Protective Effect of Alpha‑Lipoic Acid against Liver Damage Induced by Cigarette Smoke: An in vivo Study

Nurhan Gumral, Rahime Aslankoc, Nurgul Senol¹, Fatma Nihan Cankara²
Departments of Physiology, ¹Nutrition and Dietetics and ²Pharmacology, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey

Abstract

Background: Long-term cigarette smoking damages the liver tissue. Alpha-lipoic acid (ALA) is used as a therapeutic agent in a number of conditions and is known to have ameliorative effects against oxidative stress in the liver.

Objective: To investigate the ameliorative effects of ALA on cigarette smoke (CS)-induced oxidative liver damage by examining histopathological, immunohistopathological changes and biochemical parameters in an animal model.

Materials and Methods: Twenty-eight female Sprague–Dawley rats were randomly divided into three groups. In the control group (n = 8), rats were exposed to fresh air twice a day and given 0.1 ml of saline by gavage once a day for 8 weeks. In the smoking group (n = 10), rats were exposed to CS for 1 h in the morning and afternoon and given 0.1 ml of saline by gavage once a day for 8 weeks. In the smoking + ALA group (n = 10), CS exposure was same as the smoking group in addition to 100 mg/kg of ALA per day for 8 weeks through gavage. Oxidative damage in the liver tissue was determined by evaluating malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) levels. Aspartate aminotransferase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP), direct bilirubin and total bilirubin levels were measured in the blood. Histopathological and immunohistochemical examinations were performed.

Results: MDA (P = 0.011), AST (P = 0.018) and total bilirubin levels (P < 0.001) were increased, while CAT activity (P = 0.009) and the efficiency of SOD (P = 0.010) were decreased in the smoking group compared with the control group. CAT activity was increased (P = 0.017) and AST (P = 0.018) and total bilirubin levels (P < 0.001) were decreased in ALA-treated group compared with the smoking group. We observed vascular dilatation and hemorrhagic areas in the smoking group. TNF-α expression was increased in the smoking group compared with the control group. However, TNF-α expression was high in some preparations in the ALA-treated group.

Conclusions: ALA can enhance antioxidant activity, but studies with different doses of ALA are required to determine the extent of its hepatoprotective effect.

Keywords: Alpha-lipoic acid, inflammation, liver, oxidative stress, smoking

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INTRODUCTION

Cigarette smoking (CS) is common worldwide, in addition to many being exposed to secondhand smoking. Cigarettes comprise approximately 4000 compounds (aromatic hydrocarbons, nitrosamines, heavy metals, aromatic amines) that have harmful effects, such as causing chronic obstructive apnea disease, cancers, cerebrovascular and heart diseases, atherosclerosis, fatty liver diseases and premature skin aging. CS also increases the production of reactive oxygen species (ROS) by damaging the mitochondria and inducing apoptosis.

Kupffer cells, which are located in liver sinusoids, secrete cytokines that first activate this cell and then interact with hepatocytes and direct their functions. The relationship between kupffer cell and hepatocytes occurs through the receptor, which directs the cells’ function in inflammation of the liver. Kupffer cells affect a large number of cell populations in the organism with the substances they synthesize. The most important function of kupffer cells is their phagocytic feature.

Alpha-lipoic acid (ALA), a very strong antioxidant, is known to prevent disease complications and also protect against oxidative damage and inflammation. ALA is used as a therapeutic agent in a number of conditions, including oxidative stress, liver diseases, alcohol-induced liver damage, fungal, metal and carbon tetrachloride poisoning and biliary cirrhosis. In addition, ALA acts as an antioxidant by activating the Nrf2/HO-1 pathway and suppresses the activation of hepatic stellate cells induced by methotrexate in rats. Several studies have demonstrated that ALA has healing effects on hepatotoxicity induced by different toxins due to its antioxidant and anti-inflammatory properties. However, there is limited data regarding the hepatoprotective effect of ALA against CS-induced oxidative damage. In an in vitro study, it was found that the hepatoprotective action of ALA was related to decreasing levels of nitric oxide (NO) and TNF-alpha (TNF-α) in Kupffer cells in lipopolysaccharide-induced hepatic damage.

This study aimed to determine the potential protective role of ALA in CS-induced oxidative liver damage by examining histopathological, immunohistopathological changes (TNF-α) and biochemical parameters in an animal model. These parameters include malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase (CAT) activities, aspartate aminotransferase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP), and total and direct bilirubin levels, which were used to determine the development and extent of CS-induced hepatic damage in rats.

MATERIALS AND METHODS

The ARRIVE (Animal Research: Reporting of In Vivo Experiments) reporting guidelines was used while preparing this manuscript.

Animals

This experimental study was conducted at the Department of Physiology, Suleyman Demirel University, Isparta, Turkey, between June 10, 2015, and June 10, 2016. Twenty-eight female Sprague–Dawley skeletal development-completed 6-month-old (200–300 g) rats were kept in standard housing facilities (temperature: 23 ± 2°C, humidity: 60 ± 5%, 12/12 h light/dark cycle). The rats were kept in Euro type 2 cages after smoking exposure. All animals were supplied with standard laboratory chow (Korkuteli Yem, Korkuteli, Turkey) and water ad libitum.

The rats were obtained from the Experimental Animal Production and Experimental Research Center of the Suleyman Demirel University, Isparta, Turkey, and their health status was checked before the study initiation. All rats were inbred in genotype and were treatment naïve before use in current study.

The experiment was approved by the Animal Experiments Local Ethics Committee of Suleyman Demirel University, Isparta, Turkey, and conducted in accordance with the National Institutes of Health’s guidelines for animal research.

Study groups

After adaptation for 1 week, the rats were randomly divided into the following three groups and a cage was created for each group:

(1) Control group (n = 8): Animals in this group were exposed to fresh air and given 0.1 ml of saline solution for 8 weeks.

(2) Smoking group (n = 10): Animals were exposed to successive periods of cigarette smoke for 1 h in the morning and 1 h in the afternoon, 7 days for 8 weeks, and were simultaneously given 0.1 ml of physiological serum orally throughout the 8 weeks. According to the product specifications, each cigarette contained 1 mg of nicotine, 10 mg of tar and 10 mg carbon monoxide (Turkey Tobacco Industrial. Co., Ltd, Tekel 2000, Turkey).

(3) Smoking + ALA group (n = 10): Animals were exposed to the continuous period of CS for 1 h in the morning and afternoon and orally administered 100 mg/kg/d
of ALA dissolved in saline solution (Thioctacid 600 mg tablets, MEDA Pharma, Istanbul, Turkey). ALA was simultaneously given orally for 8 weeks before exposure to CS.

The experimental application was started from the control group. Each session was completed in 15 minutes for one rat. The same apparatus was used in the sessions including the control group.

There were no problems with the general health of the animals before and after the test. Twenty-four hours after the last ALA application, all rats were euthanized intraperitoneally with a mixture of 80 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylazine. Blood samples were collected from each animal for biochemical analyses. Then, serum samples were obtained by centrifugation at 4000 rpm for 10 min and stored at −80°C until analyzed. Another half of the liver samples was stored at −80°C until analyzed for MDA level, SOD and CAT activities.

The liver tissue was divided into two parts: the first part was placed in a 10% formaldehyde solution for histopathological and immunohistochemical examinations, and the second part was homogenized.

**CS apparatus and exposure details**
The smoking apparatus consists of three main parts. The first part contains a glass cube containing square blocks. On the top of the square cube, there is a valve that directs air in one direction. The third part consists of a structure holding the cigarette in front of the rotating ventilation device [Figure 1].

A cigarette holder device both holds the cigarette and burns it every 10 minutes. It also contains a syringe that transfers CS into a large cage and a part that controls the smoke flow with a valve rotating in one direction. During the experiment, the temperature in the device was maintained between 19 and 23°C, and the carbon monoxide levels were kept within the range of 310–380 ppm, which was measured in real time during CS exposure.

Animals were placed in the apparatus for 1 hour in the morning and 1 hour in the afternoon 7 days a week for 8 weeks. Each period lasted 90 minutes, with 10 minutes burning and 5 minutes ventilation time. The rats were exposed to six CS periods for 1 h twice/day until the end of the experiment.

**Biochemical analyses**
The liver tissues were homogenized in a motor-driven tissue homogenizer (IKA Ultra-Turrax T25 Basic; Labortechnic, Staufen, Germany) and sonicator (UW 2070 Bandelin Electronic, Germany) with phosphate buffer (pH 7.4). PBS was used to dilute the samples. The solution was then centrifuged at 4°C with a rotational speed of 1000 g per minute for 10 minutes and separated from unbroken cells, cell debris and nuclei. After the homogenization, the amount of protein in the obtained supernatant was determined according to the Bradford protein assay used as a standard. The amount of MDA in the tissue was determined using the Drapper and Hadley MDA method (two-step method). An autoanalyzer was used to determine CAT and SOD enzyme activity as well as MDA amount (Olympus AU 2700, Melville, NY). A spectrophotometer was used for quantification (Shimadzu UV-1601; Shimadzu, Kyoto, Japan). An autoanalyzer (Beckman Coulter AU 680, California, and USA) was used to measure the values of AST, ALT, ALP and direct and total bilirubin.

**Histopathological analysis**
The liver tissues fixed in 10% formalin solution were washed thoroughly in running tap water overnight to remove fixatives. Then, the routine histological tissue tracking procedure was performed. The tissues taken for histological examination were first placed in paraffin. Afterwards, 5-µm sections were taken from the obtained blocks. The sections were stained with hematoxylin-eosin, and histopathological evaluations were performed. Microscopic findings and the lesions were evaluated, examined and blindly graded by an independent pathologist. The sections were analyzed for hyperemia, edema, degenerated and necrotic cells under a microscope (Leica DM 500, Leica Microsystems, Wetzlar, Germany).

**Immunohistochemical analysis**
To perform immunohistochemical staining, 5-µm sections were taken from the liver tissue. The sections were
placed in the polylysine-coated slides. At the end of the deparaffinization process, the obtained preparations were washed with tap water. The preparations placed in 0.05% Tween 20 citrate buffer (pH 7.6), and left in the microwave for 30 minutes. The slides kept at room temperature were washed with PBS after cooling. Subsequently, 100 ml of 0.3% hydrogen peroxide (H₂O₂) solution was applied to the slides. The primary antibody was then incubated with polyclonal TNF-α antibodies at room temperature for 60 min. After that, it was washed again with PBS solution. Then, the sections were incubated with biotinylated anti-polyspecific for 30 minutes. Finally, they were incubated with streptavidin-peroxidase for 30 minutes. Staining was completed by soaking the sections in chromogen/substrate for 15 minutes. To exclude possible effects of the system, the control was performed using only the secondary antibody, followed by streptavidin–peroxidase and a chromogen peroxide incubation, without primary antibodies. All primary and secondary antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA). All the sections were evaluated in a blinded manner by an independent researcher. The intensity of cytoplasmic staining and extent of immune response were semi-quantitatively scored from 0 to 3, where “0” indicates no staining, “1” slight staining, “2” moderate staining and “3” severe staining.

Statistical analysis
The data obtained from the laboratory analyses were evaluated using IBM SPSS 20.0 (IBM SPSS Inc, Chicago, IL, USA). The variables obtained from the evaluation were calculated, and reported as frequency, percentage, average and standard deviation. The results obtained from immunohistochemical analyses were analyzed with the Mann–Whitney U test. The results of biochemical analyses were analyzed using the ANOVA test, post hoc least significant difference Bonferroni and Tukey tests. The results of histopathological analyses were assessed with Kruskal–Wallis test used to find significant differences between groups. P < 0.05 was considered statistically significant.

RESULTS
Oxidant/antioxidant status in the liver tissue
Oxidative stress indicators and MDA levels were significantly increased in the liver tissue in the smoking and ALA-treated groups (P = 0.01 and 0.012, respectively) compared to the control group. ALA treatment did not decrease MDA levels. In contrast, CAT activity was decreased in the smoking group compared with the control group (P = 0.009). CAT and SOD activities were increased in the ALA-treated group compared to the smoking group, but the difference was only significant for the CAT activity (P = 0.010) [Table 1].

Blood biochemistry
The levels of liver enzymes in serum, including AST and ALT, were significantly increased in the smoking groups compared with the control group (P = 0.018, and 0.010, respectively). ALA treatment significantly reversed the increased levels of both markers compared with the smoking group (P < 0.05). ALP and direct bilirubin levels were not significantly different between the three groups. In contrast, total bilirubin levels were significantly increased in the smoking group compared with the control group (P < 0.001) and were significantly decreased in ALA-treated group compared with the smoking group (P < 0.001) [Table 2].

Histopathology of liver tissue
The liver tissues of the control group demonstrated normal histological appearance [Figure 2]. We observed moderate sinusoidal and vascular dilatation as well as hemorrhagic and necrotic areas in the smoking group [Figure 2]. Similarly, necrotic areas, sinusoidal and vascular dilatation were detected in ALA-treated groups. In addition, formation of connective tissues was found in the same group [Figure 3].

Immunohistochemistry of the liver tissue
Immunohistochemical analysis showed an increased TNF-α expression in the smoking group compared with the control group (P = 0.000). Similarly, a dense TNF-α expression was detected in some preparations in ALA-treated group [Figure 4]. No significant differences were found between the smoking group and ALA-treated group (P = 0.764). However, a statistically significant difference was found between the smoking + ALA and control groups (P = 0.000) [Table 3].

DISCUSSION
Long exposure to CS causes adverse effects such as increased oxidative stress, which plays a significant role in tissue damage and cell apoptosis. It has also been found that CS-induced ROS overproduction inhibits

| Table 1: Oxidant/antioxidant levels of all groups for liver tissue |
|---------------------------------------------------------------|
| **Groups** | **MDA (µmol/mg protein)** | **CAT (ku/mg protein)** | **SOD (U/µg protein)** |
| Control group | 0.24±0.01 | 1.10±0.83 | 0.84±0.06 |
| Smoking group | 0.27±0.03<sup>a</sup> | 0.36±0.46<sup>a</sup> | 0.70±0.14<sup>a</sup> |
| Smoking + ALA group | 0.27±0.01<sup>b</sup> | 1.01±0.25<sup>b</sup> | 0.81±0.08 |

Values were expressed as mean±SD. The relationships between groups were evaluated by Bonferroni one-way ANOVA. <sup>a</sup>P < 0.05 compared with control group; <sup>b</sup>P < 0.05 compared with the smoking group. ALA = Alpha-lipoic acid; MDA = Malondialdehyde; SOD = Superoxide dismutase; CAT = Catalase; SD = Standard deviation.
enzymes such as CAT and glutathione peroxidase that are responsible for its removal and also induces inflammation in other organs.\textsuperscript{20,26–28} In this study, CS increased MDA levels in the animal models, indicating occurrence of oxidative stress in the liver of smokers, while also causing a decrease in CAT and SOD activities. However, ALA treatment did not decrease MDA levels, suggesting that ALA does not provide complete immediate protection to the liver against the negative effects of CS. Nonetheless, as ALA has antioxidant properties given that it increases the activity of CAT and SOD, it may provide hepatoprotection against CS exposure over a period of time and using different doses for early effects.

Salahshoor \textit{et al.}\textsuperscript{29} investigated the effects of nicotine on liver damage. The researchers found that nicotine (2.5 mL/kg) caused an important rise in the mean values of ALT, AST and ALP enzymes compared with the control group. Similarily, this study found that several specific biochemical markers, especially AST, ALT and total bilirubin values increased in the smoking group. These biochemical markers were improved after ALA treatment.

Sinusoidal dilatation, vascular dilatation, necrotic areas, connective tissue formation and TNF-\(\alpha\) immunopositive cells were observed in the smoking and ALA-treated groups. According to these histopathological and immunohistochemical findings, ALA does not protect liver against CS-induced damage, but longer treatment and different doses may have been needed, as mentioned previously. The inability of ALA in reducing TNF-\(\alpha\) level can be attributed to its different anti-inflammatory action at the dose used in the current study. For example, Abdel-Zaher \textit{et al.}\textsuperscript{30} investigated the ameliorative effects of pretreatment with ALA (100mg/kg) in acetaminophen-induced hepatotoxicity. They found that acetaminophen induced a pivotal elevation of NO and lipid peroxidation levels by reducing GSH-Px activity and decrease intracellular GSH level in the liver. After ALA pretreatment, these markers were reduced. They concluded that inhibition of NO overproduction and maintenance of intracellular antioxidant status by ALA may play an important role in the protection against acetaminophen-promoted hepatic damage.

In studies conducted on animals treated with ALA, ALA treatment has been found to upregulate IL-6 expression.
and glycoprotein 130 mRNA expression. Moreover, it was observed that IL-6 mediated regulation through ALA by receiving and transmitting the signal.\textsuperscript{[33]} Alternatively, the dose of 1000 μM demonstrated a significant decrease in TNF-α levels in a study that aimed to analyze the ameliorative effects of ALA against H₂O₂-induced oxidative stress and apoptosis in human lymphoid cells. It was found that ALA has protective effects against oxidation, inflammation and apoptosis.\textsuperscript{[32][33] Mirtaheri et al., in their study including 65 rheumatoid arthritis patients treated with ALA, did not find a significant difference in the levels of hs-CRP, TNF-α, IL-6 and MMP-3 in serum between the groups with and without ALA treatment. These studies suggest that ALA acts as an anti-inflammatory agent by decreasing TNF-α levels at lower doses and decreasing other markers at higher doses.\textsuperscript{[33]} In another study in which the effects of ALA on acetaminophen-induced liver damage were investigated, centrilobular necrosis, vascular degeneration and inflammatory cell infiltration were found in rat livers. In addition, the researchers showed that rats treated with ALA showed less pathological changes, but the liver was not fully protected from acute toxicity due to acetaminophen.\textsuperscript{[34]}

Limitations

Although an experimental study, the number of animals, cigarettes and therapeutic doses of ALA used in this study were limited, and thus future studies are required to circumvent this limitation, especially pertaining to different doses of ALA in the treatment.

CONCLUSIONS

This study found that cigarette smoke exposure leads to an increase in TNF-α expression, MDA, AST, ALT and total bilirubin levels, which are associated with liver damage, and that ALA has a mild ameliorative effect on the liver damage induced by cigarette smoke. Studies using different ALA doses and routes of administration are needed to fully understand the healing effect of ALA against liver damage caused by CS.

Ethical considerations

The experiment was approved by the Animal Experiments Local Ethics Committee of Suleyman Demirel University, Isparta, Turkey (Ref No: 06; Date: December 04, 2014), and conducted in accordance with the National Institutes of Health’s guidelines for animal research.

Peer review

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Conflicts of interest

There are no conflicts of interest.

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