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ORIGINAL PAPER

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The effects of chronic exposure to ethanol and cigarette smoke on the level of reduced glutathione and malondialdehyde 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). Apart from the control group, these were treated with ethanol and/or cigarette smoke for 6 months. The animals were killed and the kidneys removed to determine the levels of reduced glutathione (GSH) and malondialdehyde (MDA) and for histopathological analysis. The levels of GSH/g wet tissue were 1.58 ± 0.09 μmol, 0.91 ± 0.05 μmol, 1.14 ± 0.06 μmol, and 0.82 ± 0.04 μmol for C, E, CS, and CS + E, respectively. In groups of E, CS, and CS + E, the GSH values were significantly lower than that of group C animals ($P < 0.05$). Although, we detected lower GSH levels in the CS + E than the CS group ($P < 0.05$), a significant difference in GSH levels between CS + E and E was not observed. The levels of MDA/g wet tissue were 40.1 ± 3.4 nmol, 71.4 ± 2.8 nmol, 64.0 ± 3.6 nmol, and 76.5 ± 4.3 nmol, for C, E, CS and CS + E, respectively. In E, CS, and CS + E, the MDA values were significantly higher than in group C ($P < 0.05$). The increase in MDA levels in CS + E were not significantly different from groups E or CS. Histopathological analysis of the kidney slices showed severe degeneration of the tissues. Advanced hydropic degeneration of kidney tubules was clearly observed in the CS group. In group E, advanced tubular and interstitial damage, mononuclear cell infiltration and tubular thyroidization were clearly visible. In group CS + E, an intense inflammatory cell infiltration was detected under the transitional epithelium. We conclude that chronic exposure to ethanol and cigarette smoke may cause an oxidative burst in rat kidney by increasing the formation of reactive oxygen species.

Keywords Cigarette smoke · Ethanol · Kidney · Rat · GSH · MDA

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Introduction

The body defends itself against many chemical compounds such as toxic and pharmacological agents and xenobiotics. The kidney plays an important role in their elimination but is also vulnerable to these compounds. Smoking and alcohol consumption, which are common habits in modern societies, may cause a wide range of acute and chronic harmful effects in several organs including the kidney [1, 2, 3]. In terms of health effects, cigarette smoking has been strongly implicated as a risk factor in chronic obstructive pulmonary diseases, a wide variety of cancers, and cardiovascular diseases [4, 5, 6]. Many of the harmful effects caused by smoking may result from oxidative damage to biomolecules such as proteins, lipids, and nucleic acids [7, 8, 9]. In recent years, smoking has also emerged as a major risk factor in renal diseases [10]. Approximately 30% of renal cell carcinoma in men, and approximately 24% in women may be caused by smoking [11]. Chronic ethanol consumption also causes toxic effects in the kidney as well as the liver. A possible link between alcoholism and glomerulonephritis was reported by Keller et al. [12]. Alcohol consumption may also

cause acute tubular necrosis [13] and renal tubular dysfunction [14].

Free radicals or reactive oxygen species (ROS) induced by ethanol and cigarette smoke are thought to be responsible for the induction of many diseases, including toxicity in the kidney. Considerable experimental evidence supports the idea that ROS play a key role in the pathophysiological progression of renal diseases and renal tissue damage [15, 16, 17, 18]. Ethanol and cigarette smoking may also cause an oxidative burst resulting from ROS at the cellular level [19, 20]. The potential harmful effects of ROS are controlled by cellular antioxidant defense mechanisms including enzymatic defense systems such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as non-enzymatic defense systems such as reduced glutathione (GSH), vitamin A, and uric acid. GSH is an important non-enzymatic molecule against ROS in the cellular defense system. The detoxification pathway of ROS involves oxidation of GSH to glutathione disulfide (GSSG). Additionally, hydroxyl radicals and singlet oxygen molecules may initiate lipid peroxidation that leads to the degradation of polyunsaturated fatty acids in the cell membrane, and results in cellular injury. An elevated concentration of malondialdehyde (MDA), a breakdown product of lipid peroxidation, is one of the markers of tissue damage [21]. Many researchers reported that ethanol consumption and cigarette smoke alter GSH and MDA levels in several organs [22, 23, 24, 25, 26].

In the present study, we examined the individual and combined effects of chronic ethanol consumption and chronic cigarette smoke exposure by measuring the levels of GSH and MDA in rat kidney. In addition, histological examination of the kidney slices was also performed to further determine the effects of ethanol intake and cigarette smoke.

Materials and methods

Study design

Young male Wistar rats (1.5 months old), weighing about 180–220 g were obtained from the Inönü University Animal Research Laboratory. The animals were housed individually in stainless steel cages and maintained under conventional conditions (temperature 20–22°C, humidity 60–70%) in a room that provided a 12:12 h light:dark cycle. The animals were divided randomly into four groups, each including seven rats. In three groups the rats were treated with ethanol and/or cigarette smoke for 6 months. In the control group (group C) animals were orally fed with a modified liquid diet (MLD; cow's milk: 925 ml, vitamin A: 5,000 IU and sucrose: 17 g) with sucrose as a caloric substitute for ethanol. Animals in the ethanol group (group E) were treated as described by Uzbay and Kayaalp [27]. Briefly, all rats consumed MLD for 7 days, after that, MLD containing 2.4% ethanol (v/v) (ethanol 95.6%, Tekel, Turkish State Monopoly) was given for 3 days. Then, the ethanol concentration was increased to 4.8% for 3 days and finally to 7.2% for 6 months. When the ethanol concentration in the diet was increased, sucrose concentration was reduced to maintain isocaloricity of the diet. Animals in the cigarette smoke group (group CS) were exposed to cigarette smoke as described by Zhu et al. [28] and fed with the same modified diet as the control group animals. Rats were kept in a closed plexiglass cage (dimensions: 0.75×1.00×0.85 m., volume: 0.64 m^3) and exposed to the smoke of 20 cigarettes per day during the experimental period. A burning cigarette was connected to the plexiglass cage by a cigarette machine which burns a cigarette in about 7 min. Air was also pumped into the plastic cage. The cigarette smoke plus ethanol group (group CS + E) animals were treated in the same manner as groups CS and E. In all groups, each rat was given an 100 ml liquid diet and the daily liquid consumption of each rat was recorded. At the end of 6 months, the rats were killed and their kidneys harvested. A part of the kidney was preserved in 10% formalin for histological examination and the remaining part was stored at −70°C until GSH and MDA analysis. All of the samples were analyzed under the same conditions and at the same time.

Kidney GSH levels

The GSH content was determined according to Ellman [29]. GSH reacts with 5,5′ dithiobis-2-nitrobenzoic acid, and the product has a maximal absorbance at 410 nm. The results were expressed as μmol/g wet tissue (gwt).

Kidney MDA levels

The kidney tissues were homogenized in cold 1.15% KCl to make a 10% homogenate. The MDA levels of the homogenate were assayed spectrophotometrically at 535 and 520 nm according to Uchiyama and Mihara [30]. A standard calibration curve was drawn by using 1,1,3,3-tetramethoxypropane. The results were expressed as nmol/gwt.

Histological analysis

Fixed tissues were embedded in paraffin, sectioned at 5 μm thickness and stained in hematoxylin-eosin (H-E). The stained sections were analyzed using a light microscope. The kidney tissues were examined for tubular degeneration, inflammatory cell infiltration and thyroidization of the tubules.

Statistical analysis

Results are expressed as means ± SEM. Data were evaluated by a one way analysis of variance, and the differences were considered significant if the P value was less than 0.05 by Tukey's multiple comparison test.

Results

Over the 6 month period, the daily average liquid diet (containing 7.2% alcohol) consumption of rats was 59.12 ± 0.90 ml and 53.57 ± 1.48 ml in groups of E and CS + E, respectively. GSH and MDA levels in rat kidney tissues in all experimental groups are shown in Table 1. In group E (0.91 ± 0.05 μmol/gwt), group CS (1.14 ± 0.06 μmol/gwt), and group CS + E (0.82 ± 0.04 μmol/gwt), GSH levels were found to be significantly lower than in group C animals (1.58 ± 0.09 μmol/gwt) ($P < 0.05$). Although, lower GSH levels were measured in group CS + E than group CS animals ($P < 0.05$), a significant difference between group CS + E and group E was not obtained. Kidney

Table 1 The effects of chronic ethanol consumption and cigarette smoke exposure on the levels of GSH and MDA in rat kidney. Control (C), ethanol (E), cigarette smoke (CS) and cigarette smoke plus ethanol (CS + E). Each group represents the mean ± SEM for seven rats

Groups	GSH (μmol/gwt)	MDA (nmol/gwt)
C	1.58 ± 0.09	40.1 ± 3.4
E	0.91 ± 0.05	71.4 ± 2.8
CS	1.14 ± 0.06	64.0 ± 3.6
CS + E	0.82 ± 0.04	76.5 ± 4.3
Statistical comparison (*P*)		
C vs E	0.000	0.000
C vs CS	0.000	0.001
C vs CS + E	0.000	0.000
E vs CS	0.053	0.531
E vs CS + E	0.690	0.781
CS vs CS + E	0.005	0.103

MDA levels in groups of E (71.4 ± 2.8 nmol/gwt), CS (64.0 ± 3.6 nmol/gwt), and CS + E (76.5 ± 4.3 nmol/gwt) were significantly higher than in group C (40.14 ± 3.4 nmol/gwt) ($P < 0.05$). The increase in MDA levels of group CS + E was not significantly different than that of groups E or CS.

Histopathological evaluation of the tissues in group C did not reveal any pathological abnormalities (Fig. 1a), however, in group CS, advanced hydropic degeneration of the kidney tubules was clearly visible (Fig. 1b). While in group E, ethanol intake mostly resulted in tubular interstitial damage, mononuclear cell infiltration and tubular thyroidization in kidney tissue (Fig. 1c), in group CS + E, excessive, intense inflammatory cell infiltration was detected under the transitional epithelium (Fig. 1d), and calcification focuses were also found in the cortical medullary line (data not shown).

Discussion

In modern societies, alcohol consumption and smoking are very common habits. For a long time, the damage caused to human health caused by chronic alcoholism and smoking was poorly established. In recent years, researchers have focused on the effects of alcohol consumption and smoking on the lungs, liver and heart, but few studies has been conducted on kidneys. Even nephrologists disregarded the effects of smoking on the kidney [11]. Today, it is widely accepted that alcoholism and smoking are harmful to human health. It has been proposed that damage to several organs can be attributed to the oxidative stress which is the main effect of alcoholism and smoking. 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 [22, 26, 31, 32, 33]. The results of other studies also concluded that ROS were the main contributors to renal disease [16, 17, 18].

Several studies on the effect of ethanol damage were conducted on organs such as the liver, stomach, central nervous system, heart and testes, which are thought to be sensitive to ethanol intake. Chronic ethanol intake resulted in an increase in lipid peroxidation and a decrease in GSH levels in such organs as the liver [34], gastric mucosa [35], and testes [36]. In these studies, the changes in antioxidant levels in addition to the ROS formation were investigated following ethanol intake, and a correlation between ethanol intake and ethanol induced ROS formation was sought. Since little information is available on the effect of ethanol and cigarette smoke on kidney tissue, we examined the individual and

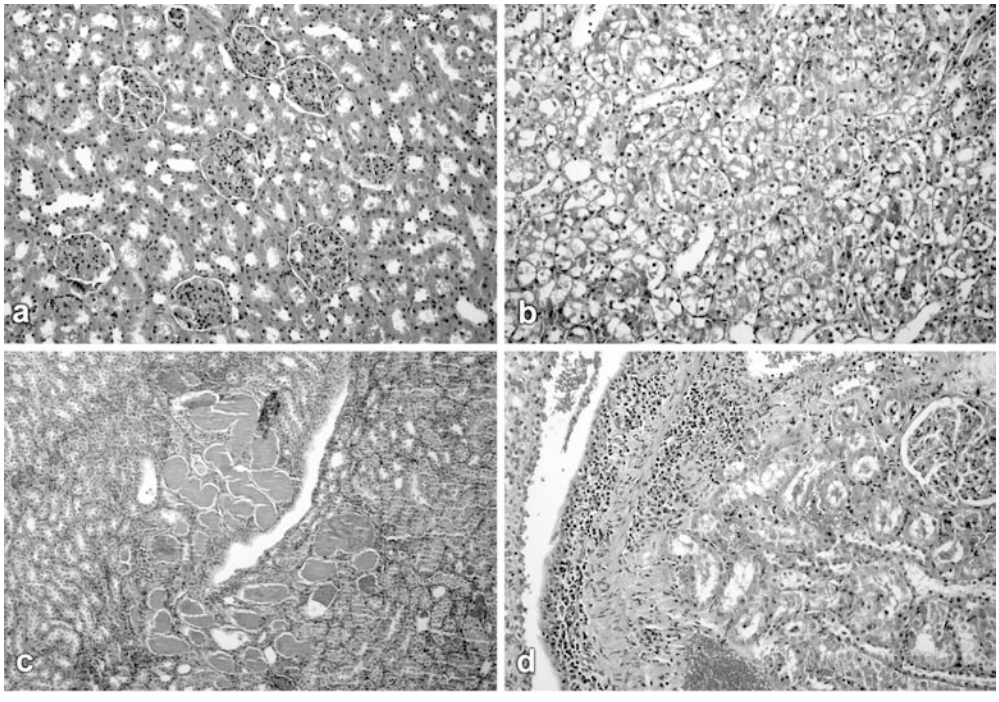

Fig. 1 a A light micrograph showing normal histoarchitecture in a group C rat kidney (H and E ×185). **b** A light micrograph showing advanced tubular hydropic degeneration of kidney in group CS (H and E ×185). **c** A light micrograph mononuclear cell infiltration in the interstitium. Hyalinized eosinophilic proteinaceous material is present in many of the tubules, "thyroidization of kidney", in group E (H and E ×85). **d** A light micrograph showing intense inflammatory cell infiltration beneath the transitional epithelium of kidney in group CS + E (H and E ×185)

combined effects of ethanol and cigarette smoke on this organ. We found that treatment of rats with ethanol and cigarette smoke for 6 months significantly reduced the GSH content and elevated the MDA level in the kidney. Other studies have also shown similar results; ethanol administration resulted in a decrease in GSH level by inhibiting GSH synthesis in the liver [32, 33] and kidney [1, 37]. Ethanol intake also increased MDA levels in rat kidney [1]. Ethanol based kidney tissue damage can be attributed to ROS, a final product of ethanol metabolism [19]. Ethanol is mainly metabolized to acetaldehyde by alcohol dehydrogenase (ADH), CAT, and a microsomal ethanol-oxidizing system in the liver [38, 39]. Rat kidney tissue also contains ADH that appears to be similar or identical to that present in the liver [40, 41]. The maintenance of physiological concentrations of GSH, an important non-enzymatic antioxidant against ROS, is essential for a large number of cellular functions. In the cell, GSH reacts directly with free radicals and is oxidized to GSSG, thus protecting essential thiol groups from oxidation. In the present study, the depletion of GSH levels in rat kidney suggests the presence of oxidative stress due to ethanol consumption. Various mechanisms have been proposed to explain ethanol-induced depletion of GSH levels in the kidney. One of these mechanisms proposes that the decreased levels observed in our study could be caused by an increased oxidation state of GSH through excessive ROS generation during ethanol metabolism. Ethanol is metabolized to acetaldehyde by ADH, and then further oxidized by acetaldehyde dehydrogenase to a final product that generates the ROS, which may oxidize GSH to GSSG. Another source of ROS which might be produced by the induced cytochrome P450 system might also oxidize GSH. Another mechanism suggests that the binding of acetaldehyde, a product of ethanol metabolism, to cysteine (a precursor of GSH) and/or GSH may contribute to the decreased GSH levels in rat kidney [38, 42].

In our study, elevated MDA levels in ethanol treated rats could be explained by the excessive ROS production due to ethanol metabolism through ADH or the induced cytochrome P450 system. MDA is the final product of a radical chain reaction of peroxidation of lipids. Membrane structure, particularly membrane fluidity, membrane cross-linking and membrane function, is affected by ROS induced lipid peroxidation of polyunsaturated fatty acids [43]. Orellana et al. [40] showed that enhanced ADH activity and a subsequent increase in acetaldehyde production in rat kidney occurred after the rats were treated with ethanol for 10 weeks. They concluded that chronic ethanol consumption increased ethanol oxidation in the rat kidney and thus gave rise to kidney damage.

Our study on rats demonstrated that cigarette smoke significantly decreased GSH levels and increased MDA levels in the rat kidney. Similarly, Anand et al. [44] examined the antioxidant status and lipid peroxidation levels of rat kidney after the rats were chronically exposed to cigarette smoke. They reported that GSH levels also decreased and MDA levels increased when compared to control animals. In another study on humans, Bingol et al. [45] compared lipid peroxidation levels in the plasma and erythrocytes of smokers and non-smokers, and found that lipid peroxidation levels in smokers were significantly higher than levels in non-smokers, both in the plasma and erythrocytes. Cigarette smoke contains about 4,000 different chemical compounds most of which are harmful to human health. This harmful effect is probably caused changes to the cellular defense systems, such as antioxidant mechanisms. Cigarette smoke includes both reactive aldehydes and a large number of oxidants which contain about 10^{14} ROS molecules with each per puff [20, 46, 47]. In addition to these reactants, nicotine in cigarettes is also oxidized and generates ROS in the body [46]. These radicals may initiate the lipid peroxidation mechanism and thus increase MDA levels in the kidney. In addition, reactive aldehydes are thought to react with protein thiols and presumably with small thiols such as GSH, thus decreasing GSH levels in the kidneys of smokers [8]. In addition to reactive aldehydes, excessive ROS, which results from cigarette smoke, might also oxidize GSH, explaining reduced GSH levels.

In the CS + E group, in which ethanol and cigarette smoke were applied together, the GSH level decreased significantly ($P < 0.05$) when compared to the CS but not to the E group. The MDA level in the CS + E group was found to be slightly higher than that in the CS and E groups (Table 1). From these results, it might be possible to conclude that the cumulative damage of cigarette smoke and ethanol was more deleterious on the kidney's antioxidant mechanism. The combination of ethanol and cigarette smoke might have induced excessive ROS production to a greater extent than ethanol or cigarette smoke alone, as suggested previously by Husain et al. [22].

Although, several of the studies discussed above support our results, contradictory results have also been reported. Husain et al. [22] measured MDA and GSH levels in rat liver, kidney, lungs and testes after 6.5 weeks of chronic ethanol, nicotine and a combination of ethanol and nicotine treatment. They reported that kidney MDA levels were significantly higher; however, kidney GSH levels remained unchanged in all experimental groups. Similarly, Park et al. [48] also showed unchanged GSH levels in rat kidney after 30 days exposure to cigarette smoke. In another study, Rodrigo et al. [49] fed rats with 12.5% (v/v) ethanol for 10 weeks and analyzed GSH, GSSG and MDA levels in the renal cortex and renal papilla. They demonstrated dramatically lower GSH levels in the renal cortex and renal papilla, however, unchanged GSSG levels in the renal cortex and substantially higher GSSG levels in the renal papilla. In these tissues, MDA levels were found to be slightly (although not significantly) elevated compared to the control groups. Thus, it was suggested that alcohol consumption decreased GSH levels in the renal cortex by preventing GSH synthesis. In renal papilla, the

decrease of GSH levels was explained by ROS induced oxidation of GSH to GSSG, which suggested the antioxidant activity of GSH. Although ethanol induced changes in GSH content supports our results, unchanged MDA levels in both the renal cortex and renal papilla are contradictory. This discrepancy might be explained by the short duration of the other study, 10 weeks only, as also stated by the authors.

As mentioned in these studies, a final conclusion can not be reached on the effects of chronic ethanol consumption and cigarette smoke exposure on the levels of GSH and MDA in the kidney. While some investigators reported no change in GSH and MDA content, others found decreased GSH or increased MDA levels. Our contradictory results can be explained due to differences in the duration of the experimental period and exposure times to ethanol and cigarette smoke, since we exposed the animals for 6 months which is about 4–7 times longer than in the other studies. It is also possible that the quantity of ethanol, the volume of inhaled cigarette smoke or different animal strains might contribute to the variation in results.

Histopathological examination of kidney slices clearly showed that, cellular damage was visible in groups of E, CS, and CS + E but not in the control group C (Fig. 1a). Detailed analyses of the tissues revealed highly advanced hydropic degeneration of the kidney tubules in group CS (Fig. 1b). In group E (Fig. 1c), mainly tubular interstitial damage, mononuclear cell infiltration and hyalinized protein deposition "thyroidization" were detected. In group CS + E, intense inflammatory cell infiltration was clearly visible under the transitional epithelium (Fig. 1d), and calcification foci were also found in the cortical medullary line (data not shown). Other studies on kidneys reported that chronic ethanol intake induced renal tubular necrosis, vacuolation in the glomeruli and degeneration of the basement membrane [50, 51]. Many studies have also demonstrated that chronic smoking induces several pathological changes in kidney tissue [52, 53, 54]. The histopathological lesions in the kidney of ethanol and cigarette smoke treated animals might be correlated to the GSH levels. We hypothesize that a ROS induced decrease in GSH levels and increase in lipid peroxidation in the kidney cells could contribute significantly to cellular kidney damage. In addition, Scott et al. [1] also suggested that the depletion of renal GSH levels (45% depletion) might be an important factor causing renal injury. We found that renal GSH levels decreased significantly by 28%, 42% and 48% in groups of CS, E and CS + E, respectively, and these results might be correlated with the severity of the tissue damage observed in the histopathological analyses of rat kidney. Moreover, ROS which is induced by other sources could also contribute to tissue damage. One of these sources might be polymorphonuclear leukocytes (PMN). The infiltration of PMN in a tissue is a characteristic feature of inflammation. Neutrophils have also been implicated as a potential source of oxygen free radicals, which are thought to be a primary cause of organ injury in inflammation. Activated neutrophils discharge tissue-damaging substances, such as superoxide anions, hydrogen peroxide, proteases and myeloperoxidase (MPO). MPO activity in a tissue has become widely accepted as a reliable estimate of PMN infiltration into inflamed tissues. Another source of ROS in a tissue is xanthine oxidase (XO). In normal tissues this enzyme can exist as xanthine dehydrogenase (XD) which uses NAD as its electron acceptor in the conversion of hypoxanthine to xanthine and uric acid. In this reaction, oxygen free radicals are not produced. In several pathological conditions, XD can be converted to XO which uses oxygen as its electron acceptor, generating superoxide radicals. Acetaldehyde resulting from the metabolism of ethanol may convert XD into the superoxide generating form XO in the tissue [55]. In another study, we also determined that MPO and XO activities increased substantially in the rat kidney after chronic ethanol administration and cigarette smoke exposure (unpublished data). These biochemical assays indicate the presence of tissue damage which supports our histopathological and biochemical results.

In summary, we suggest that chronic ethanol consumption and cigarette smoking may cause excessive ROS production in the kidney, which destroys the oxidant/antioxidant balance mechanisms and thus may initiate ROS induced tissue damage. Additionally, the combined intake of ethanol and cigarette smoke may give rise to increased tissue damage.

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