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Research Article

Efficacy of α-lipoic Acid Against Oxidative Stress and Histopathological Changes Induced by Dimethylnitrosamine in Liver Male Mice

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Astract

Background and Objective: Dimethylnitrosamine (DMN) is a representative chemical of a family of N-nitroso compounds and has been found in processed meats and industrial products. Alpha Lipoic Acid (ALA) found naturally in plants and animals is involved in many different antioxidant functions in all body tissues. So, the study was designed to find out the efficacy of ALA in reducing the DMN-toxicity in male mice by measuring some biomarkers functions of liver as well as oxidative stress parameters and histopathological changes. **Materials and Methods:** Animals were divided into 6 groups (n = 8) and received their treatment for 4 weeks as follows: Groups 1-4 served as control, ALA-treatment (16.12 mg kg^{-1}), DMN-low dose treatment and DMN-high dose treatment, respectively. Groups 5 and 6 were received ALA before DMN-low and DMN-high dose, respectively. **Results:** The treatment of the mice with DMN caused significant changes in serum biomarker enzymes of liver, lipid profile, protein content and myeloperoxidase as well as hepatocytes lipid peroxidation, superoxide dismutase, catalase, glutathione peroxidase, nitric oxide, xanthine oxidase and total antioxidant capacity. Histopathological analysis of liver confirmed the oxidative stress results. **Conclusion:** Concomitant administration of ALA with DMN significantly protected most of the changes induced by DMN suggesting its protective efficacy.

Key words: Dimethylnitrosamine, α-lipoic acid, liver, myeloperoxidase, oxidative stress, histopathology

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dimethylnitrosamine (DMN) is a representative chemical of a family of N-nitroso compounds and has been found in processed meats and industrial products as tobacco smoke, whisky, smoked, salted and dried fish, cheese and alcoholic beverages[1]. Nitrosamines are resulted from nitrites and secondary amines that found in the proteins of foods especially under strongly acidic conditions as in human stomach or at high temperatures, as in frying[2]. Jayakumar *et al.*[3] found that nitrosamines increased the oxidative stress and cellular damage due to the formation of different types of oxygen free radicals during its metabolism and increasing the lipid peroxidation (LPO)[4]. The DMN could be caused hepatocellular necrosis, carcinogenicity, neoplastic changes and tumor formation due to the oxidative stress[5]. Superoxide and other ROS generated due to metabolism of the nitrosamine by CYP2E1 in mouse liver stimulated Kupffer cells[6].

The DMN-induced hepatocellular carcinoma in rats therefore, they are widely used to study hepatocarcinogenesis[7]. Moreover, metabolism of nitrosamines in human livers similar to rat liver[8]. The liver is often target by xenobiotic, poor eating habits and over the counter drug use, which can damage the liver and eventually leads to liver disease[9].

Alpha Lipoic Acid (ALA) found naturally in plants and animals is involved in many different antioxidant functions in all body tissues[10]. Besides its roles in scavenging and eliminating the radical effects of ROS, it has also been identified as a participant in the formation of the other cellular antioxidants, as it recycles vitamin C, vitamin E, as well as increasing the intracellular glutathione (GSH) concentrations by increasing sulfur levels in its elemental form[11]. Moreover, its major metabolite dihydrolipoic acid is a potent antioxidant[12]. The antioxidant role of ALA has been implicated in hepatitis, diabetes, atherosclerosis and urolithiasis[13].

Myeloperoxidase (MPO) is an oxidative enzyme with antimicrobial activity, which uses H_2O_2 to produce hypochloric acid and other toxic substances in neutrophil phagolysosomes. It has been reported that MPO plays important roles in chronic processes such as neurodegenerative diseases and atherosclerosis[14].

Xanthine Oxidase (XO) catalyzes the oxidation of hypoxanthine to xanthine and subsequently to uric acid[15] in the purine nucleotides catabolism. Its re-oxidation involves molecular oxygen which acts as electron acceptor and during this reaction, superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are produced[16]. The O_2^- is transformed into H_2O_2 and O_2 either spontaneously or by the catalytic action of superoxide dismutase (SOD). Thus, the over-activity of XO leads to the deposition of uric acid in the susceptible tissues and this triggers the inflammatory pathways with a concomitant release of ROS.

Nitric Oxide (NO) which has both antioxidative and pro-oxidative properties, particularly increases in inflammatory diseases. It also plays an important role in the chronic stage of various diseases[17]. It has recently been demonstrated that NO acts in conjunction with hydrogen sulfide (H_2S) to carry out regulation of angiogenesis and endothelium-dependent vasorelaxation[18] and cytoprotective signaling mediated by H_2S was found to be dependent on NO generated by endothelial nitric oxide synthase (eNOS)[19].

Therefore, the present study was designed to assess the efficacy of ALA in reducing the DMN-toxicity in male mice by measuring some biomarkers functions of liver as well as oxidative stress parameters and histopathological changes.

MATERIALS AND METHODS

Chemicals: Sodium nitrite and other chemicals for measurements were purchased from Sigma Chemical Company, St., Louis, MO, USA. The nitrosamine precursors; chlorpromazine drug ($C_{17}H_{19}C_1N_2S$; a drug with amine hydrochlorides) were used as precursors for generation of endogenous nitrosamine and was obtained from the Misr Company for Pharmaceutical Industries, Cairo, Egypt. Commercial pharmaceutical preparations containing ALA was provided by EVA Pharma for Pharmaceuticals and Medical Appliances, Cairo, Egypt.

Dose and routes of administration: The dosages of the two chemicals were determined according to the Food and Drug Administration (FDA) guideline. The FDA has suggested that the extrapolation of animal dose to human dose can be correctly performed by normalization to Body Surface Area (BSA), which is generally represented in mg m^{-2}. The human dose equivalent can be more appropriately calculated by using the equation:

$$\text{Human equivalent dose (HED mg kg}^{-1}) = \text{Animal does (mg kg}^{-1}) \times \left(\frac{\text{Animal } K_m}{\text{Human } K_m}\right)$$

where, K_m factor is weight (kg)/BSA (m^2).

Hence, the appropriately calculated dose in rats is also achievable in humans. It can be easily calculated that ALA dosage can be used as 16.21 mg kg^{-1} calculated according to the formula for a person weighing 60 kg[20]. Sodium nitrite ($NaNO_2$) was given daily in drinking water (0.05%)[21]. Chlorpromazine was given orally by gastric tube at two doses,

a dose of 2 mg kg^{-1} b.wt., day^{-1} (low dose; LD) and another was 4 mg kg^{-1} (high dose; HD) as reported by File[22].

Animals and experimental design: Forty eight adult male mice weighing 45-60 g were used in this study. They were obtained from the animal house of Faculty Veterinary Medicine, Zagazig University, Egypt. The animals were allowed to free access of standard diet and water *ad labium* throughout the experimental period. We have followed the European community directive (86/609/EEC) and national rules on animal care that was carried out in accordance with the NIH guidelines for the Care and Use of Laboratory Animals 8th edition. Animals were kept in well ventilated cages in a temperature-controlled room at 22-25°C, maintained under specific pathogen-free conditions on a 12 h light, dark cycle.

After 2 weeks of acclimation, animals were divided into 6 equal groups with 8 mice each and received their respective treatment for 4 weeks as follows: Group 1 served as an untreated control group, group 2 was treated with ALA (16.12 mg kg^{-1}), group 3 was received nitrosamine precursors (sodium nitrite (0.05% in drinking water)) and given orally chlorpromazine at LD, group 4 was treated with nitrosamine precursors (sodium nitrite (0.05% in drinking water)) and given orally chlorpromazine at HD, groups 5 was received nitrosamine precursors, LD of chlorpromazine plus ALA and finally group 6 was treated with nitrosamine precursors, HD of chlorpromazine plus ALA.

Blood collection and tissue homogenate: At the end of the experimental periods (4 weeks), overnight fasting mice were anesthetized using diethyl ether and sacrificed by cervical dislocation. Blood samples were collected from the medial retro-orbital venous plexus immediately with capillary tubes (Micro Hematocrit Capillaries, Mucaps)[23]. Then, the blood was centrifuged at 3000 rpm for 15 min and serum was collected for different biochemical analyses.

Liver tissue (about 0.25 g) was used for the analysis of oxidative stress parameters. Prior to dissection, tissue was perfused with a 50 mM (sodium phosphate buffer saline (100 mM Na_2HPO_4/NaH_2PO_4) (pH 7.4) and 0.1 m ethylenediaminetetra acetic acid (EDTA) to remove any red blood cells and clots. Then, tissues were homogenized in 5 mL cold buffer g^{-1} tissue and centrifuged at 5000 rpm for 1/2 h. The resulting supernatant was transferred into Eppendorf tubes and preserved in a deep freeze until used for various oxidative assays.

Liver biomarkers: Serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) activities were determined with kits from SENTINEL CH (via Principe Eugenio 5-20155 MILAN-ITALY). The γ-glutamyl transpeptidase (γ-GT) activity was estimated by method of Orlowski and Meister[24]. Lactic dehydrogenase (LDH) activity was measured using kinetic kits[25]. The total protein content was determined by the method described by Bradford[26] using bovine serum albumin as the standard.

Lipid profile: The serum cholesterol and triglycerides were determined by the method of Carr *et al.*[27]. High density lipoprotein-cholesterol (HDL-c) was determined according to the methods of Warnick *et al.*[28]. Low density lipoprotein-cholesterol (LDL-c) level was calculated according to Friedewald *et al.*[29] with equation:

$$\text{LDL}-\text{c} = ¼\ \text{Total cholesterol levels} - \left(\frac{\text{Triglyceride concentration}}{5}\right) - \text{HDL}-\text{c concentration}$$

Determination of oxidative parameters: The SOD activity was measured according to Marklund and Marklund[30] and calculated as the amount of protein that caused 50% pyrogallol auto oxidation inhibition at 440 nm for 3 min. A blank without homogenate was used as control for non-enzymatic oxidation of pyrogallol in tris-EDTA buffer (50 mmol L^{-1} tris, 10 mmol L^{-1} EDTA and pH 8.2). The SOD activity is expressed as U g^{-1} tissue.

Catalase (CAT) activity was determined according to Aebi[31] where the hydrolysis of H_2O_2 and the resulting decrease in absorbance at 240 nm over a 3 min period at 25°C. Samples were diluted 1:9 with 1% (v/v) triton X-100 and CAT activities expressed as U g^{-1} tissue.

The GPx activities were determined as described by Hafeman *et al.*[32] and expressed in mmol GSH consumed min^{-1} g^{-1} tissue.

The extent of LPO was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA)[33]. The MDA concentrations were determined using 1,1,3,3-tetraethoxypropane as standard and expressed as nmol g^{-1} tissue.

Xanthine Oxidase (XO) activity was assayed spectrophotometrically by the reaction of enzyme with xanthine, as a substrate[34]. In two test tubes, 15 µL phosphate buffer (0.1 M, pH 7.4) and 30 µL water (control) or substrate (test; xanthine 38 mM, dissolved in distilled water by adding alkali and gentle heating) was added to 0.5 mL liver homogenates and incubated for 40 min at 37°C. Then, 0.1 mL of this solution was mixed with 0.1 mL Na-tungstate 40%, 0.5 mL water and 0.1 mL 2 N H_2SO_4 complete to 1 mL and incubated for 1 h at 37°C then centrifuged at 3000 rpm for 10 min. About 0.15 mL of supernatant was mixed with 0.75 mL water, 0.3 mL Foline reagent and 1.5 mL saturated

Na-carbonate solution. The absorbance was measured at 650 nm after 10 min. The XO activity was determined using the following equations:

$$\text{Concentration of xanthine in control or test} = \frac{\text{A (sample)}}{\text{A (standard)}} \times \text{Concentration of standard} \times 48$$

$$\text{Xanthine oxidase activity (U g}^{-1}\text{ tissue)} = \frac{\text{Concentration of test concentration of control}}{0.284}$$

The levels of nitrite, a stable end-product of Nitric Oxide (NO) production were measured based on the Griess reaction[35], in which equal volume of deproteinized tissue homogenates were mixed with acidic Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). The absorbance was measured at 546 nm.

Total antioxidant capacity was determined using ferric reducing antioxidant power assay[36]. Ferric Reducing Antioxidant Power (FRAP) reagent (300 mmol L^{-1} acetate buffer, pH 3.6, 10 mmol L^{-1} 2,4,6-tripyridyl-s-triazine, 99 %) in 40 mmol L^{-1} HCl and 20 mmol L^{-1} $FeCl_3.6\ H_2O$ in the ratio of 10:1:1 was prepared. The FRAP reagent (1.5 mL) was added to 50 µL of homogenate liver tissues and incubated at 37°C for exactly 5 min. The change in absorbance was measured at 593 nm due to the formation of a blue colored FeII 2,4, 6-tripyridyl-s-triazine complex from the colorless oxidized FeIII form by the action of electron donating antioxidants. The absorbance of the sample was read against reagent blank (1.5 mL FRAP reagent and 50 µL distilled water) at 593 nm.

Myeloperoxidase (MPO) activity assay kit (Fluorometric) provides a rapid, simple and reliable method to study MPO activity[37]. Briefly, 10 mL sample were combined with 80 mL, 0.75 mM H_2O_2 (Sigma) and 110 mL TMB solution (2.9 mM TMB in 14.5% DMSO (Sigma) and 150 mM sodium phosphate buffer at pH 5.4) and the plate was incubated at 37°C for 5 min. The reaction was stopped by adding 50 mL 2 M H_2SO_4 (Sigma) and absorption was measured at 450 nm to estimate MPO activity.

Histological evaluation: For histological examination, a portion of liver was fixed in 10% neutral buffered formalin embedded in paraffin, sectioned and stained with haematoxylin and eosin[38].

Statistical analysis: Analysis was performed using SPSS for Windows version 17.0. Data was given in the form of arithmetical Mean values±Standard Error (SE). Differences between groups were evaluated by one-way ANOVA according to $p<0.05$ and *post hoc* Duncan test.

RESULTS

Serum activates of ALT, AST, ALP, LDH and γ-GT were found unchanged in ALA-treated group as compared to control animals (Fig. 1). All the previous mentioned enzymes increased by increasing the dose of DMN that was administrated to mice. The concurrent administration of ALA with DMN-HD improved the biomarkers enzymes activities of the liver as compared to DMN-HD group.

The treatment of the mice with DMN caused significant increase in serum levels of TC, TG and LDL-c and significant decrease in HDL-c and total proteins levels (Table 1). Results revealed that there are no significant changes in total proteins levels of mice treated with ALA only as compared to control animals. However, the presence of ALA with DMN can alleviate the adverse effects of DMN and the alterations of lipid profiles restored to approximately the normal values in dose dependent manner.

Table 2 shows that treatment the animals with DMN caused a significant decrease in the activity of SOD of liver in dose dependent manner. Administration of ALA only did not cause change in the activity of SOD of liver as compared with those of control mice. In addition, a significant recovery relating to SOD was observed in response to the presence of ALA with DMN. However, co-administration of ALA with DMN-LD or DMN-HD increased the SOD activity as compared with its relative groups.

The CAT activity decreased after DMN treatment in two doses (Table 2). The administration of ALA with DMN-HD significantly increased the CAT activity in liver tissue as compared with its related group.

The activity of GPx was significantly decreased in liver tissue of rats treated with DMN-LD and DMN-HD by 48.32 and 53.86% as compared with the control group, respectively (Table 2). The presence of ALA with DMN minimized the observed alterations in examined enzyme activity induced by DMN intoxication in liver tissue.

The results indicated that the levels of LPO were significantly increased in two tested doses of DMN (Table 2). Co-administration of ALA with DMN reduced the elevation in LPO as compared with its related group of DMN.

There was no any change between the control and ALA-groups in XO activity (Table 2). The XO activities were increased by 2.0 and 2.4 fold in DMN-LD and DMN-HD groups, respectively. Co-administration of ALA with DMN reduced the elevation in XO as compared with its related group of DMN.

The NO levels were increment in all groups that treated with two doses of DMN and then decreased with ALA administrated before DMN treatment (Table 2).

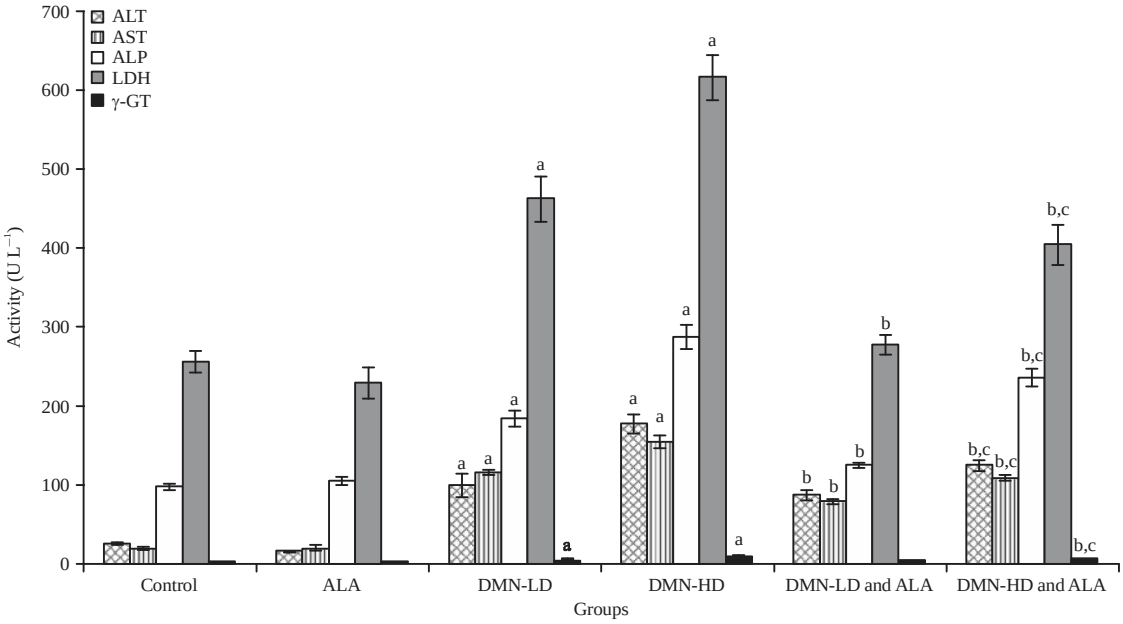

Fig. 1: Effect of α-lipoic acid and different doses of dimethylnitrosamine on serum enzymes activities of male mice, ALA: α-lipoic acid, DMN-LD: Dimethylnitrosamine-low dose, DMN-HD: Dimethylnitrosamine-high dose, ALT: Aanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, γ-GT: Gamma-glutamyl transferase, values are expressed as Means±SE, n = 8 for each treatment group, [a]Significant difference as compared to control, [b]Significant difference as compared to its related DMN group and [c]Significant difference as compared to DMN-LD with ALA group

Table 1: Effect of α-lipoic acid and different doses of dimethylnitrosamine on lipid profile and total proteins of male mice

Parameters (mg dL^{-1})	Control	ALA (16.21 mg kg^{-1} day^{-1})	DMN-LD (2.0 mg kg^{-1} day^{-1})	DMN-HD (4.0 mg kg^{-1} day^{-1})	DMN-LD and ALA	DMN-HD and ALA
TC	71.56±6.39	95.57±4.18^{a}	135.33±4.65^{a}	221.61±9.79^{a}	101.05±3.72^{b}	148.64±13.37^{b,c}
TG	133.78±4.44	83.27±3.59^{a}	131.01±3.99	187.70±8.53^{a}	112.74±3.99^{b}	135.69±6.92^{b,c}
HDL-c	34.29±1.30	34.44±0.59	25.21±1.86^{a}	20.33±1.42^{a}	29.25±2.31	27.82±1.63^{b}
LDL-c	31.92±0.84	97.27±3.96^{a}	139.84±3.31^{a}	201.30±3.37^{a}	111.80±9.52^{b}	152.95±6.50^{b,c}
VLDL-c	27.95±1.66	16.65±0.72^{a}	24.48±1.55	37.54±1.71^{a}	23.06±0.95	25.13±0.59^{b}
TP	7.94±0.27	7.61±0.33	6.17±0.28^{a}	4.69±0.25^{a}	5.96±0.12	5.33±0.18^{c}

ALA: α-lipoic acid, DMN-LD: Dimethylnitrosamine-low dose, DMN-HD: Dimethylnitrosamine-high dose, TC: Total cholesterol, TG: Triglyceride, HDL-c: High density lipoprotein-cholesterol, LDL-c: Low density lipoprotein-cholesterol, VLDL-c: Volatile low density lipoprotein-cholesterol, TP: Total protein, values are expressed as Means±SE, n = 8 for each treatment group, [a]Significant difference as compared to control group, [b]Significant difference as compared to its related DMN group and [c]Significant difference as compared to DMN-LD with ALA group

Table 2: Effect of α-lipoic acid and different doses of dimethylnitrosamine on some oxidative stress parameters in liver of male mice

Parameters	Control	ALA (16.21 mg kg^{-1} day^{-1})	DMN-LD (2.0 mg kg^{-1} day^{-1})	DMN-HD (4.0 mg kg^{-1} day^{-1})	DMN-LD and ALA	DMN-HD and ALA
SOD (U g^{-1})	23.71±0.82	22.87±1.33	13.74±1.22^{a}	12.05±1.69^{a}	15.35±1.36	17.64±1.32^{b}
CAT (U g^{-1})	4.33±0.25	3.98±0.32	2.90±0.25^{a}	3.40±0.38^{a}	3.53±0.27^{b}	3.84±0.14^{b}
GPx (mmol g^{-1})	51.78±3.68	37.73±3.13^{a}	26.76±1.47^{a}	23.89±1.46^{a}	32.30±1.57^{b}	31.15±0.65^{b}
MDA (nmol g^{-1})	37.99±4.58	36.21±1.92	87.13±4.14^{a}	182.79±99.66^{a}	72.67±2.28^{b}	118.15±9.76^{b,c}
XO (U g^{-1})	17.01±0.40	18.27±2.57	34.53±1.64^{a}	40.56±0.70^{a}	25.69±1.09^{b}	29.85±1.20^{b,c}
NO (μmol g^{-1})	0.69±0.05	0.67±0.09	1.15±0.13^{a}	2.27±0.21^{a}	0.72±0.09^{b}	1.30±0.19^{b,c}
TAC (%)	94.20±1.16	93.60±1.17	83.79±1.86^{a}	76.33±1.66^{a}	85.72±2.11	84.04±2.02^{b}

ALA: α-lipoic acid, DMN-LD: Dimethylnitrosamine-low dose, DMN-HD: Dimethylnitrosamine-high dose, SOD: Superoxide dismutase, CAT: Catalase, MDA: Malondialdehyde, XO: Xanthine oxidase, NO: Nitrite, TAC: Total antioxidant capacity, values are expressed as Means±SE, n = 8 for each treatment group, [a]Significant difference as compared to control group, [b]Significant difference as compared to its related DMN group and [c]Significant difference as compared to DMN-LD with ALA group

The TAC levels were significantly decreased in liver tissue of mice treated with two doses of DMN (Table 2). However, the presence of ALA with DMN minimized the observed alterations in the TAC level induced by DMN intoxication.

Effect of ALA and different doses of DMN on serum MPO activity of male mice was presented at Fig. 2. The MPO activities were increased by 1.48 and 1.89 fold in DMN-LD and DMN-HD as compared to control group, respectively. The administration of ALA before DMN was decreased the MPO activity by 30.2 and 31.7% for DMN-LD and DMN-HD, respectively.

Figure 3 shows the histological sections of liver of experimental mice that were observed under light microscope. In the control group, hepatic lobular architecture was intact. The laminae, sinusoids, portal canals of the rat liver were very distinct (Fig. 3a). Those structures were found to be well preserved in the mice treated with ALA (Fig. 3b). The mice treated with DMN-LD showed infinitesimal damage in rat liver tissue, cholestasis (CH) with swollen hepatocytes with foamy finely granular cytoplasm (FS) (Fig. 3c). Besides, another important observation in DMN-HD group was markedly focal hepatocytes necrosis (FN) with drop off hepatocytes (Fig. 3d). However, partial improvement in the hepatocytes structure with some congested hepatic sinusoids (CS) in DMN-LD with ALA group was observed (Fig. 3e). The restoration of normal appearance and structure of hepatocytes with dilated mildly congested central veins (DV) and showing ground glass hepatocytes were noticed in DMN-HD with ALA group (Fig. 3f).

The hepatic necrosis and inflammation were significantly increased in the mice treated with DMN in dose dependent manner than to the control group and the scoring of histological damage was displayed in Table 3. Improvement results were observed in liver section from animals treated with ALA. Treatment the mice with ALA has significantly reduced liver injury and fibrosis compared to the respective DMN groups.

Table 3: Grading of the histopathological changes in the liver sections of mice treated with α-lipoic acid and different doses of dimethylnitrosamine

Finding	Control	ALA (16.21 mg kg^{-1} day^{-1})	DMN-LD (2.0 mg kg^{-1} day^{-1})	DMN-HD (4.0 mg kg^{-1} day^{-1})	DMN-LD and ALA	DMN-HD and ALA
Normal hepatic lobule	++++	++++	---+	-----	++++	-+++
Normal nuclei	++++	++++	---+	---+	-++	-+++
Pyknotic nuclei	-----	-----	-+++	++++	-----	-++
Focal hepatic necrosis	-----	-----	-+++	++++	-----	-++
Dilated congested central vein	-----	-----	-+++	++++	-----	---+
Swollen hepatocytes with foamy finely granular cytoplasm	-----	-----	++++	-++	---+	---+
Dilated congested sinusoids	-----	-----	-+++	++++	-+++	-++
Glass hepatocytes	-----	-----	++++	+++-	---+	-++

-----: Absence of the change in the animals of the studied group, ++++: A change which was often found in all the studied animals of a group, +++-: A change which was observed in almost all the studied animals of a group, ++-: A change not so often observed in all animals of a group and -+: A change which was rare within a group, The study was done for eight mice of each group

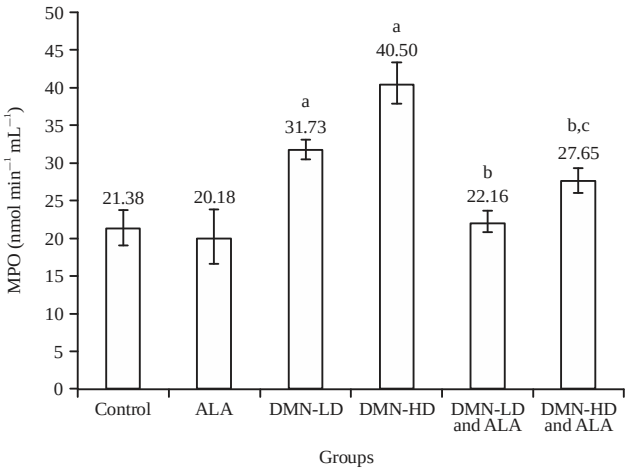

Fig. 2: Effect of α-lipoic acid and different doses of dimethylnitrosamine on serum myeloperoxidase (MPO) activity of male mice, ALA: α-lipoic acid, DMN-LD: Dimethylnitrosamine-low dose, DMN-HD: Dimethylnitrosamine-high dose, values are expressed as Means±SE, n = 8 for each treatment group, [a]Significant difference as compared to control, [b]Significant difference as compared to its related DMN group and [c]Significant difference as compared to DMN-LD with ALA group

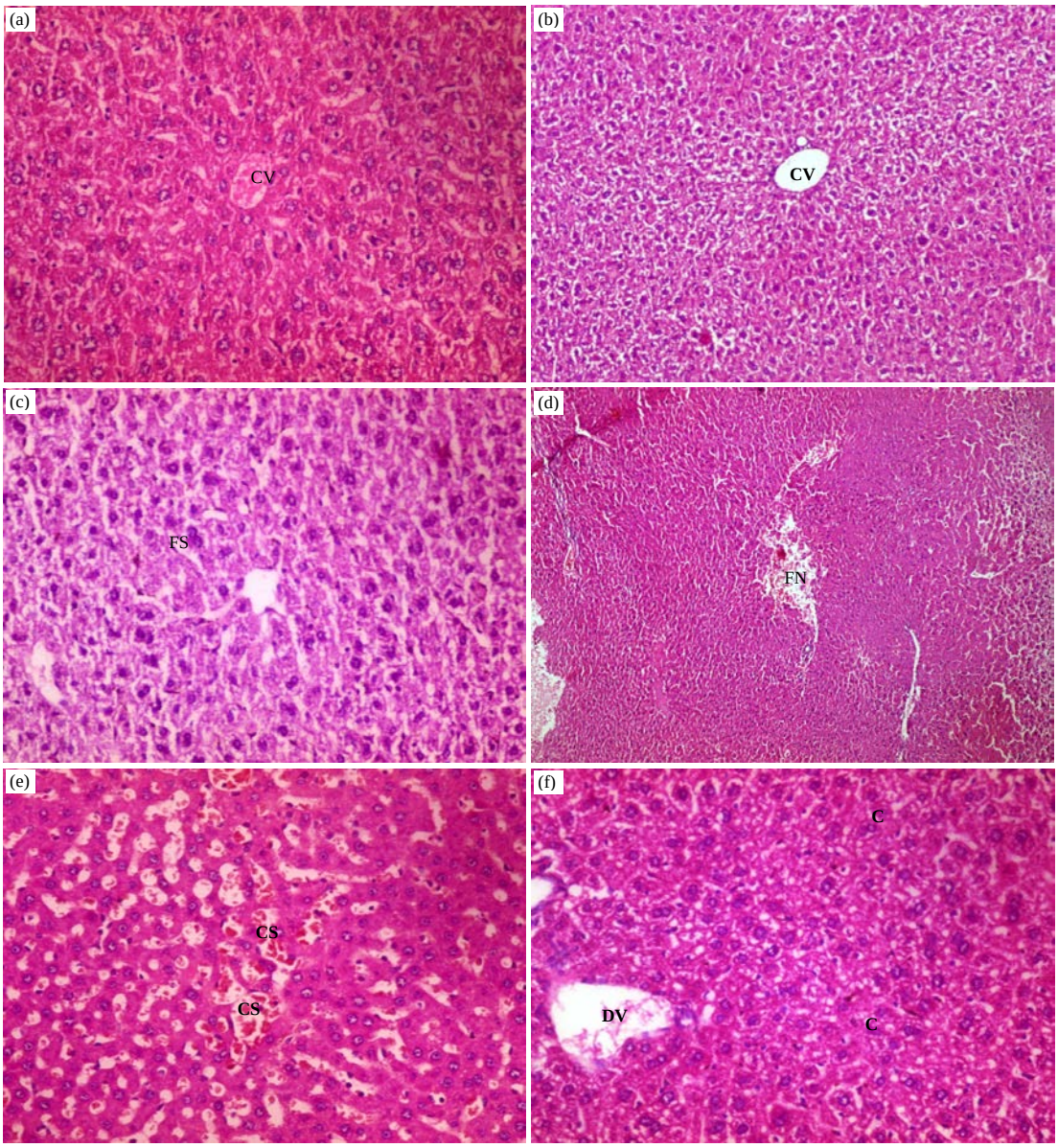

Fig. 3(a-f): Effect of α-lipoic acid (ALA) and different doses of dimethylnitrosamine (DMN) on liver tissue, (a) Histological section from control with normal Central Vein (CV) and normal hepatic lobule, (b) Hepatic tissues appear to be normal in ALA group with normal Central Vein (CV) and normal hepatic lobule, (c) Cholestasis (CH) with swollen hepatocytes with foamy finely granular cytoplasm (FS) in DMN-LD group, (d) Hepatic tissues showing focal hepatocytes necrosis (FN) with drop off hepatocytes in DMN-HD, (e) Partial improvement in the hepatocytes structure with some congested hepatic sinusoids (CS) in DMN-LD with ALA group and (f) Restoration of normal appearance and structure of hepatocytes with dilated mildly congested central veins (DV) and showing ground glass hepatocytes (C) DMN-HD with ALA group (H and E, x400)

DISCUSSION

The present study was carried out to elucidate the protective effect of ALA against the oxidative and histopathological changes induced in mice by DMN. The ALA is reported to quench ROS and nitrogen species, hydroxyl radicals, peroxyl radicals and peroxynitrite[39]. However, there is no data available on the efficacy of ALA against DMN specially concern the MPO, XO and NO. Exposure of mice to DMN revealed a statistically significant positive correlation between

the daily dose of DMN and all the hepatic enzymes-tested. The biochemical and histopathological alternation in DMN-treated groups were dose dependent.

In present study, the effect of DMN on mice liver biomarkers could be similar to other studies that were carried on diethylnitrosamine (DEN) that one of nitrosamin. Ramalingam and Vaiyapuri[40] reported that TC and TG were significantly increased after treated rats with DEN. Nitrosamine is absorbed in intestine and enters the liver and hepatocytes by portal venous system and hampers the detoxification system of liver by interfering in cytochrome P450 enzymes, so, the liver gently proceeds to cirrhosis and cancer[41]. When DEN is formed, it leads to LPO of the polyunsaturated fatty acid in cell membranes, break down of membrane-structure and leading to the release of microsomal corboxyal esterase and other enzymes, such as amino transferases in to the extra cellular compartments including serum[42]. These results agree with previous studies of Atakisi *et al.*[43] who found that antioxidant enzymes activities (GPx, CAT and SOD) levels were decreased significantly following injected DEN exposure.

Plasma transaminases are sensitive indicators of liver cell injury[44] which elevated in DMN-treated mice may be due to either direct hepatocyte damage or due to oxidative stress leading to apoptosis of hepatocytes. These data indicate that DMN causes liver dysfunction.

The administration the two doses of DMN caused a significant increase in the levels of lipid profile parameters (TC, TG, LDL-c and VLDL-c) and decrease of HDL-c in a dose-dependent manner. These observations are consistent with the presence of liver cell damage that was seen in the DMN-exposed mice by light microscopy. The LPO and oxidative modification of LDL-c are implicated in the development of atherosclerosis[45].

The DMN caused significantly decreased in serum total protein. The present results agree with Ahmed *et al.*[46] who found that injection of DEN for 2 months to rats showed significantly decreased the level of total protein and albumin. Decreasing the total protein could be due to impaired liver function.

The DMN induced profound elevation of NO production, increasing XO and MPO activities, oxidative stress of LPO along with reduction of antioxidant enzymes as SOD, CAT, GPx and depletion of TAC level in liver. Hepatic injury induced by DMN was associated with oxidative stress due to DMN-induced free radical production and toxic[3]. Oxidative stress plays an important role in chronic complications of injected with DMN and is postulated to be associated with increased LPO[4]. The cytotoxic action of DMN is associated with the generation of ROS causing oxidative damage. The free radicals may react with polyunsaturated fatty acids in cell membranes leading to LPO. Lipid peroxide-mediated damage has been observed in the development of injected with DEN[47].

The highly oxidative enzyme MPO is abundant in granules of inflammatory cells such as activated neutrophils, macrophages and monocytes. The MPO acts as a master enzyme in the generation of a range of ROS by catalyzing the conversion of hydrogen peroxide (H_2O_2) to species including OH and NO_2. The MPO-derived ROS can then modify lipids, lipoproteins and proteins. Inflammatory cells recruited into the vascular wall release MPO-derived ROS that can in turn promote endothelial dysfunction by reducing the bioavailability of nitric oxide[48] and modify HDL-c, impairing its function in cholesterol efflux. Elevated circulating MPO levels have been found to be associated with the presence of DMN.

The DMN has been shown to elevate ROS in the liver[49] and the present data also demonstrated increasing LPO after DMN administration. Though, NO could be scavenged by radicals such as superoxide to produce peroxynitrite[50], this suggests that the DMN treatment alters steady-state NO levels. The NOS isoform involved in constitutive NO generation is endothelial NOS (eNOS), the hepatic levels of which have been shown to be altered in various liver disorders and hepatic ischemia[51].

Inhibitors of XO activity are used clinically for the treatment of hyperuricaemia and gouty arthritis; as they help in reducing the levels of uric acid in circulation and vascular oxidative stress[52]. In the present study, it is the first time to investigate the effect of DMN on OX activity where it is causing severs elevation.

This histological picture was related to the exhaustion of antioxidants and overproduction of oxidants and in the long term led to tissue damage in the liver mice that treated with DMN. The changes in the redox status of the cell may lead to apoptosis, cell proliferation or transformation as observed in histological evaluation. This may explain significantly increased leukocyte apoptosis following DMN administration in the present study. The histological evaluation showed that the hepatic lobular architecture was found to be distorted in the mice treated with DMN. Histopathological study of tissue damage DMN in the liver confirmed previous finding that, it causes inflammation of the liver capsule, necrosis and steatosis[49]. These changes are entirely consistent with the changes in various oxidative and antioxidant parameters that were also observed. Such liver damage may arise from the toxic effects of DMN, which disturbs the detoxification mechanisms of the liver.

When antioxidants react with ROS, the antioxidant is converted to a form that is no longer able to function and is said to be consumed. Therefore, this oxidized product needs

to be recycled to its native form to function again. There exists a network of antioxidants in which ALA can interact and replenish to maintain both lipid and aqueous phase antioxidant status. The ALA supplementation increases tissue ubiquinol content, in turn recycles vitamin E. The ALA treatment (10-100 mM) induced an increase in the cellular level of GSH by 30-70%[53].

Treatment DMN-mice with ALA exhibited improvement in liver functions compared to DMN-treated mice. Moreover, our results provide evidence that ALA inhibited NO overproduction and maintaining intracellular antioxidant status play a pivotal role in the treatment of DMN that induced alterations in the above parameters. Saad *et al.*[54] investigated the protective effect of ALA against isoniazid and rifampicin combination induced hepatotoxicity by elevation of serum hepatic enzymes. Co-administration of ALA significantly ameliorated isoniazid and rifampicin combination induced hepatic damage.

In present study, ALA inhibited XO activity. This could be as allopurinol, it inhibited XO by binding at its purine binding site[55], thereby blocking the ultimate formation of uric acid. In addition to their ability to inhibit XO, phenolics are well-known for their antioxidant activities[56] and ability to inhibit Fe^{2+}-induced LPO[57].

CONCLUSION

The free radicals produced by DMN causing oxidative stress and play an active role in liver damage at biochemical and cellular levels. The study is the first time to demonstrate that ALA has an impressive hepatoprotective effect on liver injuries induced by DMN, which might be considered to be therapeutic effect in clinical situations. The ALA may be protective against DMN-induced oxidative injury by inhibiting neutrophil infiltration and subsequent activation of inflammatory mediators that induce LPO and NO. In addition, hepatic SOD, CAT, GPx, CAT and XO activities and MPO activity were found to be improved following ALA administration.

SIGNIFICANCE STATEMENTS

- The DMN elevated NO production, increasing XO and MPO activities
- The DMN increased the LPO along with reduction of antioxidant enzymes as SOD, CAT, GPx in liver
- Treatment DMN-mice with ALA exhibited improvement in liver functions

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