gastric mucosa of rats and in neutrophils of human. However, combined administration of ethanol and cigarette smoke to the rats significantly increased MPO activity of gastric mucosa [42]. Discrepancy among these studies could be due to the differences in the doses administered and the duration of the experiments.

Reactive oxygen species in the cells of glomerulus and proximal tubules could be produced during normal cellular events, though production of ROS in kidneys is prominently enhanced in several disease conditions through the action of PMN leukocytes. The basis of the tissue damage is associated with the activation of PMN leukocytes and monocytes leading to the exhaustion of cellular oxygen leading to generation of $O_2^{\bullet -}$ radicals where the process is collectively called respiratory burst. The activated neutrophils produce ROS and hypochlorous acid (HOCl), which are the major causes of peripheral tissue injury [44]. In addition, MPO itself could lead to injury in the kidneys. It was shown that oxidants generated by MPO could cause cell and tissue damage [45]. Tepperman *et al.* [46], reported that the increase in MPO activity might be responsible for the initiation of gastric mucosal lesions seen in ethanol-treated rats.

Xanthine oxidase could be another source of ROS. Under pathological conditions, ROS could be generated by the action of XO enzyme that is produced from XD via proteolytic breakdown, or oxidation of thiols [7]. The results of our study demonstrated that exposure to chronic ethanol treatment with or without cigarette smoke increases XO activity of kidney tissue in rats compared to the control group ($P < 0.05$). Though there was an increased activity of XO in the kidney tissue of rats exposed to cigarette smoke, the increase was not statistically significant ($P > 0.05$). Accordingly, other studies on the effect of ethanol and cigarette are in agreement with the results of the present study. Sultatos [47], reported that acute ethanol treatment in rats increased hepatic XO level through a conversion process from XD. In addition, Abbondanza *et al.* [48], studied the effects of chronic ethanol treatment on XO status of rat liver and they found that repeated ethanol administrations resulted in an increased XO level in the rat liver. Similarly, exposure to cigarette smoke may also cause alterations in XO levels. For example, Deliconstantinos and Villiotou [9], showed that XO activity of synaptosomes was increased by tobacco smoke. In another study Chow *et al.* [49], treated male Sprague–Dawley rats with ethanol and cigarette smoke, and they found that XO and MPO activity of gastric mucosa were increased in the group exposed to ethanol plus cigarette smoke group compared to control. However, Dinu *et al.* [50], reported that ethanol did not change XO level in kidney tissue of rats exposed to ethanol for 30 weeks.

Ethanol and cigarette smoke-induced increase in XO level observed in our study could be due to many factors. Acetaldehyde produced from ethanol metabolism could also favor the conversion of XD into XO by interaction with free sulfhydryl groups of XD. It may be possible that chronic ethanol and cigarette smoke exposure could reduce tissue GSH level that provides the conversion of XD to XO. McKelvey *et al.* [51], reported that the formation of XO from XD was associated with the decreased GSH level in ischemic rat liver and kidney. In our previous work [34], we demonstrated that chronic ethanol and cigarette smoke exposure resulted in decreased levels of GSH in the kidney. It is possible that ROS produced by cigarette smoke could result in conversion of XD to XO. Cigarette smoke includes both reactive aldehydes and a large number of oxidants that contain about 10^{14} ROS molecules with each per puff. In addition to these reactants, nicotine in

cigarettes is also oxidized and generates ROS in the body [52]. Although we did not observe any significant increase in XO levels of CS group, CS treatment along with ethanol resulted in an increased level of XO. CS+E-induced ROS production could result in oxidation of XD that could be a cause for increased XO level. Excessive production of $ONOO^-$ could also be involved in the mechanism of increased XO activity. $ONOO^-$ is formed by the incorporation of NO with $O_2^{\bullet -}$. Sakuma *et al.* [53], reported that XD was converted into XO where sulfhydryl groups of XD are reversibly oxidized through the action of $ONOO^-$. In our present study, we showed that $ONOO^-$ production was greatly increased in E and CS+E groups as revealed by immunohistochemical examinations (Figs. 8 and 9). In addition, PMN infiltration in the kidney tissue leading to overproduction of ROS could result in the conversion of XD to XO. The increase in XO activity of E and CS+E groups in this study may be due to an increased production of oxygen free radicals by PMN leukocytes.

We found that, kidney NO levels in groups of E and CS+E were significantly lower than in groups of CS and C ($P < 0.05$). However, no difference was observed in NO level between group E and group CS+E ($P > 0.05$). Rats treated with cigarette smoke had decreased level of NO compared with that of control, but the decrease in NO level was not statistically significant ($P > 0.05$). It was reported that cigarette could decrease NO level in endothelial cells and plasma [54, 55]. Another report showed that cigarette smoke had no effect on NO level [23]. In another study, Fataccioli *et al.* [56], treated rat cerebellar homogenates with various concentrations of ethanol (25–200 mM) and measured NOS activities. They found that ethanol significantly decreased NOS levels in cerebellar cytosolic fractions. In the same study, addition of tetrahedrabiopterin and L-arginine into the medium prevented ethanol-induced NOS inactivation. The authors speculated that ethanol could cause conformational change in NOS possibly via alterations in the binding regions of tetrahedrabiopterin and/or L-arginine. However, some studies demonstrated that alcohol caused increased NO level. For example, Uzun *et al.* [57], showed that plasma and liver NO levels of rats treated with ethanol were higher than that of control rats. In our present study, we have found that NO level decreased in the kidneys of rats in groups of E and CS+E, and exposure to cigarette smoke for 6 months resulted in no significant alteration in NO level of kidney tissue. According to the results of our study, we can speculate that reduced levels of NO could be due to the elevation of ROS especially $O_2^{\bullet -}$ that was induced by ethanol and cigarette smoke. The increase in superoxide radical production might subsequently result in decreased level of NO leading to the formation of $ONOO^-$. Determination of endogenous $ONOO^-$ through immunohistochemical methods has been dependent on the detection of nitrotyrosine formation, which was considered to be a specific marker for nitrosative stress. The method depends on the formation of $ONOO^-$ that is produced by the reaction of molecular oxygen or superoxide with NO. Indeed, the results of our immunohistochemical examination support the results

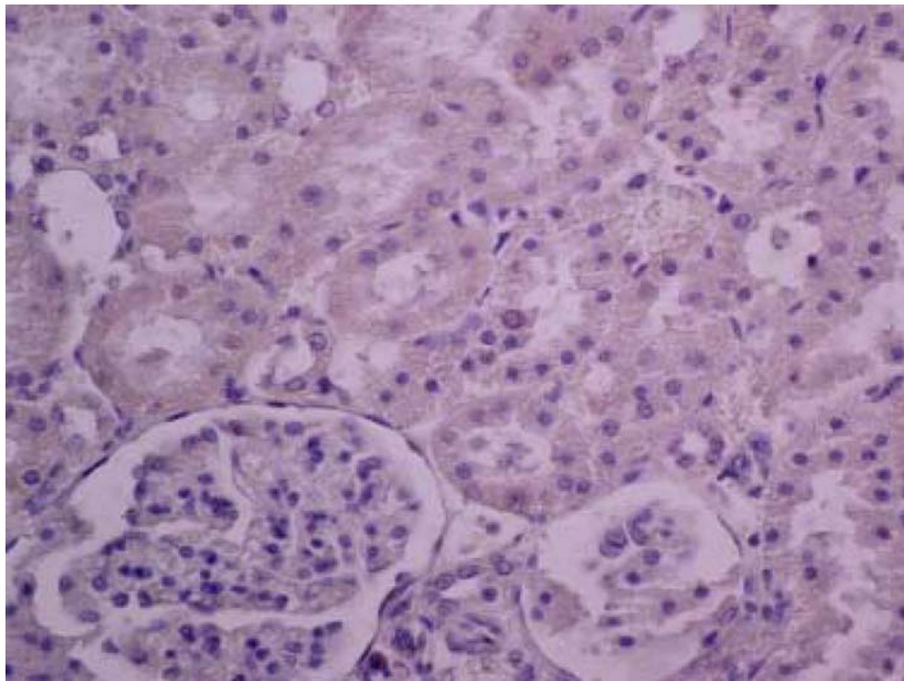

Fig. 7. Little immunostaining for anti-nitrotyrosine antibody in the proximal tubular epithelial cells in group CS (×370).

obtained from NO measurements in that we found decreased NO levels in E and CS+E groups.

Immunohistochemical investigation of kidney tubule epithelia revealed little immunoreactivity against anti-nitrotyrosine antibody in CS group (Fig. 7). However, there were moderate to strong immunostaining in group E (Fig. 8). On the other hand, the immunoreactivity increased when ethanol was given together with cigarette smoking (Fig. 9). $ONOO^-$ is able to oxidize many molecular structures such as proteins, resulting in nitrotyrosine formation. It was

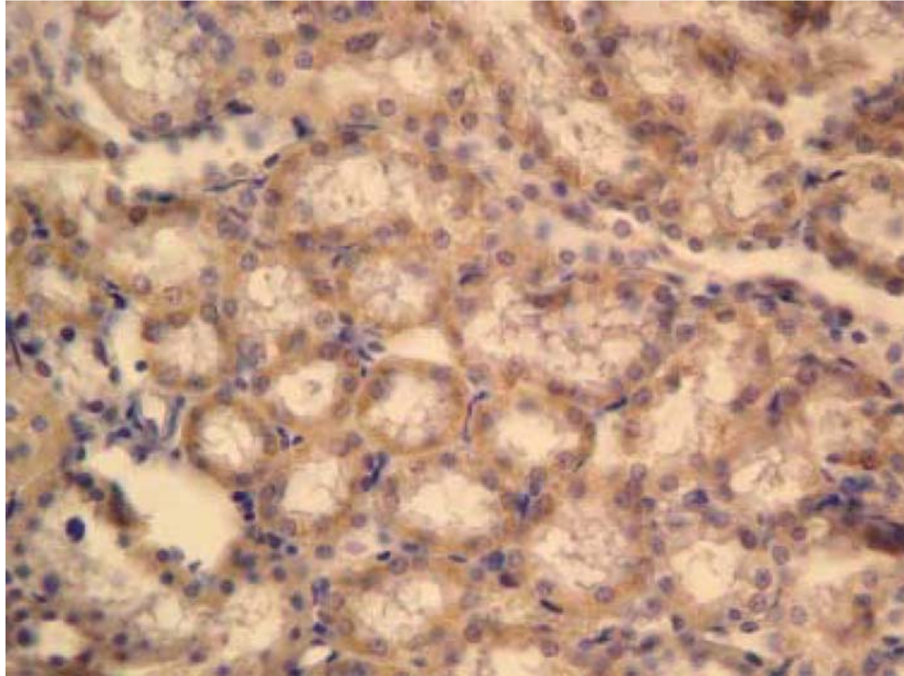

Fig. 8. Moderate immunostaining for anti-nitrotyrosine antibody in the proximal tubular epithelial cells in group E (×370).

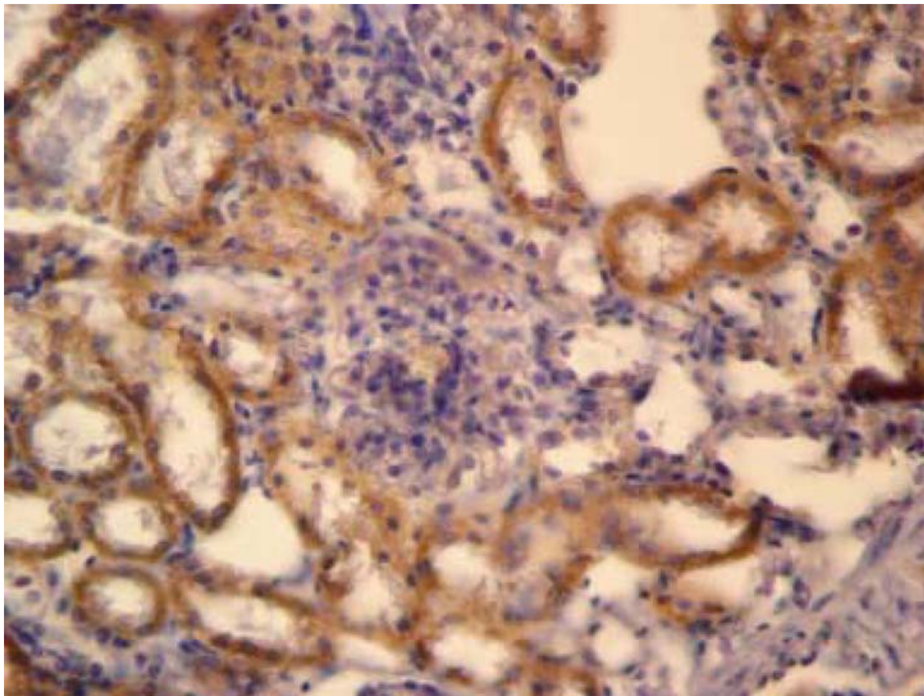

Fig. 9. Strong immunostaining for anti-nitrotyrosine antibody in the proximal tubular epithelial cells in group CS+E (×370).

suggested that presence of nitrosative stress could also result in injury of the kidney tissue [58]. In this study, the injury observed in the kidney tissues of group E and CS+E could be due to the increased nitrosative stress. Venkatraman *et al.* [59], showed that mice treated with an ethanol-containing diet for 5 weeks had increased 3-nitrotyrosine in the liver tissue as evidenced by immunohistochemical examination. In another study, Nanji *et al.* [60], also reported that administration of ethanol to the rats for 8 weeks increased formation of nitrotyrosine in the liver tissue. Nitration reaction could be achieved in two different ways. First, NO can react with superoxide to generate peroxynitrite, and the second one is associated with the reaction of NO with HOCl by the action of enzyme MPO [61, 62]. According to the results of our study, a possible cause for the decreased level of NO observed in the kidney tissues of group E and CS+E could be due to the action of MPO. Indeed, we found that NO levels of group E and CS+E decreased along with a proportional increase in MPO activity.

As a conclusion, in this study, the biochemical and immunohistochemical findings clearly demonstrated that chronic ethanol administration and cigarette smoke exposure cause excessive ROS formation which is leading to oxidative stress, nitrosative stress and a decrease of NO level, which might have caused renal tissue damage and renal function disorders.

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