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ORIGINAL STUDY

Long-term Alcohol Consumption Increases Pro-Matrix Metalloproteinase-9 Levels via Oxidative Stress

Tulay Koken · Fatih Gursoy · Ahmet Kahraman

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Abstract Matrix metalloproteinases (MMPs) play an important role in alcoholic liver disease. In this study, we evaluated the relationship between pro MMP-9 (pMMP-9) and oxidative stress in plasma of rat exposed to chronic alcohol consumption. Twenty four rats were divided into four groups. Rats in the control group (n=6) were subjected to physiologic saline by intragastric (i.g.) route. Group Ethanol (n=6) was given 1 ml of 80% ethanol (v/v) in distilled water through i.g. route. Group Vitamin E (Vit E), (n=6) was given vitamin E (100 mg kg^{-1} day^{-1}) by intra peritonealy. Group Vitamin E+Ethanol (n=6) was given vitamin E 2 h before the administration of ethanol. At the end of 4 weeks, blood samples were taken and plasma malondialdehyde (MDA), protein carbonyls (PCs), aspartate aminotransferase (AST), tumor necrosis factor-α (TNF-α) and pMMP-9 levels were measured. Chronic ethanol administration increased the AST, MDA, PCs, TNF-α and pMMP-9 levels when compared to those in control group (p<0.05, p<0.01, p<0.01, p<0.05, p<0.05, respectively). Vitamin E treatment was found to decrease lipid peroxidation and protein oxidation (p<0.01, p<0.01, respectively). Also TNF-α and pMMP-9 levels returned to normal by vitamin E treatment. Within all subjects, there was positive correlation between pMMP-9 levels and MDA, PCs levels (p=0.045, r=0.454; p=0.004, r=0.574, respectively). We conclude that since antioxidant supplementation decreases the alcohol-induced pMMP-9 levels, oxidative stress could be one of the mediators of the generation of MMP-9.

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Introduction

A number of hypotheses regarding to the mechanisms of alcohol causes cell injury have been suggested. Endotoxic theory is currently viewed as a major mechanism of alcoholic liver injury [1]. Ethanol increases gut permeability which can cause an influx of microflora derived endotoxins into the blood [2]. These endotoxins activate Kupffer cells to induce nitric oxide [3], superoxide [4], and cytokines such as tumor necrosis factor-α (TNF-α), finally leading to liver damage.

Also, ethanol is able to induce an oxidative stress by generating oxygen radicals, inhibiting glutathione synthesis and inducing cytochrome P-450 2E1 (CYP2E1) [5].

The matrix metalloproteinases (MMPs) are a family of proteinases that can degrade extracellular matrix proteins. MMP activity is controlled at least at three levels: transcription, proteolytic activation of the zymogen form, and inhibition of the active enzyme by a host of natural inhibitors. MMP-9 is also called gelatinase B, the majority of which is secreted by Kupffer cells in liver. The link between alcohol and MMPs has been suggested by multiple studies. Dynamic changes in the expression of matrix metalloproteineses and their inhibitors are occurred during alcohol-induced hepatic fibrosis [6]. Also in an animal study, activation of matrix degrading enzymes are elevate in the lung of alcohol-treated rats [7].

Investigations of oxidative stress have demonstrated involvement of MMPs, in particular, MMP-9 and MMP-2 were detected in the different tissue. Downregulation of MMPs by oxidative stress has been reported in a photochemical oxidative stress model of cataract [8]. In another study, Uemura et al. demonstrated that MMP-9 expression

and activity in endothelial cells are upregulated during hyperglycemia by oxidative stress [9]. Also, increased oxidative stress activates MMPs and decreases fibrillar collagen synthesis in cardiac fibroblasts [10]. In this study, we examined plasma pro MMP-9 (pMMP) levels in ethanol induced oxidative stress.

There are several animal models used in the study of alcoholic liver disease (ALD). Animals have been administered ethanol chronically by various methods in attempts to develop liver lesions resembling those seen in human ALD [11–15]. But researchers have not been able to develop a suitable paradigm that exactly replicates the human prototype for reasons that are not understood as yet, but which probably involve genetic differences between humans and other animals [16]. For this reason, we chose the simple rat model [17, 18]. For the chronic ethanol treatment, ethanol was given by gavage and the control received the same volume of vehicle. All animals were fed with standard rat chow and allowed to drink water ad libitum.

Accordingly, this study has two goals. We first investigated whether ethanol-induced oxidative damage is correlated with pMMP levels. Second, we tested the effect of vitamin E as an antioxidant on pMMP levels.

Methods

Animal Model Twenty four male Spraque Dawley rats (250-300 g body weight and 3-4 month of age) were used in our study. The animals were housed in the facilities of the Laboratory Animal Services Centre of the Afyon Kocatepe University. They were fed a standard rat chow and allowed to drink water ad libitum but they were deprived of food for 12 h before the ethanol administration. They were housed in a single temperature controlled (20-25°C) cages with a 12-h dark and 12-h light cycles. The study was approved by the University Ethical Committee. Unnecessary animal suffering was avoided throughout the study.

This study was designed as an experimental, randomized, controlled trial with blind assessment of outcome. The rats were randomly assigned to one of the four groups listed below:

Group control (C), (n=6) was given physiologic saline by gavage. Group ethanol (E), (n=6) was given 1 ml of 80% ethanol (v/v) in distilled water by gavage. Group vitamin E (vit E), (n=6) was given vitamin E (Aksu Farma, Istanbul, Turkey; 100 mg kg^{-1} day^{-1}) by intra peritonealy. Group ethanol+vitamin E (E+vit E), (n=6) was given vitamin E intra peritonealy 0.5 h before the administration of 1 ml 80% ethanol by gavage. After 30 days, blood samples were collected from all groups. They were drawn into heparinized tubes. Plasma was separated by centrifugation at 800 g and +4°C for 10 min.

Biochemical Assays

All chemicals were purchased from Sigma Chemical Co. (St. Lois, MO, USA).

Malondialdehyde (MDA) MDA was measured by thiobarbituric acid method describe by Ohkawa et al. [19]. This method measures several aldehydes derived from lipid hydroperoxide and also known as thiobarbituric acid reactive substance. MDA constitutes one indicator of oxidative stress, since it arises from the breakdown of lipid peroxyl radicals. MDA is also important in that it can cause further oxidative injury by oxidizing protein molecules [20].

Protein Carbonyls (PCs) Protein oxidation was determined by using a colorimetric assay measuring the protein carbonyl content after reacting the plasma with dinitrophenylhydrazine, as described by Levin et al. [21]

TNF-α TNF-α levels were determined by using a commercially available rat ELISA kit (Biosource Europe SA. Nivelles, Belgium) and the results were expressed as picograms per deciliter.

pMMP-9 pMMP-9 levels determined by using a commercially available rat ELISA kit (R&D Systems, Inc. Minneapolis, USA) and the results were expressed as picogram per liter.

Aspartat Aminotransferase (AST) AST enzyme activities were determined in Hitachi 917 automated analyzer by using commercial kits supplied from Roche Diagnostic (Manhaime Germany).

Statistical Analysis

The results are expressed as mean ± SD (standard deviation of mean). Statistical comparisons between groups were performed by non-parametric Mann-Whitney U test and the difference was considered to be significant when $P<0.05$. The correlations were performed by Spearman's correlation test.

Results

The results are graphically displayed in Figs. 1, 2, 3, 4, and 5. Changes in lipid peroxidation expressed as MDA levels were found to be increased in ethanol group when compared to control group (0.497±0.088 μmol/L vs. 0.304±0.035 μmol/L; $p<0.01$). Vitamin E treatment

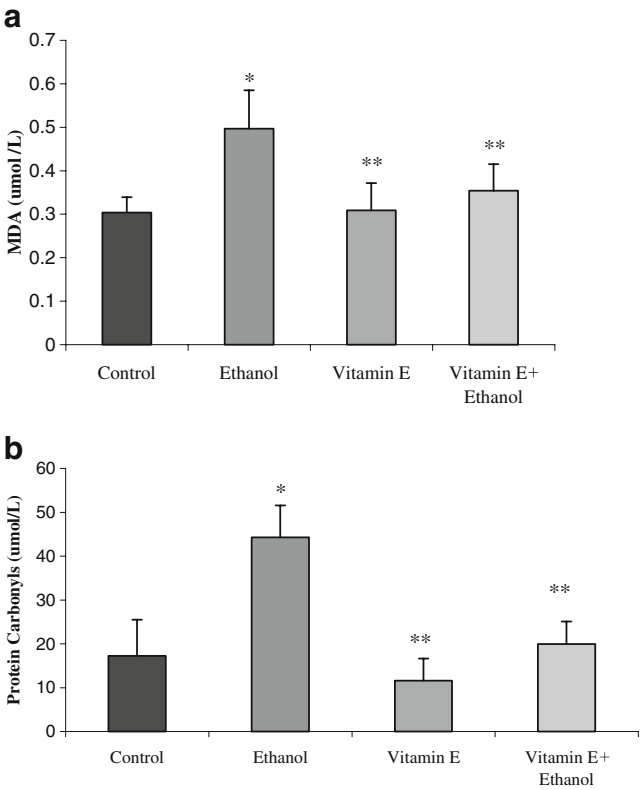

Fig. 1 The effect of ethanol and vitamin E treatment on plasma MDA levels (**a**) as an index of hepatic lipid peroxidation and plasma protein carbonyl levels (**b**) as an indices of protein oxidation. Values represent mean ± SD. *$p<0.01$ compared with control group. **$p<0.01$ compared with ethanol group

before ethanol administration resulted in a moderately but significantly decreased MDA level (0.354±0.088 μmol/L) when compared to ethanol group ($p<0.01$). Only vitamin E treatment did not affect MDA levels (Fig. 1a).

As shown in Fig. 1b, the PCs, as an index of protein oxidation was significantly higher in the ethanol group than those in the control group (44.25±7.3 μmol/L vs. 17.24±

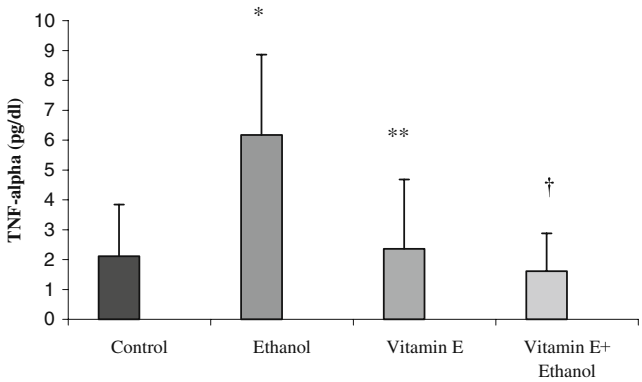

Fig. 2 The effect of ethanol and vitamin E treatment on plasma TNF-α levels: Values represent mean ± SD. *$p<0.05$ compared with control group. **$p<0.05$ compared with ethanol group. †$p<0.01$ compared with ethanol group

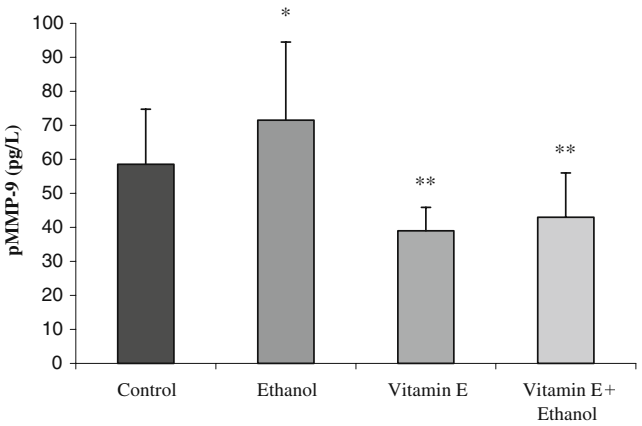

Fig. 3 The effect of ethanol and vitamin E treatment on plasma pMMP-9 levels: Values represent mean ± SD. *$p<0.05$ compared with control group. **$p<0.01$ compared with ethanol group

8.26 μmol/L; $p<0.01$). After Vitamin E treatment, increase in plasma PCs levels were reversed and almost control levels were achieved (19.93±5.15 μmol/L).

After ethanol administration, plasma TNF-α levels increased approximately threefold (6.17±2.69 pg/dl). Also under conditions of vitamin E treatment, ethanol-induced TNF-α release was significantly reduced (1.61±1.27 pg/dl; $p<0.01$; Fig. 2).

pMMP-9 levels were found to be increased in ethanol group when compared to control group (64.83±7.11 pg/L vs. 48.57±10.61 pg/L; $p<0.05$). Vitamin E treatment before ethanol administration significantly decreased pMMP-9 levels (39.00±6.87 pg/L) when compared to ethanol group ($p<0.01$). Only vitamin E treatment did not affect pMMP-9 levels (Fig. 3).

Ethanol significantly increased AST levels when compared to control group (153±34 U/L vs. 108± 24 U/L; $p<0.05$). Vitamin E treatment before ethanol administration decreased AST levels but there is not statistically important (122±21 U/L; Fig. 4).

A statistically relevant positive correlation was observed between the pMMP-9 and MDA ($r=0.532$, $p<0.05$, $n=20$; Fig. 5a) and PCs ($r=0.709$, $p=0.01$, $n=20$; Fig. 5b).

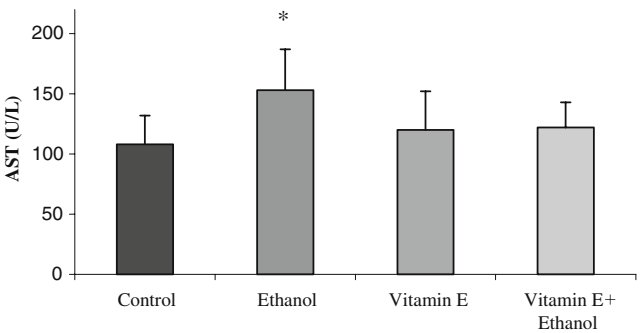

Fig. 4 Plasma AST levels as an index of hepatic injury. Values represent mean ± SD. *$p<0.05$ compared with control group

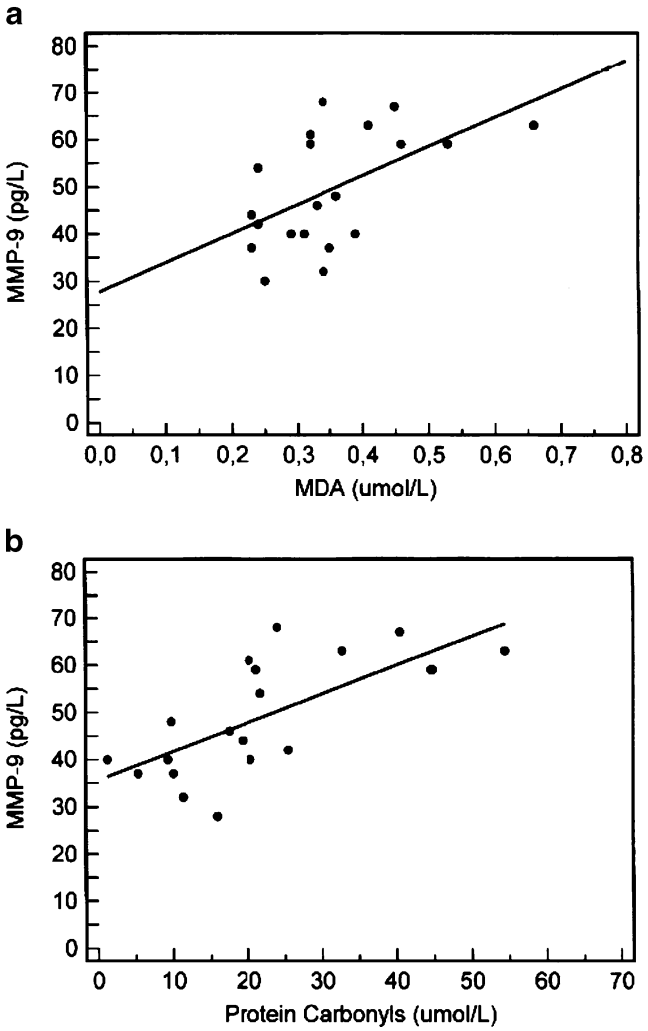

Fig. 5 **a** Association between concentrations of protein carbonyls and pMMP-9: r=0.574, p=0.004, n=24. **b** Association between concentrations of MDA and pMMP-9: r=0.454, p=0.045, n=24. Statistical analyses were performed using Spearman's correlation coefficient

Discussion

There is a clear link between oxidative stress and ethanol-induced hepatic injury. Three major pathways for alcohol metabolism exist in the liver: alcohol dehydrogenase, microsomal ethanol oxidizing system, and aldehyde oxidase. All these pathways result in the generation of reactive oxygen species, including superoxide hydroxyl radical, and hydrogen peroxide [22, 23]. Also, endotoxemia has long been known to be associated with ethanol exposure in both animal models and patients with alcoholic liver disease [24, 25]. Thurman et al. [1] proposed the endotoxic theory as a major mechanism of alcoholic liver injury. Gut permeability is increased by the taken ethanol and the microflora-derived endotoxin may leak into the bloodstream. These endotoxins activate Kupffer cells to induce nitric oxide [3] superoxide [4] and cytokines such as TNF-α, which activate nuclear translating factors such as nuclear factor kappa B (NF-κB) [26], finally leading to liver damage.

In the present study, we found that long-term alcohol consumption was associated with increased amounts of lipid peroxidation, protein oxidation, and levels of AST. Importantly, treatment with vitamin E prevented both alcohol-induced lipid and protein oxidations. But decreases of AST levels were not statistically important.

Also after ethanol administration, plasma TNF-α levels increased approximately threefold. TNF-α plays a critical role in the initiation and development of alcoholic hepatitis [27]. Kupffer cells are the main source of TNF-α. Oxidative stress has been implicated in TNF-α production. For example, hypoxia per se stimulates NF-κB activation and TNF-α gene transcription in macrophages [28]. Investigation with antioxidants demonstrated that treatment with allopurinol [29] and ebselent [30] results in the attenuation of NF-κB activation and TNF-α expression. Also, this study has clearly demonstrated that inhibition of oxidative stress by vitamin E leads to attenuation of TNF-α production. A lot of studies suggest that oxidative stress plays an important role in alcohol induced TNF-α production in the liver.

In this study, we found significant correlation between pMMP-9 and lipid peroxidation and protein oxidation. Regulation of MMPs by oxidative stress has been shown in nonhepatic cells. Hydrogen peroxide (H_2O_2) and xanthine oxidase (XO) increases total MMP activity in neonatal and adult cardiac fibroblast [10]. H_2O_2 increases pMMP2, pMMP9, and MMP14 proteins and activates pMMP2 in human venous endothelial cells [31]. Hyperoxia increases expression of pMMP2 and pMMP9 in rat lung [32]. XO increases expression of pMMP2 and decreases expression of tissue inhibitor matrix metalloproteinase 2 in dermal fibroblast [33]. Some studies have demonstrated that many metabolites of ethanol and cytokines could be effected MMPs activities. For example, Casini et al. have shown acetaldehyde, an immediate metabolic product of alcohol, has the ability to upregulate MMP-2 gene expression in cultured liver cells [34]. In our study, the rats were treated with vitamin E to understand whether the increase in pMMP-9 levels were as a result of ethanol itself or ethanol induced oxidative stress. Vitamin E decreased pMMP-9 levels in alcohol treated rat. On the other hand, we found significant correlation between pMMP-9 levels and lipid peroxidation and protein oxidation.

In conclusion, this study demonstrates that alcohol administration causes oxidative damage and alteration in plasma TNF-α and pMMP-9 levels. Vitamin E supplementation leads to attenuation of TNF-α and pMMP-9. Since antioxidant supplementation decreases the alcohol-induced pMMP-9 levels, oxidative stress could be one of the mediators of the generation of MMP-9. This study also

suggests that inhibition of oxidative stress may be an important strategy in the prevention of alcohol-induced liver injury.

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