Potential effects of L-NAME on alcohol-induced oxidative stress

Hafize Uzun, Gonul Simsek, Seval Aydin, Ethem Unal, Yesari Karter, Nermin Karaturan Yelmen, Suphi Vehid, Asli Curgunlu, Safiye Kaya

Abstract

AIM: Nitric oxide (NO) is a highly reactive oxidant synthesized from L-arginine by nitric oxide synthase (NOS). NO may cause injury through the generation of potent radicals. Nω-nitro-L-arginine methyl ester (L-NAME) is a non-selective inhibitor of NOS. We aimed to evaluate whether L-NAME treatment had protective effects against oxidative stress in rats intragastrically fed with ethanol during a 4 wk-period.

METHODS: Thirty-six male Wistar rats were divided into 3 equal groups: group 1 (control group-isocaloric dextrose drinking water-given group). Animals were sacrificed at the end of 4 wk-experimental period, and intracardiac blood and liver tissues were obtained. Biochemical measurements were performed both in plasma and in homogenized liver tissues. Alanine amino transferase (ALT), aspartate amino transferase (AST), malondialdehyde (MDA), NO, superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) levels were measured by spectrophotometry.

RESULTS: ALT and AST in group 2 (62 U/L and 128 U/L, respectively) were higher than those in group 1 (24 U/L and 38 U/L) and group 3 (37 U/L and 81 U/L) (\(P<0.001\) for both). Plasma and tissue levels of MDA in group 2 (4.66 \(\mu\)mol/L and 0.55 nmol/mg protein) were higher than in group 1 (2.65 \(\mu\)mol/L and 0.34 nmol/mg protein) and group 3 (3.43 \(\mu\)mol/L and 0.36 nmol/mg protein) (\(P<0.001\) for both). Plasma and liver tissue levels of NO in group 2 (54.67 \(\mu\)mol/L and 586.50 nmol/mg protein) were higher than in group 1 (34.67 \(\mu\)mol/L and 435.33 nmol/mg protein) and group 3 (27.50 \(\mu\)mol/L and 412.75 nmol/mg protein) (\(P<0.001\) for both). Plasma and liver tissue SOD activities in group 2 (15.25 U/mL and 5.38 U/mg protein, respectively) were lower than in group 1 (20.00 U/mL and 8.13 U/mg protein) and group 3 (19.00 U/mL and 6.93 U/mg protein) (\(P<0.001\) for both). Plasma and liver tissue CAT activities in group 2 (145 U/mL and 37 U/mg protein, respectively) were lower than in group 1 (176 U/mL and 73 U/mg protein) and group 3 (167 U/mL and 61 U/mg protein) (\(P<0.001\) for both). Meanwhile, erythrocytes and liver tissue levels of GSH in group 2 (4.12 mg/g Hb and 5.38 nmol/mg protein, respectively) were lower than in group 1 (5.52 mg/g Hb and 4.49 nmol/mg protein) and group 3 (5.64 mg/g Hb and 4.18 nmol/mg protein) (\(P<0.001\) for both).

CONCLUSION: Our findings show that L-NAME may produce a restorative effect on ethanol-induced liver damage via decreasing oxidative stress and increasing antioxidant status.

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INTRODUCTION

Normal cellular metabolism involves the production of reactive oxygen species (ROS)[1]. Low level of ROS is vital for many cell signaling events and essential for proper cell functioning[2-4], while excessive in vivo generation of ROS can adversely affect cell functioning[5]. In some clinical setting, such as obesity, liver transplantation, hepatic surgery, and hemorrhagic shock, as an outgrowth of ischemic-reperfusion injury in liver, microcirculatory derangement, energy depletion, production of ROS and lipid peroxidation occur[5-7]. ROS causes inflammation and cell death through modulation of signal transduction pathways by affecting redox-sensitivity enzyme and transcription factors, by supporting protease activity, and by stimulating the expression of inflammatory mediators and adhesion molecules[8-10]. In the recovery of hepatocellular function after severe traumas, free oxygen radicals should be kept in the normal range[11,12].

The chronic consumption of alcoholic beverages is the major cause of liver injury, and the development of serious liver diseases[10,11]. The mechanisms of liver injury may include the effects of oxygen radicals on hepatocytes[12]. Increased oxygen radical production leads to lipid peroxidation by inducing cytochrome P4 502E[13,14].

Oxidative stress is known to play an important role in the pathogenesis of ethanol-induced liver injury[15,16]. Oxidative damage correlates with the amount of ethanol consumed[17]. Recently, it has been demonstrated that nitric oxide (NO) is an important mediator of hepatotoxicity, and the changes in its generation or actions may contribute to pathologic states[18,19]. It has been proposed that the high production of NO causes injury, perhaps through the generation of potent radicals[20]. An increase in NO production has been reported in monocytes of patients with chronic liver disease[21] and in the livers of rats chronically fed with ethanol[22]. However, in some models of
inflammation, it has been shown that inhibition of NO increases tissue dysfunction or injury\(^2\(^3\)).

The role of NO seems to be controversial, and furthermore, the prooxidant and/or antioxidant effects of NO inhibition in alcoholism have not been studied before. In the present study, we tested whether nitric oxide synthase (NOS) inhibition attenuated alcohol-induced oxidative stress in a rat model. For NOS inhibition Nω-Nitro-L-arginine methyl ester (L-NAME)-a nonselective inhibitor- was used.

**MATERIALS AND METHODS**

**Experimental procedure**

Thirty-six male Wistar-Albino rats weighing 240-300 g were used. Animals were fed ad libitum on a standard diet and had free access to water. All studies were performed in accordance with the National Institutes of Health Criteria for Care of Laboratory Animals.

The rats were divided into three groups, and were given isosoronic dextrose (group 1), ethanol (ETOH) (group 2), or both ethanol and L-NAME (ETOH + L-NAME) (group 3) for 4 wk. Ethanol was given intragastrically at a dose of 6 g/(kg/d) L-NAME was added to drinking water of the rats (500 mg/L).

All rats were sacrificed after 1 mo with Na-pentobarbital anesthesia (35 mg/kg, i.p.). After exploration of the thorax, blood was taken by intracardiac puncture. Then, a laparotomy was done, liver tissue was excised, and stored at -70 \(^{\circ}\)C. Upon arrival, plasma was separated by centrifugation (+4 \(^{\circ}\)C, 3 000 r/min, 10 min), and divided into 0.5-1.0 mL aliquots, placed in cryovials, and stored at -70 \(^{\circ}\)C until analyzed. Erythrocytes were washed three times in 5 mL saline, hemolyzed by diluting 4-fold with water and glutathione (GSH) was studied in erythrocytes on the same day. Each plasma sample was divided into 4 aliquots; alcohol, ALT and AST were studied immediately in 1\(^{\text{st}}\) aliquot; 2\(^{\text{nd}}\) aliquot was saved until analysis of plasma NO within 2 wk, the other two aliquots were used for estimation of plasma MDA, SOD, CAT on a later date (within 1 mo).

The liver tissues were weighed, washed in 0.9 % NaCl, and homogenized in ice-cold 0.1 M KCl 100 g/L. Homogenates of 20% were obtained and sonicated twice at 30 s intervals at 4 \(^{\circ}\)C. Homogenates were centrifuged at >10 000 g for 15 min at 4 \(^{\circ}\)C. All biochemical parameters in homogenates were studied on Olympus AU800 analyzer.

**Biochemical analysis**

Blood samples collected in heparinized vacutainer tubes were immediately transported to the laboratory in a cooler with ice. Upon arrival, plasma was separated by centrifugation (+4 \(^{\circ}\)C, 3 000 r/min, 10 min), and divided into 0.5-1.0 mL aliquots, placed in cryovials, and stored at -70 \(^{\circ}\)C until analyzed. Erythrocytes were washed three times in 5 mL saline, hemolyzed by diluting 4-fold with water and glutathione (GSH) was studied in erythrocytes on the same day. Each plasma sample was divided into 4 aliquots; alcohol, ALT and AST were studied immediately in 1\(^{\text{st}}\) aliquot; 2\(^{\text{nd}}\) aliquot was saved until analysis of plasma NO within 2 wk, the other two aliquots were used for estimation of plasma MDA, SOD, CAT on a later date (within 1 mo).

**ALT and AST activities**

Plasma ALT and AST activities were measured by enzymatic methods using commercial kits (Olympus, Hamburg, Germany) on Olympus AU800 analyzer.

**Lipid peroxidation**

MDA, an end product of fatty acid peroxidation, was measured in plasma and liver homogenates by the thiobarbituric acid reactivity assay as previously described\(^2\(^4\)). The total protein concentration was measured by the method of Lowry et al\(^2\(^5\)).

**Nitric oxide**

Plasma and tissue concentrations of NO were measured through its stable metabolites nitrate and nitrite. Nitrate was first reduced by nitrate reductase to nitrite and then nitrite was determined spectrophotometrically by the Griess reaction\(^2\(^5\)). Griess reagent, the mixture (1:1) of 0.2 % N-(1-naphthyl)-ethylene-diamine and sulfanilamide in 5% phosphoric acid, gave red-violet diazo dye with nitrite, and was measured in the visible range at 540 nm.

**Cu-Zn-superoxide dismutase** (Cu-Zn SOD)

Plasma and tissue Cu-Zn SOD activities were determined by the method of Sun et al\(^2\(^6\)) by inhibition of nitroblue tetrazolium reduction with Xanthine/Xanthine oxidase used as a superoxide generator. One unit of SOD was defined as the amount of protein that inhibits the rate of NBT reduction by 50%.

**Catalase:**

Catalase activity was measured by the breakdown of hydrogen peroxide catalysed by catalase enzyme\(^2\(^7\)).

**Glutathione**

Erythrocyte and tissue glutathione (GSH) concentrations were determined according to the method of Beutler et al\(^2\(^8\)) using metaphosphoric acid for protein precipitation and 5'-5'-dithiobis-2-nitrobenzoic acid for color development.

**Alcohol**

Serum alcohol level was measured by fluorescent polarizing immunoassay using commercial kits (Abbott TDx, Cat No:378190100).

**Statistical analysis**

All results are expressed as mean±SD. The groups were compared with Anova-Tukey HSD. \(P<0.05\) was considered to be statistically significant.

**RESULTS**

Values of the analysed parameters and the statistical significances in the groups are shown in Tables 1 and 2. Plasma alcohol levels of groups 2 and 3 were comparable (210±42 mg/100 mL and 196±30 mg/100 mL). ALT and AST in group 2 were higher than those in group 1 and group 3 (\(P<0.001\) for both). Similarly, the values were higher in group 3 in comparison with group 1 (\(P<0.001\)).

Lipid peroxidation levels as assessed by MDA in plasma and tissue were found to be significantly higher (\(P<0.001\)) in group 2 in comparison to groups 1 and 3. Plasma MDA level was significantly higher in group 3 in comparison to group 1 (\(P<0.001\)); however, there was no difference between these groups in tissue MDA level (\(P>0.05\)).

Plasma and tissue levels of NO in group 2 were higher than in groups 1 and 3 (\(P<0.001\) for both). Plasma NO level was lower in group 3 in comparison with group 1 (\(P<0.001\)).

Each of plasma and tissue antioxidant components (SOD, CAT and GSH) in group 2 was lower than in groups 1 and 3.

| Table 1 | Plasma transaminase activities, prooxidant and antioxidant status in groups |
|---------|---------------------------------------------------------------|
|         | Control (Group 1) | ETOH (Group 2) | ETOH + L-NAME (Group 3) | \(P^1\) | \(P^2\) | \(P^3\) |
| ALT (U/L) | 24.25±4.07 | 62.00±6.73 | 37.00±4.73 | <0.001 | <0.001 | <0.001 |
| AST (U/L) | 38.92±6.42 | 128.42±16.98 | 81.33±14.90 | <0.001 | <0.001 | <0.001 |
| MDA (μmol/L) | 2.65±0.30 | 4.66±0.64 | 3.43±0.35 | <0.001 | <0.001 | <0.001 |
| NO (μmol/L) | 34.67±4.69 | 54.67±9.19 | 27.50±4.34 | <0.001 | <0.001 | <0.001 |
| SOD (U/mL) | 20.00±1.86 | 15.25±0.97 | 19.00±1.54 | <0.001 | <0.001 | NS |
| CAT (U/mL) | 176.0±11.23 | 145.33±9.23 | 166.83±7.27 | <0.001 | <0.001 | <0.05 |
| GSH (mg/g Hb) | 5.52±0.41 | 4.12±0.32 | 5.64±0.52 | <0.001 | <0.001 | NS |

\(^1\)Group 1 vs group 2; \(^2\)Group 2 vs group 3; \(^3\)Group 1 vs group 3. NS: Not-significant.
were significantly higher in group 3 in comparison with group 1.

However, tissue SOD, CAT, and plasma CAT levels were significantly higher in group 3 in comparison with group 1 (P<0.01, P<0.01, and P<0.05, respectively).

### DISCUSSION

It is now generally accepted that oxidative stress plays an important role in the pathogenesis of ethanol toxicity[13,14]. The close relation between ethanol and liver is due to the fact that more than 80% of ingested alcohol is metabolized in the liver without a feedback mechanism. Early phase, oxygen and NO-radicals derive from the complete oxidation of ethanol, and aldehyde exceeding in excess markedly alters the intracellular redox status, induces fat deposits, and triggers the inflammatory and immune response[29]. The progression of liver damage is also affected by generation of additional products between acetate and cytochrome oxidase and/or P450 2E1[30,31]. Excessive production of reactive oxygen species (ROS), depletion of GSH and ATP, adducts with acetaldehyde, rise of lipid peroxidation markers, are all documented findings in alcoholics[31,33].

In this study, we found that chronic ethanol administration causes a significant rise in plasma and hepatic MDA, and erythrocyte GSH levels, hepatic GSH levels, SOD and catalase activity. Some authors have reported an increase in various parameters of lipid peroxidation such as MDA, diene conjugates and lipid hydroperoxides after alcohol intake[28,33], whereas others have shown opposite effects of alcohol drinking[26,27]. We also observed the presence of increased oxidative stress and decreased antioxidant status in the plasma and liver of ethanol-treated rats in our previous study[34].

In the present study, alcohol-induced hepatotoxicity was manifested as an increase in the activities of ALT and AST enzymes and a decrease in GSH level, SOD and catalase activities. These findings pointed out an obvious change in prooxidant-antioxidant balance in the liver of rats following chronic ethanol administration. However, Husain et al[35] have reported that GSH levels decreased, and SOD activity increased in liver in chronic alcoholism.

NO is a highly reactive oxidant. It is produced both by parenchymal and nonparenchymal cells in the liver[40,41]. Supplementation of the NO precursor L-arginine has been shown to exacerbate damage in models of inflammation and injury[42]. In vitro and in vivo studies have also shown NO down-regulates cytochrome P-450 and suppresses liver protein and DNA synthesis, and induces apoptosis and necrosis, all of which may contribute to liver failure[40,43]. NO also inhibits catalase activity, suggesting that it may alter the detoxification of cytotoxic free radicals, and react with superoxide anions to form peroxynitrite, which can react with sulphhydril residues in cell membranes leading to lipid peroxidation[40].

In this study, we found that there was a significant increase in plasma and liver NO concentrations in ethanol-induced rats. Our findings suggest that NO may have an important role in cellular damage seen in alcoholism. Wang et al have shown that serum NO concentration increased in cirrhotic rats. Also in some studies, it has been shown that there is an increase in NO production in monocytes of patients with chronic liver disease[44], and in livers of rats chronically fed with ethanol[36]. On the other hand, Sergent et al[37] reported that NO biosynthesis in hepatocytes protects them from ethanol-induced oxidative stress. Joshi et al[38] suggested that low-level NO acts as an antioxidant and higher level as a pro-antioxidant. They proposed that the mechanism of low concentration of NO’s protection may involve diminished metal- catalyzed lipid peroxidation and the high concentration of NO’s potentiation of oxidative stress may involve mitochondrial dysfunction. The significant rise in NO concentration that was seen in our study might be the reason for oxidative stress-stimulated lipid peroxidation.

TNF-α administration could lead to hepatocyte apoptosis and liver failure[47] TNF-α has been considered a mediator of cell injuries in liver caused by alcoholism, reperfusion, primary graft nonfunction, graft rejection and endotoxic insult[48,49]. It is expressed by both infiltrating inflammatory cells and hepatocytes in chronic liver injuries, and has been proposed to play an important role during tissue damage. The role of IL-6 during chronic liver injuries and fibrogenesis remains to be clarified. Some reports provided evidences for an important role of IL-6 in reducing CCl₄-induced acute and chronic liver injury and fibrosis[50,51]. However, some other data showed that the serum level of IL-6 was associated with hepatic necroinflammatory activity in patients with chronic hepatitis and cirrhosis[32]. It has been suggested that IL-6 might be vitally involved in fibrotic changes, partly by modulating intrahepatic expression of other cytokines[32,33]. On the other hand, another study has suggested that proinflammatory cytokines increase as a result of alcohol-induced cellular damage[35], and this causes an increase in NO production by stimulating inducible NOS (iNOS)[52]. Hink et al[53] have claimed that H₂O₂ actively stimulates iNOS expression. In this case, we can assume that the increase in NO concentration and the contribution of NO to cellular damage could be secondary; however, simultaneous administration of ethanol and L-NNAME in our study was shown to decrease oxidative stress parameters, and increase antioxidant parameters. These findings suggest that the oxidative damage in alcoholism can be mediated by NO.

The results of our study also show that co-administration of L-NNAME diminishes oxidative stress, by increasing antioxidant enzymes. This restoration of oxidant/antioxidant balance is reflected by lower levels of transaminases.

Liver contains different forms of NO-synthase: the neuronal form (nNOS) in the peribiliary plexus; the iNOS form in hepatocytes, cholangiocytes, Kupffer cells, and stellate cells; and the endothelial form (eNOS) in the endothelial cells[56,57]. Over-production of NO in the liver from L-arginine via iNOS[58] has been implicated as an important part of the cascade of events that takes place in the pathogenesis of septic shock and in various forms of hepatic injury, inflammation and acute liver injury[59].

### Table 2 Hepatic prooxidant and antioxidant status in groups

|                  | Control (Group 1) | ETOH (Group 2) | ETOH + L-NAME (Group 3) |
|------------------|------------------|----------------|-------------------------|
| MDA (nmol/mg protein) | 0.34±0.05       | 0.55±0.08       | <0.001                  |
| NO (nmol/mg protein)   | 435.33±35.64     | 586.50±34.79    | 412.75±38.67            |
| SOD (U/mg protein)     | 8.13±0.79        | 5.38±0.75       | 6.93±0.81               |
| CAT (U/mg protein)     | 72.58±7.37       | 36.50±6.65      | 61.17±7.81              |
| GSH (nmol/mg protein)  | 4.49±0.51        | 3.55±0.36       | 4.18±0.26               |

*Group 1 vs group 2; Group 2 vs group 3; Group 1 vs group 3. NS: Not-significant.*
hepatic failure.

In conclusion, NO can be held responsible for alcohol-induced free radical damage, and NOS inhibition can decrease oxidative stress seen in alcoholism. However, further studies should be done to detect which type of NOS is stimulated by ethanol. Findings of this study suggest a role of NOS inhibition in the management of ethanol-induced liver damage.

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