Chronic alcohol ingestion is associated with pronounced detrimental effects on the renal system. In the current study, the protective effect of ginger extract on ethanol-induced damage was evaluated through determining 8-OHdG, cystatin C, glomerular filtration rate, and pathological changes such as cell proliferation and fibrosis in rats' kidneys. Male wistar rats were randomly divided into three groups and were treated as follows: (1) control, (2) ethanol and (3) ginger extract treated ethanolic (GETE) groups. After a six weeks period of treatment, the results revealed proliferation of glomerular and tubular cells, fibrosis in glomerular and peritubular and a significant rise in the level of 8-OHdG, cystatin C, plasma urea and creatinine. Moreover, compared to the control group, the ethanol group showed a significant decrease in the urine creatinine and creatinine clearance. In addition, significant amelioration of changes in the structure of the kidneys, along with restoration of the biochemical alterations were found in the ginger extract treated ethanolic group, compared to the ethanol group. These findings indicate that ethanol induces kidneys abnormality by oxidative DNA damage and oxidative stress, and that these effects can be alleviated using ginger as an antioxidant and anti-inflammatory agent.

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1. Introduction

The growing body of evidence suggests that chronic ethanol exposure induces functional alterations and structural damage in the kidneys [1,2]. From a functional perspective, after ethanol exposure, disorganization of proximal tubules, disorientation of microvilli and luminal casts, expansion of the mesangial matrix of glomeruli, occlusion of proximal convoluted tubules, as well as partial degeneration of proximal tubule cells along with reduced height of the cells have been observed in previous studies [3,4]. In functional future, a wide range of changes, such as filtration defects, BUN and creatinine enhancement in the circulation, a significant increase in the amount of protein in urine, hyper-acetylation of mitochondrial protein, and retention of renal Na⁺ with elevated plasma aldosterone concentration, following ethanol consumption, has recently been reported [5,6]. Additionally, chronic ethanol exposure results in acute and chronic kidney disease and incidence of end stage renal disease with a high risk of mortality [7,8]. Despite the finding of different futures of functional and structural abnormalities in kidneys, due to ethanol consumption, little is known about the precise underlying molecular and cellular mechanisms through which ethanol exerts its susceptibility to this organ. Our recent works and the literature suggest that ethanol can induce tissue damage by oxidative and inflammatory stress [9,10]. Controversy exists, however, on whether alcohol has any direct or indirect nephrotoxic effects. Studies from some laboratories support the idea that ethanol ingestion is not nephrotoxic, and ethanol induced kidney injury is secondary and associated with the mortality of alcoholic liver disease [5,11]. According to this conception, ethanol oxidation in the liver by cytochrome P450 2E1(CYP2E1) monoxygenase forms free radicals, such as superoxide (O₂⁻) radical and then H₂O₂; substances that prompt tissue oxidative stress and injury [12]. In contrast, Latchoumycandane et al. in a recent study demonstrated that ethanol induced direct nephrotoxic injury in kidneys in many ways. Approximately, five to 10% of CYP2E1 express in kidneys and metabolism of ethanol by CYP2E1 in kidneys generated reactive
oxygen species (ROS). Subsequently, it generated ROS and induced peroxidation of polyunsaturated phospholipids in kidneys [3]. In addition, oxidative fragments of polyunsaturated lipids generated PAF-like truncated phospholipids that stimulated formation of PAF receptors (PTAFR) in kidneys. PTAFR then stimulated the infiltration and activation of neutrophil. Activated neutrophils generated abundant myeloperoxidase. Moreover, myeloperoxidase, following ethanol exposure, caused oxidative injury to kidneys [3].

Regardless of the idea that ethanol-induced susceptibility in the kidney is direct or indirect, the beneficial effect of antioxidant therapy during alcohol exposure in kidneys and other tissues, support the idea that ethanol may exert its deleterious effects mainly via oxidative stress [9,13]. Based on the findings of previous studies, a wide range of plant-derived active agents has been reported to have beneficial effects against ethanol-induced tissue pathogenesis [9,14]. Among plant-derived active agents, ginger, due to its antioxidant as well as anti-inflammatory properties, is found to be unique [15]. Oxidative nature of ethanol, on the one hand and beneficial effects of antioxidant therapy during alcohol exposure on the other hand, tempted us to re-examine the theory that if deleterious effect of chronic ethanol consumption on kidneys is entirely or partially mediated by oxidative stress and inflammatory reaction, ginger treatment should alleviate or protect kidneys from susceptibility to ethanol. Accordingly, in the current study, to test this hypothesis, the possible protective effects of ginger extract against ethanol-induced structural and functional alteration were investigated in the kidneys of male rats.

2. Materials and methods

All procedures for rats were conducted in accordance with the Principles of Laboratory Animal Care* (NIH publication no. 85-23, revised 1985) and were approved by the Urmia University of Medical Sciences Animal Care Committee. Twenty four male wistar rats with an initial body weight of 210 ± 10 gr were divided into three groups, namely, 1- control, 2- ethanol, and 3- ginger extract treated-ethanol (GETE) groups.

Rats in the ethanol group received ethanol with a dose of 4.5 g/kg body weight (Merck KGaA, Darmstadt, Germany) saluted in tap water (20% w/v) intragastrically once a day, by gavage for six weeks. GETE rats received BW hydro-alcoholic extract of ginger with a dose of 50 mg/kg body weight intragastrically by gavage, in addition to their regular daily diet and the same amount of ethanol. The control group was treated only with tap water. To prepare ginger extract, a dried ginger rhizome (originally Chinese) was purchased from a local market. In an electric grinder, a sufficient quantity of rhizome was powdered. Hydro-alcoholic extract of ginger was prepared by mixing three kg of powder with six liters of ethanol 70% in a suitable container. It was then left for 72 h in room temperature. Next, the extract was filtered through filter paper and then concentrated using a rotary evaporator. The yield of the extract was kept in a refrigerator until the time of use.

After six weeks of treatment, the rats were anesthetized by 10% chloral hydrate (0.5 mL/kg body weight, IP), and the depth of anesthesia was confirmed by pinching a hind paw. For urine samples collection, 24 h before anesthetizing, rats were kept in metabolic cages singly, and urine was collected. Urine samples were immediately centrifuged to remove debris or extra substances before storage at −20 °C.

After weighing, the blood samples were collected directly from the heart and mixed with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Blood samples were then centrifuged at 4000 × g for 20 min within 30 min of collection. Furthermore, the yielded plasma was stored at −80 °C without repeated freeze-thaw cycles. After blood sampling, the abdominal cavities were opened, and both kidneys were dissected. Excised kidneys were freed from adventitial tissues, fat, and blood clots and were subsequently weighed. The right kidney was divided into two parts. For the purpose of histopathological investigations, a part of the kidney was immediately fixed in 10% buffered formalin and then after standard dehydration steps were taken, it was embedded in paraffin.

To perform biochemical analysis, other parts of the kidneys were washed with ice-cold physiological saline and then dried on filter papers. Subsequently, an ice-cold extraction buffer (10% wt./vol), containing a 50 mM phosphate buffer (pH 7.4), was added. It was then homogenized using Ultra Turrax (T10B, IKA, and Germany). Next, the homogenates were centrifuged at 10,000 × g at 4 °C for 20 min. As the last step, the supernatant sample was collected and stored at −80 °C until the time of analysis.

2.1. Cystatin C

The amount of plasma Cystatin C was measured by the quantitative sandwich enzyme immunoassay method using a commercial rat cystatin C Elisa kit (CUSABIO, CHINA). Briefly, micro-plates were pre-coated with a specific cystatin C antibody. Then, standards and samples were pipetted into the wells, and any cystatin C present was bonded by an immobilized antibody. After removing all unbound substances, a biotin-conjugated Horseradish Peroxidase (HRP) was added to the wells. Moreover, any unbound avidin-enzyme was removed by washing. A substrate solution was added to the wells and a color was developed in proportion to the amount of cystatin C bounded in the initial step. The color development was stopped and the intensity of the color was measured.

2.2. 8-OHdG

The amount of 8-OHdG was measured by the quantitative sandwich enzyme immunoassay method using a commercial rat 8-hydroxy-desoxynosine Elisa kit (CUSABIO, CHINA), according to the manufacture guidelines.

2.3. Plasma and urine creatinine and urea

Plasma and urine creatinine and urea levels were measured by using urea and a creatinine commercial kit (Pars Azemoon, Karaj, IRAN).

2.4. Glomerular filtration rate

Glomerular filtration rate (GFR), as a best overall index of kidneys function, was estimated by calculating creatinine clearance (GFR=\(\frac{UCr \times V}{Scr}\)) and using plasma and urine creatinine concentrations and the urine flow rate or volume.

2.5. Histopathological examinations

To assess the glomerular and tubular cells proliferation rates, proliferation cell nuclear antigen (PCNA) anti-body staining protocol was used, according to the kit instructions and our recent method. In brief, tissue sections (4-μm thick) from the paraffin-embedded kidney tissues were stained by Monoclonal anti-PCNA antibody (Dako Denmark A/S, Denmark). This was performed after taking tissue processing steps such as deparaffining, rehydrating by gradual ethanol passage, and finally washing them in Tris buffer.

Optimal results were achieved using the EnVision™ visualization system (Dako Denmark A/S, Denmark). Furthermore, hematoxylin was used as a counterstain. Appropriate negative controls

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were included in the assessment. Moreover, all the slides were independently inspected by two expert pathologists. In addition, PCNA-positive indices were considered as indicators of cell proliferation.

To assess the percentage of PCNA-positive indices, all cells contained in each cross section (3 to 5 cross sections) of the kidneys were scored. The criteria for quality scoring of PCNA-positive indices were as follows: normal, PCNA-positive indices present in less than 5% of glomerular and tubular cells; mild, PCNA-positive indices present in less than 25% of glomerular and tubular cells; mild to moderate, PCNA-positive indices present in 25–50% of glomerular and tubular cells; moderate to severe, PCNA-positive indices present in 50–75% of glomerular and tubular cells; and severe, PCNA-positive indices present in 75–100% of glomerular and tubular cells [16]. To evaluate the glomerular and peritubular fibrosis in kidneys, and following the manufacturer’s instructions (asiaajocsh, Amol, IRAN), 4 μm kidney tissue sections were stained with Masson trichrome. The severity of tissue fibrosis was assessed using a semi-quantitative method explained by O’Shea et al. [17]. A score ranging from zero (normal heart) to eight (total fibrosis) was allocated. The criteria for scoring the heart fibrosis were as follows: grade 0 = normal heart, grade 1 = minimal fibrosis thickening of the myocardial or coronary vessels walls, grade 2 and 3 = moderate thickening of the myocardial or coronary vessels walls without obvious damage to the heart tissue structure, grade 4 and 5 = increased fibrosis with definite damage to the heart architecture and formation of fibrosis bands or small fibrosis masses, grade 6 and 7 = severe structural distortion and large fibrosis areas, and 8 = total fibrotic obliteration [17].

2.6. Statistical analyses

Normal distributions of data within each group were verified by the Kolmogorov-Smirnov test. Statistical differences between the groups were tested by performing a one-way ANOVA, followed by the Tukey’s post hoc test. In each test, the data were expressed as the mean ± S.D., and p = 0.05 was considered to be statistically significant.

3. Results

The data presented in Table 1 detail the impact of 42 days chronic ethanol consumption on urine, plasma, and kidney parameters. Exposure to ethanol resulted in a significant increase (p < 0.05) of the kidneys’ 8-OHdG content compared to that of the control group. Co-administration of ginger extract along with ethanol reduced the 8-OHdG content significantly compared to that of the ethanol group (p < 0.05). The amount of cystatin C in the plasma of the ethanol group was increased significantly, as compared with that in the controls (p < 0.05). In the GETE group, plasma cystatin C level showed a significant decrease, compared with that in the ethanol-treated group, but it was still significantly higher than that in the control group (p < 0.5). Urea levels in the plasma and urine of the ethanol-treated group were significantly higher than those in the control group (p < 0.05). In the GETE group, urine urea levels decreased significantly compared with those in the ethanol-treated group, but they were still significantly higher than those in the control group (p < 0.05). There were no significant differences in urea levels in the urine between the GETE and the control groups (p < 0.3). The Plasma creatinine level showed a significant increase in the ethanol treated group compared to that in the control group (p < 0.05). Ginger extract administration along with ethanol reduced the plasma creatinine level significantly compared to the ethanol group (p < 0.05); however, it was still significantly higher than that in the control group (p < 0.05). In contrast to plasma, ethanol treatment reduced creatinine level in the urine, compared to the control group (p < 0.05). There was no significant difference in terms of the urine creatinine level between the GETE and the control group. In the ethanol treated group, the creatinine clearance, as an indicator of glomerular filtration rate, was significantly lower than that in the control group (p < 0.05). Ginger extract treatment significantly increased the creatinine clearance compared to that in the ethanol group (p < 0.05), but it was still significantly lower than that in the control group (p < 0.05).

The ratio of serum cystatin C to serum creatinine increased significantly in the ethanol treated group compared to that in the control group (p < 0.03). There were no significant differences found between the GETE and control group in terms of cystatin C/creatinine ratio.

The ratio of proliferated cells (PCNA-positive indices) in different parts of nephron is given in Fig. 1. The ratio of proliferated cells in the glomerulus part of nephron was 1, 3.65 ± 6, and 1.5 in the control, ethanol, and GETE groups, respectively. The PCNA-positive indices were dramatically increased in the ethanol treated group, compared to that in the control group (p < 0.05). There were no significant differences between the GETE and the control group. In the proximal section of nephron tubules, the ratio of PCNA-positive indices showed a significant increase in the ethanol group (56.5 ± 2.5%), compared to that in the control group (1%) (p<0.05). PCNA-positive indices did not differ between the GETE group (1.5%) and the control group. In the distal portion of nephron tubules, the percentage of PCNA-positive indices of ethanol treated group (43.3 ± 7.3%) was significantly higher than that in the control group (1%) (p < 0.001). There were no significant differences between the GETE group (1.5%) and the control group in terms of PCNA-positive indices percentage.

Fig. 2 shows microscopic fibrosis scores in different parts of nephron tubules and peritubular vessels indifferent groups. There were no lesion score in the glomerulus, proximal tubules, distal tubules, as well as around peritubular vessels in the control group (grade 0). The microscopic lesion score in the glomerulus, proximal tubules, distal tubules, and peritubular vessels was 4 to 5 which is an indication of increased fibrosis with definite damage to the kidney’s architecture and formation of fibrosis bands or small fibrosis masses. There were no significant differences between the GETE and the control group.

4. Discussion

The main findings of the current study are as follows: long term administration of ethanol to rats impairs kidneys function which is manifested by a significant increase of plasma creatinine, urea, cystatin C levels, ratio of cystatin C/creatinine, as well as a significant decrease in creatinine clearance as an indicator of glomerular filtration rate in the ethanol treated group compared to
that in the control group. As a biomarker of oxidative DNA damage, significant higher levels of 8-OHdG were observed in kidney tissue of the rats in the ethanol treated group. Structural alterations, such as moderate to severe cell proliferation in glomerulus and different parts of nephron tubules as well as increased fibrosis with definite damage to the kidney architecture and formation of fibrosis bands or small fibrosis masses were also found in kidneys of ethanol treated rats compared to the control rats. Significant amelioration of urea, creatinine, cystatin C levels, 8-OHdG amount, and restoration of structural alterations, similar to those of the control group animals, were observed in the GETE group animals.

Although alcoholic disease is uniformly considered as a consequence of alcoholic hepatitis [17,18], recently, the direct association between alcoholism and renal dysfunction and not the underlying hepatitis has attracted researchers’ attention [5]. Our recent works and others have indicated that oxidative stress plays a key role in ethanol toxicity of different organs [6]. In addition, recent studies have identified oxidative stress as a key player in ethanol nephrotoxicity [5,11,19]. For example, the study by Murta et al. indicated that the exposure to ethanol in rats models promoted the antioxidant system depression due to the increase of lipid peroxidation and decrease of GSH amount in the kidneys [20].

Here, as the first study, we suggest that chronic ethanol ingestion leads to a significant increase in oxidative DNA damage to kidney tissue. This damage is manifested by a high level of 8-OHdG in the ethanol group compared to the control group. The 8-OHdG level, as a biomarker of oxidative DNA damage, reflects the average of oxidative DNA damage [21]. Metabolism of one molecule ethanol produces four molecules hydroxyl radicals. These radicals have a key role in causing damage to DNA [22]. While 8-OHdG has the slowest repair kinetics among DNA based oxidation products, it is the most common adduct produced in DNA following oxidative stress [23]. Moreover, it can be said that accumulation of 8-OHdG is the result of serious ROS damage accompanied with chronic ethanol consumption.

In the present study, ethanol administration caused a reduction in creatinine excretion and increased plasma creatinine levels. Plasma cystatin C also increased in the ethanol treated group. A recent study by Saravanan et al. showed that chronic ethanol administration to rats resulted in significantly elevated levels of serum urea, creatinine and uric acid as well as kidney thiobarbituric acid reactive substances, as indicators of lipid peroxidation, compared to control rats [24]. Plasma creatinine and cystatin C are two predictor markers of kidney function. The estimation of
glomerular filtration rate by calculating creatinine clearance and elevated plasma cystatin C levels indicated that ethanol consumption affected renal function in rats receiving ethanol. Because the levels of serum creatinine and cystatin C are affected by different non-renal factors such as body weight, higher white blood cell count, and increased markers of inflammation [25,26], Grubb et al. suggested the ratio of cystatin C/creatinine be used as an indicator of alterations in the glomerular filtration quality [27]. The enhancement of serum cystatin C/creatinine ratio occurs in a condition called 'shrunken pore syndrome', due to shrinkage of glomerular pores. Such pathophysiological phenomenon in glomerular barrier with shrinkage of glomerular pores influences the composition of glomerular filtrate as follows: A small reduction in pore size is identified by increased serum concentrations of large molecules such as cystatin C, but further pore shrinkage causes retention of smaller molecules such as urea and creatinine, which in turn leads to their accumulation in the blood [10,28]. Cystatin C is 100 times larger than creatinine and is retained more than

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**Fig. 2.** Photomicrographs of kidney tissue staining by Masson Trichrome staining show that microscopic lesion score in the glomerulus, proximal tubules, distal tubules, and as well as peritubular vessels was 4 to 5 that indicates increased fibrosis with definite damage to the kidney architecture and formation of fibrosis bands or small fibrosis masses. There were no significant differences between the GETE and control groups. A) Glomerulus B) proximal section of nephron tubule C) Distal section of nephron D) peritubular vessels. (Original magnification × 400). Fibrosis Bond (--).
creatinine in a lower degree of pore shrinkage. Therefore, the ratio of cystatin C/creatinine can serve as an indicator of kidney disease at an earlier stage [29]. Interestingly, results of the current study showed a significant increase in the cystatin C/creatinine ratio concurrent with a high plasma creatinine and urea level in ethanol treated group compared to those in the control group. To our knowledge, this is the first in vivo study showing that alcohol exposure influences the kidney function by shrinking glomerular pores and documenting a rise in the cystatin C/creatinine ratio.

Another important result of the current study was tubulointerstitial fibrosis and cell proliferation in ethanol fed rats. Our recent work and others have indicated that ethanol exposure of rats leads to fibrosis and cell proliferation in tissues such as the heart, aorta, pancreas, liver and lung [16,30–32].

Although cell proliferation was found in all parts of the nephron tubules and interstitial tissue, the severity of proliferation was more pronounced in proximal segments than in other parts of tubules. Fibrosis is caused by the progressive accumulation of interstitial matrix proteins followed by a maladaptive wound repairing; consequently, leading to gradual destruction of renal tubules and nephron functions [33]. The mechanism through which ethanol induced cell proliferation and fibrosis is not fully understood, but it may have resulted from oxidative stress and inflammatory reactions. Recently, we and others reported that chronic ethanol consumption caused cell proliferation and fibrosis in the heart and aorta tissue and was mediated by oxidative stress and inflammation [10,34]. In addition, accumulating evidence suggested that reactive oxygen species yielded from oxidative stress and inflammatory cytokines played a key role in the kidney tubular proliferation and kidney fibrosis [35]. Ethanol ingestion caused infiltration and activation of leukocyte in kidneys [5,33]. Infiltrated neutrophils and monocytes recruited and activated leukocytes during the initial phase of inflammation by expression of PAF receptors. In response to PAF, neutrophils generated PAF-like phospholipids, such as phosphatidyl choline in the kidneys of ethanol-fed rats, and the kidneys of ethanol-fed rats showed an inflammation infiltrate by enhanced leukocyte CD18 and CD64 mRNA [5]. The study conducted by Harris et al. showed that beside the inflammatory reaction, ethanol exerted deleterious effects on kidneys by oxidative stress [6]. Numerous research studies have shown that ROS induces cell proliferation in many types of mammalian cells through mitogen-activated protein kinases (MAPKs), and that this substance plays an important role in cell proliferation, oncogenesis, differentiation, inflammation, and stress responses [36,37]. Based on the results of the current study and the previous ones, we suggest that ethanol induces oxidative stress, and that inflammatory cytokines may trigger fibrotic and proliferative signaling pathways and contribute to the proliferation and fibrosis of kidneys and nephropathy.

The second issue addressed in the current study was the rescue effect of ginger extract against kidney functional alterations, structural changes, DNA oxidative damage, and plasma cystatin C elevation induced by ethanol exposure in the kidney tissue. Multiple lines of evidence have highlighted that the ginger rescue effect is due to its antioxidant and anti-inflammatory properties. As an anti-inflammatory agent, ginger supplementation inhibits inflammation process by suppressing pro-inflammatory cytokine expressions such as TNF-α, arachidonic acid cascade, IL-1β, and macrophage chemoattractant protein-1 (MCP-1). Furthermore, it inhibits prostaglandin and leukotriene biosynthesis via suppression of 5-lipoxygenase synthetase activities [38,39]. Our previous work and others have also shown that ginger supplementation increases the total antioxidant capacity and reduces lipid and protein oxidation as two main ROS generator sources in diabetics and other oxidative stress conditions [40,41]. Accordingly, if chronic ethanol consumption induces some functional and structural abnormalities in kidneys through oxidative stress and inflammation, as confirmed by present study and some previous ones, the rescue effects of ginger supplementation on these abnormalities will be due to antioxidant and anti-inflammatory properties of ginger.

In conclusion, our work launches three novel points. First, the exposure to chronic ethanol leads to shrinkage of glomerular pores manifested by elevation of cystatin C/creatinine ratio in ethanol treated rats. It is also manifested as an increase in plasma levels of urea, creatinine, and cystatin C. The second point is that ethanol-induced kidney dysfunction correlates with oxidative DNA damage and also with kidney proliferation and fibrosis. The third point we established is that ginger extract ingestion concurrent with ethanol nearly eliminates functional and structural alteration-induced by ethanol. Based on our results, we conclude that ethanol exerts its deleterious effect on the kidney which is mediated by oxidative stress and inflammatory reaction. However, further research is still required to elucidate the comprehensive details of subjects and whether ethanol acts as a direct nephrotoxin to induce kidney injuries, whether kidney injuries related to ethanol consumption are secondary to ethanol – induced hepatitis.

Conflict of interest
None

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