Influence of zinc sulfate intake on acute ethanol-induced liver injury in rats

Sema Bolkent, Pelin Arda-Pirincci, Sehnaz Bolkent, Refiye Yanardag, Sevim Tunali, Sukriye Yildirim

Abstract

AIM: To investigate the role of metallothionein and proliferating cell nuclear antigen (PCNA) on the morphological and biochemical effects of zinc sulfate in ethanol-induced liver injury.

METHODS: Wistar albino rats were divided into four groups. Group I; intact rats, group II; control rats given only zinc, group III; animals given absolute ethanol, group IV; rats given zinc and absolute ethanol. Ethanol-induced injury was produced by the 1 mL of absolute ethanol, administrated by gavage technique to each rat. Animals received 100 mg/kg per day zinc sulfate for 3 d 2 h prior to the administration of absolute ethanol.

RESULTS: Increases in metallothionein immunoreactivity in control rats given only zinc and rats given zinc and ethanol were observed. PCNA immunohistochemistry showed that the number of PCNA-positive hepatocytes was increased significantly in the livers of rats administered ethanol + zinc sulfate. Acute ethanol exposure caused degenerative morphological changes in the liver. Blood glutathione levels decreased, serum alkaline phosphatase and aspartate transaminase activities increased in the ethanol group when compared to the control group. Liver glutathione levels were reduced, but lipid peroxidation increased in the livers of the group administered ethanol as compared to the other groups. Administration of zinc sulfate in the ethanol group caused a significant decrease in degenerative changes, lipid peroxidation, and alkaline phosphatase and aspartate transaminase activities, but an increase in liver glutathione.

CONCLUSION: Zinc sulfate has a protective effect on ethanol-induced liver injury. In addition, cell proliferation may be related to the increase in metallothionein immunoreactivity in the livers of rats administered ethanol + zinc sulfate.

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Key words: Liver; Ethanol; Zinc sulfate; Metallothionein; Proliferating cell nuclear antigen; Rat

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INTRODUCTION

The liver is the main site of ethanol biotransformation and it plays a key role in zinc metabolism[1,2]. Acute ethanol exposure causes liver injury in experimental animals. The mechanisms of ethanol-induced liver injury are not fully elucidated[3,4]. Metallothionein is an intracellular protein capable of binding metals and can scavenge reactive oxygen species[5,6]. The mechanism of action for metallothionein is also unknown. Although all tissues are able to synthesize metallothioneins, the main place of synthesis is in the liver[7]. Ethanol is a potent inducer of liver metallothionein[8-12]. Proliferating cell nuclear antigen (PCNA) is an essential protein in both DNA replication, DNA repair and possibly cell-cycle control[13,14]. Zinc is an essential nutrient that is required in humans and animals for many physiological functions, including antioxidant functions. Zinc has been shown to be essential for the structure and function of a large number of macromolecules and is also essential for over 300 enzymatic reactions[15]. Zinc is an anti-oxidative element and it probably mediates the protective action of metallothionein[16]. Changes in the metabolism of some elements such as zinc can lead to disorders in the antioxidant defense system of liver[17,18]. It has been shown that zinc plays an important role in the maintenance of glutathione[19]. Zinc deficiency is important in liver damage, although it is unclear whether zinc supplementation has a place in the treatment of ethanol-induced liver injury[19,20].
Although some studies have addressed the relationship between zinc-metallothionein and ethanol toxicity, the results obtained from these studies are in conflict\textsuperscript{3,21,22}. The aim of this study was to examine the role of metallothionein and PCNA, and the effects of zinc sulfate on ethanol-induced liver injury morphologically and biochemically.

**MATERIALS AND METHODS**

**Experimental design and treatment of animals**

In this study, 2.5-3 mo-old male Wistar albino rats ($n = 41$) from DETAM (Istanbul University, Centre for Experimental Medical Research and Application) were used. The experiments were reviewed and approved by the Local Institute’s Animal Care and Use Committees. The animals were fed with pellet chow and tap water ad libitum. The animals were randomly divided into four groups. Group I ($n = 8$) was intact control animals. The control rats of group II ($n = 8$) were treated only with 100 mg/kg per day zinc sulfate (ZnSO$_4$.7H$_2$O) (Merck) for 3 d, by gavage technique. The animals of group III ($n = 14$) received 1 ml of absolute ethanol once, by the same method. The animals of group IV ($n = 11$) were treated with zinc sulfate and absolute ethanol at the same dose and time. After 2 h from the time when the last dose of zinc sulfate was given, acute ethanol toxicity on the liver of rats was produced by administration of absolute ethanol. The animals were sacrificed by ether 2 h after treatment with absolute ethanol.

**Morphological study**

On the 3rd day of the experiment, all of the animals were fasted overnight and sacrificed under ether anesthesia. Liver samples were taken from the animals for morphological studies. The tissues were fixed with Bouin fixative for light microscopic studies and subsequently a routine paraffin embedding method was used. Liver sections of 5 µm thickness were stained by HE and Masson’s triple dyes (Masson), and examined under Carl Zeiss Ultraphot II microscope. Liver samples were fixed in 20 g/L glutaraldehyde and post-fixed in 10 µg/L of phosphate-buffered osmium tetroxide. The samples were dehydrated in ethanol and embedded in epon. Specimens were examined with JEM 1011.

**Immunohistochemical study**

Sections were dewaxed and rehydrated. The tissues were rendered permeable with 3 g/L Triton-X 100 for 10 min and then rinsed in phosphate-buffer saline (10 mmol/L, pH 7.5). For antigen retrieval, the slides were kept in 0.01Mol/L citrate buffer for 10 min in a microwave oven. Endogenous peroxidase was blocked with 30 mL/L hydrogen peroxide. An Ultra Vision Detection System for streptavidin-biotin-peroxidase technique (Lab Vision, USA) was employed. Sections were covered with blocking serum for 10 min to block non-specific binding sites. They were then incubated with metallothionein antibody for 1 h (Zymed Laboratories, USA) at room temperature with 1:50 dilution. Slides were then incubated with PCNA mouse monoclonal antibody for 30 min (Neomarkers, USA) at room temperature with 1:50 dilution. They were incubated for 15 min with biotinylated secondary antibody and then incubated with the streptavidin-peroxidase conjugate for 15 min. The enzyme activity was developed using aminoethylcarbazole (AEC) and then the sections were counterstained with hematoxylin. Negative control sections were prepared by substituting the metallothionein or PCNA antibodies with phosphate-buffer saline. Hepatoctyes were viewed using a light microscope (Olympus, CX41) at a magnification of 400X. Approximately 400 cells from 10 randomly selected fields (0.0506 mm$^2$ per field) of vision were counted. The PCNA and metallothionein labeling indices were expressed as a percentage of positive stained cells relative to the number of counted cells. (i.e., PCNA or metallothionein labeling index = PCNA or metallothionein positive hepatocytes/total hepatocytes per high power field $\times 100$).

**Biochemical study**

In this study, biochemical investigation was carried out in serum, blood, and liver tissues. The blood samples were taken by a syringe from the heart. Blood glutathione (GSH) levels were measured according to the method of Beutler, Duron and Kelly using Ellman’s reagent\textsuperscript{23}. Serum alkaline phosphatase activities (ALP) were determined by the Two Point method\textsuperscript{24}. Serum aspartate transaminase (AST) was assessed by the Reitman-Frankel method\textsuperscript{25}. The liver tissues were homogenized in cold 9 g/L saline by means of a glass homogenizer to make up a 100 g/L homogenate. The homogenates were centrifuged and the clear supernatants were used for GSH, lipid peroxidation (LPO) and protein levels. Liver GSH levels were determined according to Beutler’s method, using Ellman’s reagent\textsuperscript{26}. Liver LPO levels were measured by Ledwozyn’s method\textsuperscript{27}, liver protein levels were assessed by Lowry’s method using bovine serum albumin as the standard\textsuperscript{28}.

**Statistical analysis**

The biochemical results were evaluated using an unpaired $t$-test and analysis of variance (ANOVA) using the NCSS statistical computer package (Kaysville, Utah, USA)\textsuperscript{29}. The microscopic results were analyzed by one-way ANOVA followed by the Scheffe and Student’s $t$-test for multiple comparisons of the control against all other groups using SPSS 13 for Windows. Data were expressed as mean $\pm$ SD. $P < 0.05$ was considered to be significant.

**RESULTS**

**Morphological results**

The livers of control and zinc-treated rats were visually normal. Acute ethanol exposure caused degenerative morphological changes in the liver. The hepatocyte of rats exposed to absolute ethanol alone had occasional diffuse vacuolar degeneration. Vacular degeneration was found in hepatocytes of zones 2 and 3. In the alcohol group, there was a mild dilation of the sinusoids and hyperemia. Moreover, mononuclear cell infiltrations were evident in this group. These alcohol-induced hepatic pathological changes were significantly inhibited in the zinc-pretreated
rats. A moderate degree of mucosal hyperemia was observed in the group given ethanol and zinc sulfate. In addition, less vacuolar degeneration was observed in this group (Figure 1).

An increase in hepatic metallothionein-producing cells in control rats given only zinc, when compared to intact controls, was observed. In addition, an increase in hepatic metallothionein-producing cells was observed in the rats given zinc and ethanol, when compared to the group given ethanol. Immunoreactive metallothionein-producing cells in the control group were rarely found to be scattered in lobular hepatocytes or around the periportal area. In the control rats given only zinc, more metallothionein-producing cells were observed in hepatocytes of zones 1 around the periportal area as compared with the control group. Metallothionein was observed significantly in the cytoplasm of cells by immunohistochemical staining in the rat livers of all groups. The intensity of immunoreactivity of metallothionein-producing cells in the group given ethanol and zinc was generally higher in hepatocytes of zones 1 compared to zones 2 (Figure 2). The metallothionein labeling index was determined to be 27.27% ± 2.02% in the group given zinc sulfate, while in intact control group the index was 2.44% ± 0.50%. The metallothionein labeling index was increased 55-fold in the group administered ethanol + zinc sulfate (26.32% ± 3.09%) compared to the group administered only ethanol (0.19% ± 0.10%) (P<0.007) (Figure 3A). However, according to semiquantitative evaluations, most of the PCNA-positive hepatocytes were in S phase of proliferation in all groups.

Ultrastructurally, proliferation of smooth endoplasmic reticulum, degenerative mitochondria, condensation of chromatin and increased lipid droplets were observed after alcohol treatment. However, administration of zinc sulfate caused a remarkable decrease in the lipid droplets, smooth endoplasmic reticulum, and mitochondria degeneration of the hepatocytes (Figure 4).

Biochemical results

Blood GSH levels are presented in Table 1. From the obtained results, values of GSH in the blood of the group administered ethanol showed a significant decrease.
when compared to the control group ($P_{t-test} = 0.0001$). Also, a marked increase in blood GSH level was observed in the group administered ethanol + zinc sulfate when compared to the group administered ethanol ($P_{t-test} = 0.0005$). According to Table 1, a significant difference in the blood GSH levels of four groups was observed ($P_{ANOVA} = 0.0001$).

Serum ALP and AST activities are given in Table 1. From the obtained results, values of ALP in the serum of the group administered ethanol showed a notable increase when compared to the control group ($P_{t-test} = 0.0001$). Also a considerable decrease was noted in the group administered ethanol + zinc sulfate when compared to the group administered ethanol ($P_{t-test} = 0.0001$). According to Table 1, a significant difference in the ALP activities of the four groups were observed ($P_{ANOVA} = 0.0001$). In this study, a statistically marked increase was observed in serum AST activity of the group administered ethanol, in comparison with the control group ($P_{t-test} = 0.0001$). On the other hand, in the group to which ethanol + zinc sulfate was administered, the AST activity decreased compared to the ethanol group. A significant difference in the serum AST activities of four groups was observed ($P_{ANOVA} = 0.0001$).

Table 2 shows the effects of zinc sulfate on liver GSH and LPO. The GSH levels were significantly reduced in the group administered ethanol as compared to the other groups ($P_{ANOVA} = 0.0001$). A significant decrease of liver GSH levels in the group administered ethanol was determined in comparison to the control group ($P_{t-test} = 0.0001$). After zinc sulfate administration to ethanol-treated rats, liver GSH levels increased greatly when compared
to the ethanol group ($P_{\text{ANOVA}} = 0.0001$). Zinc sulfate had no significant effect on liver GSH levels in the control group ($P_{\text{ANOVA}} = 0.589$). An eminent difference in the liver LPO levels of the four groups was observed ($P_{\text{ANOVA}} = 0.0001$). A significant increase of liver LPO levels in the group administered ethanol was determined in comparison to the control group ($P_{\text{ANOVA}} = 0.0001$). After the administration of ethanol + zinc sulfate, the liver LPO levels decreased notably when compared to the ethanol group ($P_{\text{ANOVA}} = 0.001$).

**DISCUSSION**

Ethanol-induced diseases in humans are important in clinical gastroenterology. Animal models in alcohol research have already been applied to study acute and chronic ethanol damage of the liver. Ethanol may accelerate oxidative stress via increased production of active oxygen species. Ethanol-induced oxidative stress plays a major role in liver injury[38]. Ethanol causes a notable fall in the level of zinc in the liver[39]. Zinc may play a key role in certain alterations observed in alcoholic patients[40]. The antioxidant defensive system of the liver might be influenced by changes in the zinc content of the liver[41]. In the present study, the administration of zinc sulfate prior to the administration of ethanol + zinc sulfate indicates that zinc ameliorates the damage to liver tissue caused by a free radical-mediated mechanism.

The present study suggests that ethanol-induced hepatic injury is related to the formation of free radicals and pretreatment of zinc prior to the administration of ethanol prevents toxicity. Ethanol treatment is accompanied by the generation of free radicals which stimulate LPO and decrease GSH levels. Therefore, zinc supplementation prevented ethanol-induced decreases in GSH and increases in LPO.

Under light and electron microscopes, the decrease of degenerative changes in the livers of rats administered ethanol + zinc sulfate indicates that zinc ameliorates the damage in the liver tissue of the group given ethanol. An increase in hepatic metallothionein-producing cells in control rats given only zinc as compared to intact controls was observed. This increase shows that metallothionein is required for high zinc levels in liver. In this study, synthesis of metallothionein was shown to increase in the livers of rats given zinc + ethanol, immunohistochemically, in accordance with previous findings[35]. Our results indicate that metallothionein induction by zinc sulfate has a protective effect against the injury of acute ethanol administration in liver. Elevation of metallothionein may maintain the integrity of the membrane of the organelles, as observed in the group given ethanol + zinc sulfate. Metallothionein expression may vary with types of tissue and physiological and nutritional factors such as zinc. There are limited studies on hepatic metallothionein levels following acute administration of ethanol to rats[41,42]. An over-expression of metallothionein serves to protect cells from the alkylating agents[43]. In the ethanol-intoxicated group, metallothionein levels increased nearly 3.5-times when compared to the control group[43]. Ebadi et al[29] also reported that the administration of zinc sulfate increased the synthesis of metallothionein mRNAs. It has been reported that hepatic metallothionein synthesis is stimulated by dietary zinc supplementation[44]. Koterov[45] reported that there was a linear dependence of the hepatic levels of metallothioneins with the dosage of alcohol. However, recent studies have shown that zinc inhibition of acute alcohol-induced liver injury is independent of metallothionein[35,36].

In the present report, the metallothionein labeling index correlated positively with the PCNA index in the rats given zinc + ethanol. It is suggested that enhanced antioxidant proteins such as metallothionein may be involved in hepatocyte proliferation. This finding supports a previous study showing an impairment of liver regeneration in metallothionein-I and metallothionein-II gene knockout mice after partial hepatectomy[27].

The liver is the most important organ in alcohol metabolism. The increase in AST and ALP activities in serum is an indicator of liver destruction. Their increase in the serum activities of these enzymes was directly proportional to the degree of cellular damage. The activity of AST in the serum of the group administered ethanol showed a statistically significant increase when compared to the control group. AST activity decreased in the group administered ethanol + zinc sulfate. This suggests that zinc pretreatment of rats prevented the elevation of serum AST. Acute ethanol exposure caused a 4-fold increase in the levels of serum AST compared with control animals[34]. The activity of ALP in the serum of the group administered ethanol has shown a significant increase when compared to the control group. The increase of alkaline phosphatase in the serum may be a result of damage to liver cells. Since zinc is a component of many enzymes, including alkaline phosphatase, a significant decrease was found in the activity of ALP in the group administered ethanol + zinc sulfate when compared to the group administered ethanol.

Glutathione plays a major role as a reductant in oxidation-reduction processes and also serves in detoxification[46]. Ethanol, or its metabolites, can alter the balance in the liver toward auto-oxidation, either acting as pro-oxidants, or reducing the antioxidant levels, or both[47]. The pathogenesis of alcohol-induced liver disease involves the adverse effect of ethanol metabolites and oxidative tissue injury[48]. The most prominent defensive system in the liver is reduced GSH. The values of GSH in blood of the rats administered ethanol have shown a significant decrease when compared to the control.
group. Metallothionein shares an important similarity with glutathione due to the fact that one-third of their amino acids are cysteines. Experimental depletion of glutathione in isolated rat hepatocytes has been shown to induce metallothionein expression and to create a new pool of thiol groups in the cell[41]. Glutathione provides a protective action against damage from reactive oxygen species and free radicals formed during drug metabolism[42,43]. On the other hand, Drescoi et al[44] reported that ethanol alone had no effect on glutathione levels. Cho et al[45] reported that ethanol and zinc sulfate administration did not affect hepatic glutathione levels in mice. It is reported that zinc supplementation prevented ethanol-induced decreases in glutathione concentration in the liver[46], in accordance with our findings.

Chronic ethanol administration induces oxidative stress and increases lipid peroxidation of the cell membrane. This leads to increased membrane fluidity, disturbances of calcium homeostasis, and finally results in cell death[47]. Oxidative stress is characterized by increased lipid peroxidation. A remarkable increase of liver lipid peroxidation levels in the group administered ethanol was determined in comparison to the control group. The present results suggest that zinc sulfate supplementation has a protective effect against lipid peroxidation in liver. Lipid peroxidation was observed to increase prominently in ethanol-fed rats after 4 and 8 wk when compared to the controls[48]. Patients and experimental animals with acute and chronic ethanol exposure have a depleted liver glutathione content, correlated with an increase in lipid peroxidation[49,50]. Zinc supplementation for 12 wk caused a decrease in lipid peroxidation, together with an increase in metallothionein concentration in rats[51]. However, 100 mL/L ethanol ingestion for 8 wk enhanced lipid peroxidation in liver[52]. Cabre et al[53] reported that zinc is an efficient hepato-protective agent against lipid peroxidation in alcoholic rats.

In conclusion, microscopic and biochemical evaluations reveal that zinc sulfate has a protective effect on ethanol-induced liver injury. Our results demonstrate that zinc acts as an antioxidant agent in hepatic antioxidant systems after acute ethanol administration. In addition, this protective effect against acute ethanol injury has also included proliferation in hepatocytes. Zinc may be a therapeutic agent in the prevention and treatment of ethanol-induced liver injury.

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