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# Effect of α-lipoic acid and N-acetylcysteine on liver oxidative stress, preneoplastic lesions induced by diethylnitrosamine plus high-fat diet

## α-Lipoik asit ve N-asetilsisteinin sıçan karaciğerinde dietilnitrozamin ve yüksek yağlı diyetin neden olduğu oksidatif stres ve preneoplastik lezyonlar üzerine etkisi

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Abstract

Aim: Oxidative stress and inflammation are important for development of nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC). High fat diet (HFD) acts as promoter and induces cancer formation by diethylnitrosamine (DEN)-initiated carcinogenesis. DEN+HFD experimental model may be suitable to investigate the relationship between diet, cirrhosis, and cancer.

Methods: Rats were injected with DEN (50 mg/kg/once a week; i.p.) for 4 weeks. After 15 days, rats received HFD with/without supplementations of α-lipoic acid (ALA; 2 g/kg chow), N-acetylcysteine (NAC; 1% w/v drinking water) and their combination for 12 weeks.

Results: DEN+HFD-treatment resulted in increase of serum hepatic damage markers, hepatic oxidative stress parameters (lipid/protein oxidation products) and fibrotic changes. However, no HCC nodule was detected. Hepatic GST-pi and Ki-67 expressions also increased. Accordingly, DEN+HFD-treatment resulted in precancerous lesions and high rate of proliferation in the liver. NAC supplementation decreased hepatic oxidative stress and formation of fibrotic and preneoplastic lesions of DEN+HFD-treated rats. However, ALA supplementation did not have a curative effect on these lesions. No synergistic effect was seen with co-administration of ALA and NAC.

Conclusion: According to present results NAC, acting as an antioxidant, has ameliorating effect on DEN+HFD-induced oxidative stress and the formation of preneoplastic lesions in liver.

Keywords: High fat diet, diethylnitrosamine, oxidative stress, α-lipoic acid, N-acetylcysteine, liver injury.

Öz

Amaç: Oksidatif stres ve inflamasyon steatohepatit (NASH), siroz ve hepatoselüler karsinom (HCC) gelişimi için önemlidir. Yüksek yağlı diyet (HFD) promotör görevi görür ve dietilnitrosamin (DEN) ile başlatılan karsinogenez modelinde kanser oluşumunu indükler. DEN + HFD uygulaması diyet, siroz ve kanser arasındaki ilişkiyi araştırmak için uygun bir deneysel model olabilir.

Yöntemler: Sıçanlara 4 hafta boyunca DEN (haftada bir kez 50 mg / kg / i.p.) enjekte edildi. 15 gün sonra, sıçanlara HFD tek başına veya α -lipoik asit (ALA; 2 g / kg yem), N-asetilsistein (NAC; içme suyunda % 1 w/v) takviyeleri 12 hafta süreyle verildi.

Bulgular DEN + HFD uygulaması serum hepatik hasar belirteçleri ve hepatik oksidatif stres parametrelerinin (lipit/protein oksidasyon ürünleri) armasına ve fibrotik değişikliklere neden oldu. Ancak, HCC nodülü tespit edilmedi. Hepatik GST-pi ve Ki-67 ekspresyonları de artmış olarak bulunduğundan dolayı, DEN + HFD uygulaması, prekanseröz lezyonlara ve karaciğerde yüksek proliferasyon oranlarına neden oldu. NAC takviyesi, DEN + HFD ile muamele edilen sıçanların karaciğerinde oksidatif stresi ve fibrotik ve preneoplastik lezyonların oluşumunu azalttı. Bununla birlikte, ALA takviyesinin bu lezyonlar üzerinde iyileştirici bir etkisi olmamıştır. ALA ve NAC'nin birlikte uygulanmasıyla hiçbir sinerjistik etki görülmedi.

Sonuç: Sonuçlarımıza göre, bir antioksidan olan NAC, karaciğerde DEN + HFD'nin neden olduğu artmış oksidatif stresi azaltmada ve preneoplastik lezyonların oluşumu üzerine iyileştirici etkiye sahiptir.

Anahtar Kelimeler: yüksek yağlı diyet, dietilnitrozamin, oksidatif stres, α-lipoik asid, N-asetilsistein, karaciğer hasarı

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) is frequent disorder linked with variety of conditions including simple steatosis (non-alcoholic steatohepatitis, NASH), fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. Several hypotheses have recommended to elucidate the pathogenesis of advancement of steatosis to serious liver pathologies. The ''two hit'' hypothesis is the most common one. According to this, the ''first hit'' is simple steatosis (initiation phase) which sensitize liver to secondary injuries to promote its advancement to NASH. Oxidative stress, following lipid peroxidation and varios cytokines are recommended to be the ''second hit'' and conduce to the progress of NASH to several serious liver pathologies [1, 2].

Diethylnitrosamine (DEN) is an environmental hepatocarcinogen and is frequently used to produce experimental liver cancer [3]. In fequently applied models, initiation and promotion phases are essential in HCC generation. DEN initiation is mostly followed by phenobarbital, carbon tetrachloride ($CCl_4$), 2-acetylaminofluorine and partial hepatectomy promotion [3,4]. High fat diet (HFD) may also have a promoter effect and induce cancer formation by DEN-initiated carcinogenesis in rats [5]. HFD is an animal model of NAFLD/NASH [6] and causes steatosis, changes in prooxidant-antioxidant balance and inflammation in the liver [7, 8]. Indeed, HFD-induced NASH was found to promote DEN-initiated hepatocarcinogenesis by increasing oxidative stress and inflammation [9-11]. So that, this animal model was used to investigate the pathophysiological mechanisms in NASH-promoted liver cirrhosis and carcinogenesis and to test reducing or preventing potential of some antioxidants against these changes in the liver [9-11].

α-Lipoic acid (ALA) and N-acetylcysteine (NAC) are sulfur-containing antioxidants. As an antioxidant ALA is competent to scavenge reactive oxygen species (ROS), regenerate various antioxidants such as glutathione (GSH), and vitamins E, C, and possess metal chelating activity [12]. Additionally, ALA treatment was noticed to be protective in oxidative stress-induced pathologies including liver damage [12-14]. On the other hand, ALA was found to suppress tumor growth in mice treated with Ehrlich carcinoma cells and improve prooxidant-antioxidant balance in the liver [15]. It has also been reported that ALA shows an anti-carcinogenic effect by suppressing HCC that develops because of DEN and thioacetamide administration [16]. Contrarily, ALA administration was detected to aggravate liver damage and stimulate the development of preneoplastic lesions in DEN-initiated and choline-methionine-deficient (MCD) diet promoted HCC model [17].

Like ALA, NAC is an antioxidant compound with free radical scavenging properties and activating enzymes responsible for the regeneration of GSH, leads to increased intracellular GSH levels [18]. NAC was found to be an alleviating compound in oxidative stress-induced conditions including liver disorders [19, 20]. In addition, there are a few studies demonstrating the antineoplastic effect of NAC [21-23].

In the light of this information, we wanted to investigate whether the supplementation of ALA and NAC and their combination have a protective role against the development of liver lesions in DEN plus HFD-treated rats.

## Material and methods

DEN, ALA, NAC used chemicals were purchased from Sigma-Aldrich (USA).

**Experimental design**

Approval for the experimental procedures used in this study was obtained from Bezmialem Vakif University Animal Experiments Local Ethics Committee (No: 2014/170). Male Sprague Dawley rats weighing 200-220 g were haused in stainless cages at 22°C on daily 12/12-hour light/darkness cycles and supplied with food (standard diet) and water ad libitum. Standard and HFD were obtained from Barbaros Denizeri A.Ş. (Kocaeli-Turkey). HFD included 34.3% fat (31% bovine oil, 3.4% corn oil), 27.3% carbohydrate, 23.5% protein, salt mixture and vitamins.

Rats were fed with standard diet and injected with DEN (50 mg/kg, once a week for a total of 4 doses) intraperitoneally. 15 days after the fourth injection of DEN, rats were divided into four groups as follows and started to receive HFD with and without antioxidants for 12 weeks. In addition, a control group was performed as the fifth group. No food and water restriction were performed in experimental period and total food and water intake were recorded.

1) DEN+HFD group (n=7): Rats were fed with HFD for 12 weeks. This group was used as model group.
2) DEN+HFD+ALA group (n=7): Rats were fed with HFD containing ALA (2 g/kg) for 12 weeks. The consumption of ALA was roughly equivalent to 100 mg/kg/day.
3) DEN+HFD+NAC group (n=6): Rats were fed with HFD and received NAC (1%; w/v) in drinking water for 12 weeks. The consumption of NAC was rougly equivalent to 500 mg/kg/day.
4) DEN+HFD+ALA+NAC group (n =7): Rats were fed with HFD containing ALA (2 g/kg) and received NAC (1%; w/v) in drinking water for 12 weeks.
5) Control group (n=6): Rats were injected with % 0.9 NaCl (once a week for a total of 4 doses) and fed on normal commercial chow during experimental period.

In our study, HFD composition [24] and the used doses of DEN [25], ALA [13,14] and NAC [19,20] were selected with respect to previous studies.

**Blood and tissue samples**

Following an overnight fast, rats were sacrificed by cardiac puncture taking their blood under sodium pentobarbital (50 mg/ kg, i.p.) anesthesia into dry tubes. Blood samples were centrifuged at 1500 x g for 10 min to obtain serum samples. Liver tissues were removed, washed in ice-cold saline and homogenized in 0.15 M KCl. A portion of homogenates was centrifuged (600xg/10 min/4°C) to remove unhomogenized tissue residues and nuclear portion. Postnuclear supernatants (PNS) were used for biochemical analyses. From another portion of supernatants (centrifuged at 10000g/20 min) postmitochondrial fractions (PMF) were obtained in which superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were measured. The materials were stored at -80 °C until analyses.

**Determinations in serum**

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH)

activities and glucose, cholesterol, triglyceride and albumin levels were measured with autoanalyzer (Cobas Integra 800, Roche Diagnostics, Mannheim, Germany).

**Determinations in the liver**

*Reactive oxygen species (ROS) levels (relative fluorescence units, RFU)*

Modified fluorometric assay was used to test ROS generation [26]. After incubation of PNS samples with 2',7'-dichlorodihydrofluorescein diacetate (37°C/30 min) the fluorescence of the formed profuct was assayed by fluorometer (Fluoroskan Ascent FL, Thermo Scientific Inc, USA) ($\lambda_{excitation}$: 485 nm and $\lambda_{emission}$: 538 nm).

*Malondialdehyde (MDA) levels (pmol/mg protein)*

Lipid peroxidation in PNS was determined by measuring the levels of MDA [27]. The formed product 1,1,3,3-tetraethoxypropane was used as a standard.

*Advanced oxidation protein products (AOPP) levels (nmol chloramine-T /mg protein)*

Measurements of AOPP were detected spectophotometrically and calibrated with chloramine-T [28].

*Advanced glycation end products (AGEs, relative fluorescence units, RFU)*

Serum samples were diluted (1:50) with phosphate-buffered saline (PBS, pH 7.4), and fluorescence intensity was measured ($\lambda_{emission}$: 440 nm; $\lambda_{excitation}$: 350 nm) [29].

*Ferric reducing antioxidant power (FRAP, nmol/mg protein)*

FRAP assay [30] was used to determine the antioxidant power in PNS samples. At low pH, the formation of ferrous-tripyridyltriazine complex was monitored at 593 nm.

*Glutathione (GSH) levels (nmol/mg protein)*

GSH levels were detected spectophotometrically with 5,5'-dithiobis-2-nitrobenzoate (DTNB) at 412 nm [31].

*Superoxide dismutase (SOD) activity (U/mg protein)*

The SOD activity was determined by its ability to increase the riboflavin-sensitized photooxidation of o-dianisidine [32].

*Catalase (CAT) activity (μmol/min/mg protein)*

CAT activity was determined spectrophotometrically at 240 nm using hydrogen peroxide ($H_2O_2$) as substrate [33]. One unit of CAT was considered the activity of enzyme needed to degrade 1 μmol $H_2O_2$ per min at 25 °C.

*Glutathione peroxidase (GSH-Px) activity (nmol/min/mg protein)*

GSH-Px activity was assessed using cumene hydroperoxide as a substrate [34]. Extinction coefficient of NADPH ($6.22x10^3\ M^{-1}cm^{-1}$) was used for calculaton of results.

*Glutathione transferase (GST) activity (nmol/min/mg protein)*

GST activity was tested using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [35].

Extinction coefficient ($9600\ M^{-1}cm^{-1}$) of the formed conjugation product (CDNB+GSH) was used for calculaton of results.

*Protein levels*

Protein levels were detected by bicinchoninic acid [36].

**Immunohistochemical analysis for GST-Pi and Ki-67**

GST-Pi and Ki-67 expressions were analyzed as markers of preneoplastic lesions and proliferation, respectively. All specimens were prefixed in 10 % buffered formalin.

Slides were rehydrated by graded ethyl alcohol, immersed in citrate buffer (Citrate Buffer, Thermo Scientific, Germany) and put in a microwave oven (20 min). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Primary antibodies of GST-Pi (#311-H; Anti-GST-P rabbit polyclonal antibody, MBL, Nagoya, Japan) and Ki-67 (#PRM-325AA, rat monoclonal antibody, Biocare, USA) were done and incubated for 60 min. Then, biotinylated second antibody (goat anti-rabbit IgG; Santa Cruz Biotechnology, Heidelberg, Germany), streptavidin peroxidase, and substrate-chromogen (AEC) solution were done, respectively. Hematoxylin was used for nuclear staining. Staining intensity for GST-Pi was assigned as a percentage and given a score in the range from (+) to (+++): 5–30% (+), 30–60% (++), and >60% (+++). Nuclear brown staining was considered as positive for Ki-67 and staining intensity was defined as a percentage. Negative and positive cells numbers were counted on Leica DM 6000 Digital microscope. For each sample, "percent expression" was calculated formula: positive cells/ total number of counted cells x100.

**Histopathological examination**

Liver tissues were prefixed in 10% buffered formalin intombed in paraffin, splited, and stained with hematoxylin and eosin (H&E). Fibrosis was evaluated by using H&E staining considering Ishak's stage [37].

**Statistical analysis**

The results were expressed as mean ± SD. One-way analysis of variance (ANOVA) with Tukey's honestly significant differece post-hoc test, Kruskal–Wallis test with post-hoc Mann–Whitney U test were used. Difference was considered significant when $p < 0.05$.

## Results

*Effects of ALA and NAC and their combination (ALA+NAC) on body and liver weights and liver index in DEN+HFD-treated rats*

Body and liver weights and liver index at the end of experiment remained unchanged in DEN+HFD-treated rats compared to control values. Similarly, NAC and ALA+NAC treatments did not affect these parameters in DEN+HFD rats. Only, decreased final body weight and increased liver index were detected in DEN+HFD-treated rats due to ALA supplementation (Table 1).

*Effects of ALA and NAC and their combination (ALA+NAC) on biochemical parameters in serum of DEN+HFD-treated rats*

Serum glucose, triglyceride and albumin levels did not alter in DEN+HFD group as compared to controls, but serum cholesterol levels increased (Table 1).

Serum ALT, AST and LDH activities increased significantly in DEN+HFD group as compared to controls. There were no changes in serum ALT and AST activities due to ALA, NAC and ALA+NAC supplementations in DEN+HFD-rats. However, these supplementations were detected to significantly decrease serum LDH activities in DEN+HFD-rats (Figure 1).

*Effects of ALA, NAC and their combination (ALA+NAC) on hepatic histopathology of DEN+HFD-treated rats*

Extensive nodular disarrangement surrounded with thin fibrotic tissue was observed in DEN+HFD group. Few inflammatory cell infiltrations were also seen in some areas (Figure 2A). ALA supplementation caused decreases in nodular structures as compared to DEN+HFD group. These structures showed the tendency to interconnect with each other and there

were thin fibrous bands around them (Figure 2B). It was seen that the number of nodular structures in the liver decreased with NAC application and they occupied less area. Fibrous bands and inflammatory response were not observed (Figure 2C). ALA+NAC administration showed similar changes to NAC administration in the liver, but nodules were higher than the NAC

Table 1. The effect of α-lipoic acid (ALA), N-acetylcysteine (NAC) and their combination (ALA+NAC) on final body weight, liver weight, liver index as well as some biochemical parameters in serum of diethylnitrosamine plus high fat diet (DEN+HFD)-treated rats (Mean±SD).

| Variable | Control (n=6) | DEN+HFD (n=7) | DEN+HFD +ALA (n=7) | DEN+HFD +NAC (n=6) | DEN+HFD +ALA+NAC (n=7) |
|---|---|---|---|---|---|
| Final body weight (g) | 310.8±24.4 | 262.8±60.0 | 247.1±34.3 [a] | 286.3±35.2 | 283.7±21.5 |
| Liver weight (g) | 7.40±0.83 | 6.41±1.15 | 7.40±1.40 | 7.08±0.65 | 7.95±0.92 |
| Liver index* (%) | 2.37±0.14 | 2.48±0.33 | 2.97±0.28 [a,b] | 2.48±0.22 | 2.79±0.16 |
| Glucose (mmol/L) | 8.83±1.66 | 11.1±2.36 | 9.71±1.32 | 11.3±1.38[a] | 10.5±1.98 |
| Cholesterol (mmol/L) | 2.45±0.37 | 3.31±0.39[a] | 2.93±0.55 | 2.87±0.21[a] | 3.22±0.45[a] |
| Triglyceride (mmol/L) | 0.72±0.11 | 0.89±0.27 | 0.77±0.33 | 0.99±0.36 | 0.98±0.30 |
| Albumin (g/dL) | 3.96±0.20 | 3.74±0.63 | 3.85±0.36 | 3.77±0.13 | 3.94±0.21 |

[a] $p < 0.05$ as compared to controls; [b] $p<0.05$ as compared to DEN+HFD.
*Liver index= Liver weight x 100 / body weight.

group and few fibrous bands and inflammatory changes were observed (Figure 2D).

*Effects of ALA, NAC and their combination (ALA+NAC) on hepatic GST-pi expression of DEN+HFD-treated rats*

GST-Pi expression was found to be (+++) in DEN+HFD group. This expression did not alter due to ALA supplementation. However, NAC and NAC+ALA supplementations caused (+) and (++) staining, respectively, in DEN+HFD-rats (Figure 3 A-D).

*Effects of ALA and NAC and their combination (ALA+NAC) on hepatic Ki-67 expression in DEN+HFD-treated rats*

Ki-67 expression in DEN + HFD group was 4%. This expression was determined as 4%, 2% and 2-3%, respectively, due to ALA, NAC and ALA + NAC supplementations in DEN+HFD rats (Figure 4 A-D).

*Effects of ALA and NAC and their combination (ALA+NAC) on hepatic oxidative stress parameters in DEN+HFD-treated rats*

Hepatic MDA and AOPP (27.3% and 43.7%, respectively) levels increased significantly in DEN+HFD rats as compared to controls. ROS (21.2%) and AGE (17.7%) levels were also increased, but these increases were not statistically significant. ALA, NAC and ALA+NAC supplementations decreased high levels of MDA and AOPP in DEN+HFD group. However, these supplementations did not alter ROS and AGE levels in the liver of DEN+HFD rats (Figure 5).

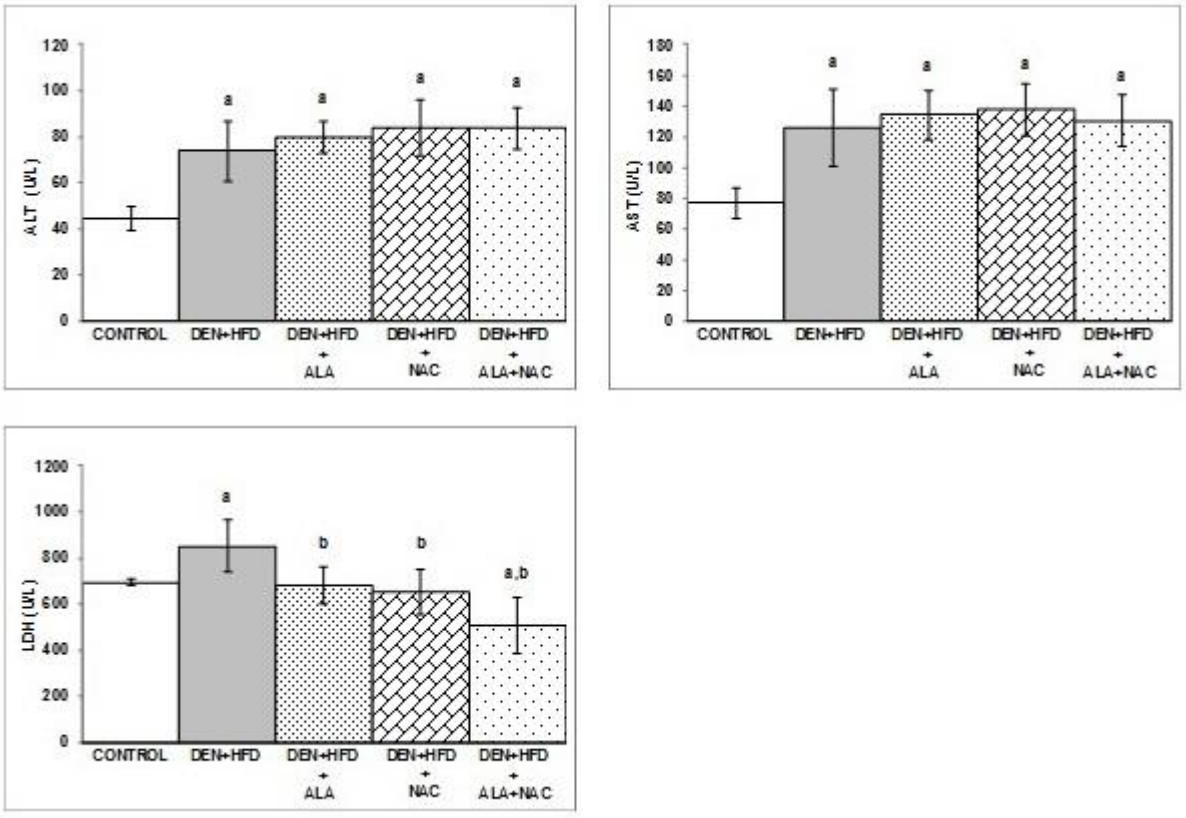



Figure 1. The effect of α-lipoic acid (ALA), N-acetylcysteine (NAC) and their combination (ALA+NAC) on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities of diethylnitrosamine plus high fat diet (DEN+HFD)-treated rats (Mean±SD). [a] $p < 0.05$ as compared to controls. [b] $p<0.05$ as compared to DEN+HFD.

Table 2. The effect of α-lipoic acid (ALA), N-acetylcysteine (NAC) and their combination (ALA+NAC) on ferric reducing antioxidant power (FRAP) and glutathione (GSH) levels, and superoxide dismutase (SOD), catalase (CA), glutathione peroxidase (GSH-Px) and glutathione transferase (GST) activities in the liver of diethylnitrosamine plus high fat diet (DEN+HFD)-treated rats (Mean±SD).

| Variable | Control (n=6) | DEN+HFD (n=7) | DEN+HFD +ALA (n= 7) | DEN+HFD +NAC (n=6) | DEN+HFD+ALA +NAC (n=7) |
|---|---|---|---|---|---|
| FRAP (nmol/mg protein) | 54.0±14.1 | 52.2±6.72 | 47.9±7.34 | 48.2±5.86 | 45.6±3.37 |
| GSH (nmol/mg protein) | 27.1±3.57 | 38.2±9.68 | 48.0±13.0[a] | 35.1±9.50 | 38.1±8.13 |
| SOD (U/mg protein) | 13.6±2.05 | 17.9±4.35 | 15.1±1.53 | 11.9±2.2[b] | 11.4±1.75[b] |
| CAT (µmol/min/mg protein) | 277.5±48.1 | 256.5±100.1 | 330.4±47.2 | 301.2±72.4 | 321.5±59.6 |
| GSH-Px (nmol/min/ mg/protein) | 564.1±87.0 | 665.2±172.5 | 816.3±94.1[a,b] | 719.3±77.4[a] | 671.0±75.3[a] |
| GST (nmol/min/ mg/protein) | 531.9±121.0 | 734.1±147.9[a] | 756.1±74.8[a] | 617.0±68.0 | 554.2±86.6[b] |

[a] $p< 0.05$ as compared to controls.
[b] $p<0.05$ as compared to DEN+HFD.

Hepatic GSH (31.3%) levels, and SOD (22.6%) and GSH-Px (17.9%) activities were found to increase in DEN+HFD rats in comparation with controls, but these increases were not significantly. FRAP levels and CAT activities remained unchanged, but GST activity (38.0%) increased significantly in DEN+HFD group. Although ALA treatment did not alter antioxidant parameters in DEN+HFD group, only GSH-Px (22.7%) activity increased significantly. Similarly, no changes in antioxidant parameters were observed, excluding SOD activity, in DEN+HFD rats due to NAC supplementation. ALA+NAC supplementation significantly decreased SOD (36.3%) and GST (24.5%) activities in DEN+HFD rats (Table 2).

## Discussion

NASH is the progressive form of NAFLD and features of NASH on liver biopsy include steatosis, inflammation and varying degrees of fibrosis. Persistent fibrosis may lead to cirrhosis and HCC. Although fibrosis is reversible, advanced stages of cirrhosis are very difficult to reverse [1, 2, 6]. The molecular basis of HCC formation in fibrotic/cirrhotic livers is not elucidated yet. Oxidative stress and inflammation has important roles in pathogenesis of NASH [1, 2]. Since dietary animal models of NAFLD such as HFD or MCD-diet require a relatively long period to produce cirrhosis/ HCC [6], new models are needed to investigate the development of NASH-induced cirrhosis/ HCC.

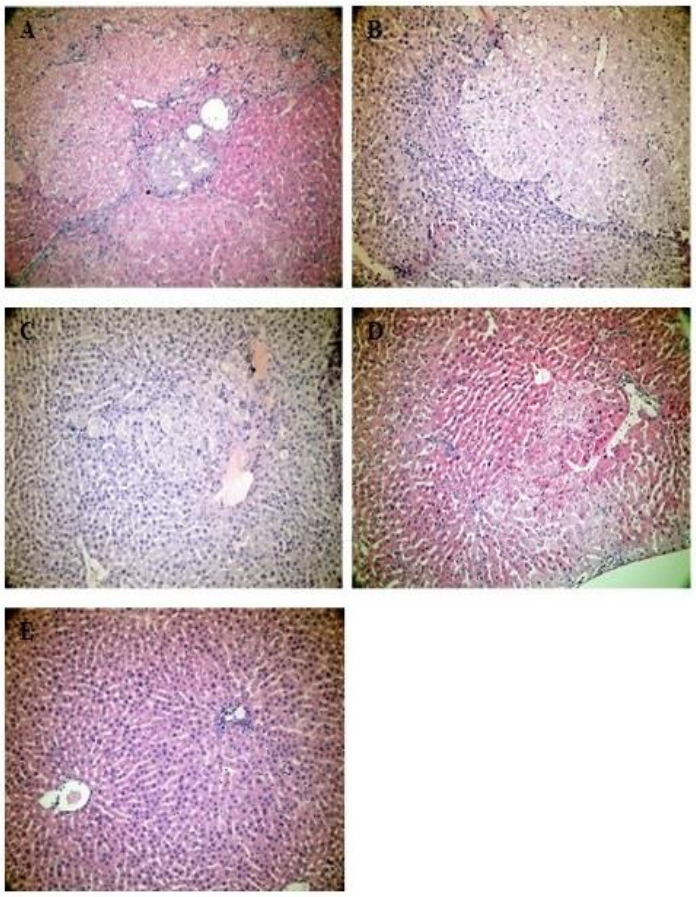



Figure 2. Histopathological appearance of liver in groups (H&Ex200). (A) Diethylnitrosamine plus high fat diet (DEN+HFD)-treated group. (B) α-lipoic acid (ALA)- supplemented DEN+HFD-treated rats. (C) N-Acetylcysteine (NAC)-supplemented DEN+HFD-treated rats. (D) ALA+NAC-supplemented DEN+HFD-treated rats. (E) Control group.

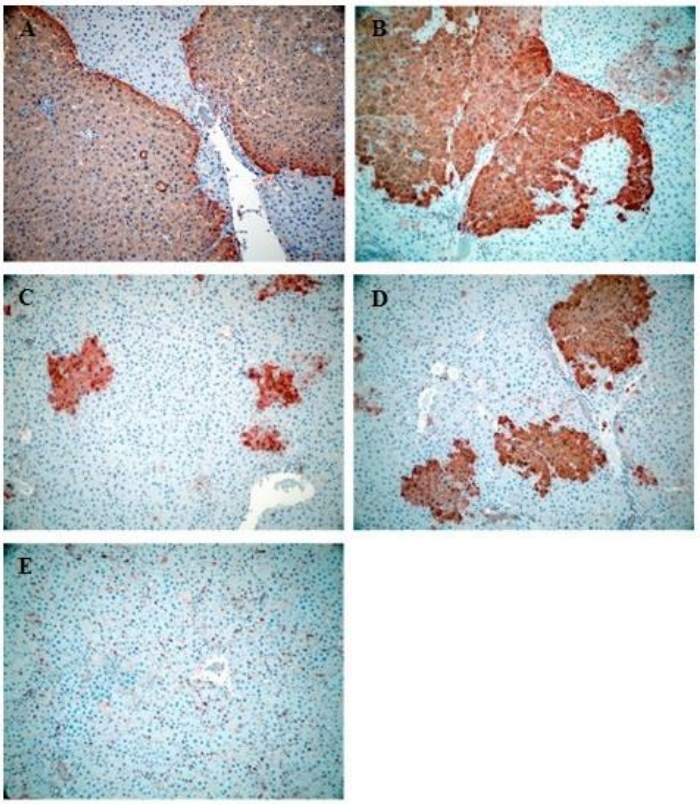



Figure 3. Immunohistochemical detection of hepatic glutathione transferase-Pi (GST-Pi) expression in groups (x200). (A) Diethylnitrosamine plus high fat diet (DEN+HFD)-treated group. (B) α-lipoic acid (ALA)- supplemented DEN+HFD-treated rats. (C) N-Acetylcysteine (NAC)-supplemented DEN+HFD-treated rats. (D) ALA+NAC-supplemented DEN+HFD-treated rats. (E) Control group.

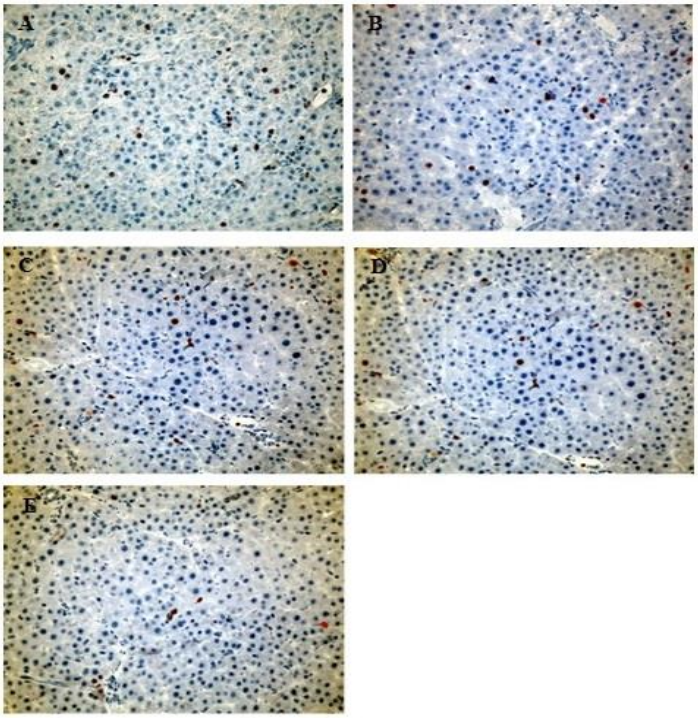



Figure 4. Immunohistochemical detection of hepatic Ki-67 expression in groups (x400). (A) Diethylnitrosamine plus high fat diet (DEN+HFD)-treated group. (B) α-lipoic acid (ALA)- supplemented DEN+HFD-treated rats. (C) N-Acetylcysteine (NAC)-supplemented DEN+HFD-treated rats. (D) ALA+NAC-supplemented DEN+HFD-treated rats. (E) Control group.

DEN is important hepatotoxin and hepatocarcinogen. DEN leads to hepatocellular necrosis and increased cell proliferation [3, 4, 38]. DEN-induced hepatic injury was observed to be related to increased ROS generation. DEN following ROS generation is related to biotransformation in cytochrome P450 system (CYPs), especially CYP2E1 [3, 39]. Meanwhyle, DEN changes the DNA structure and forms alkyl DNA adducts in the rat liver [3, 4, 38].

DEN has been used to initiate the liver cancer either alone or in combination with promoters. The initiation can be brought either by administering a single necrogenic dose of DEN or by administering a lower dose of DEN in combination with promoters [3, 4, 38]. On the other hand, previous studies in experimental animals have shown that repeated subnecrogenic doses of DEN without promoters cause progressive liver fibrosis and cirrhosis followed by HCC [40, 41].

HFD has been reported to have a promoter effect on DEN-induced hepatocarcinogenesis [5]. Bioactivation of DEN is controlled by CYP2E1. Increase in CYP2E1 activity is a probable risk factor in the progression of hepatofibrosis to hepatocarcinogenesis [39]. Since HFD elevates hepatic CYP2E1 activity [42], it may increase NAFLD progression and carcinogenesis [39]. According to this, HFD combined DEN exposure may be a suitable model to investigate the relationship between diet and liver cirrhosis and cancer formation.

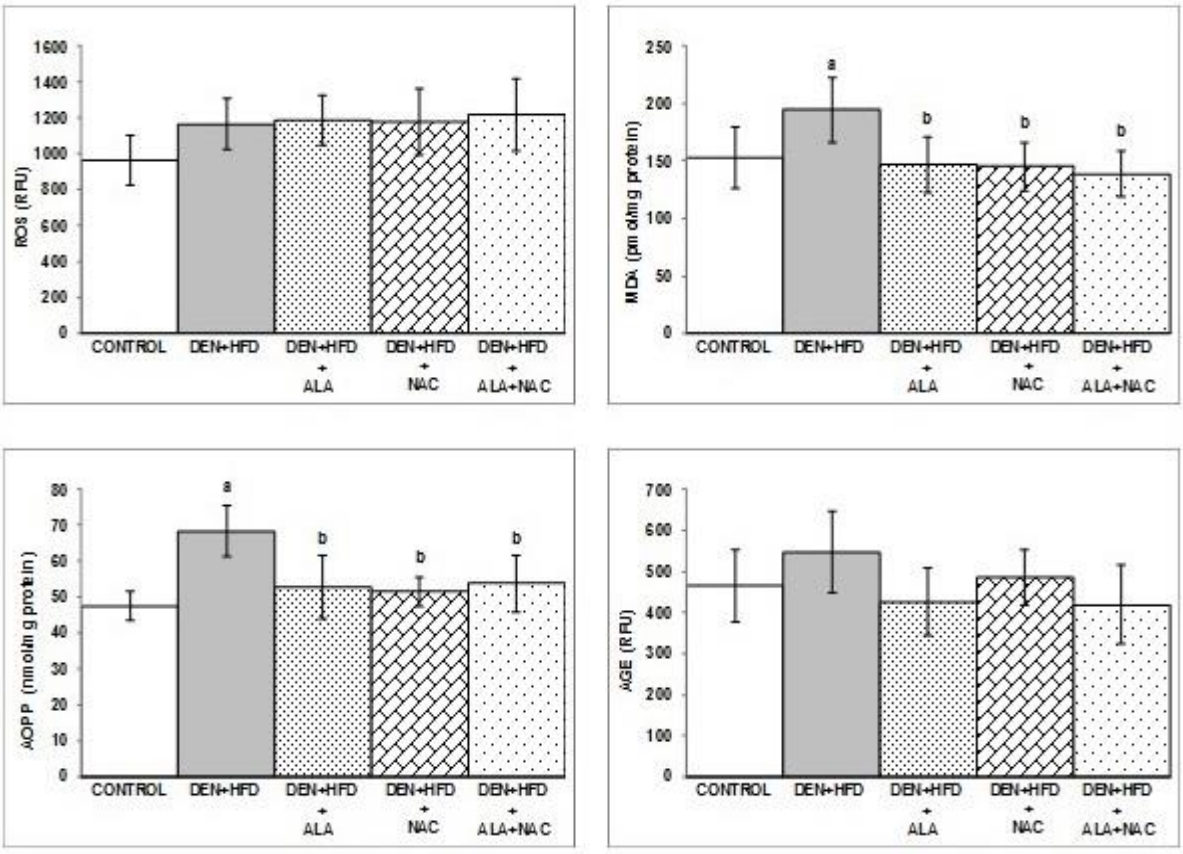



Figure 5. The effect of α-lipoic acid (ALA), N-acetylcysteine (NAC) and their combination (ALA+NAC) on reactive oxygen species (ROS) formation, malondialdehyde (MDA), advanced oxidation protein products (AOPP) and advanced glycation end products (AGE) levels in the liver of diethylnitrosamine plus high fat diet (DEN+HFD)-treated rats (Mean±SD). [a] $p< 0.05$ as compared to controls. [b] $p<0.05$ as compared to DEN+HFD.

In studies using DEN+HFD model, the subnecrogenic dose of DEN was followed by HFD application with different composition and duration. However, liver damage resulting in fibrosis and finaly to HCC was found to be different depending on some factors such as DEN dosage, examination time, HFD composition and application period [8-10]. In the present study, rats were injected with DEN (50 mg/kg; i.p.) 4 times with an interval of one week, and then fed a HFD for 12 weeks. This DEN+HFD protocol elevated serum ALT, AST and LDH activities and caused fibrotic changes in the liver. Serum aminotransferases such as ALT and AST are sensitive markers of hepatocellular injury. Increases in ALT and AST activities are also well described in hepatic fibrosis progression. However, no HCC nodule was detected macroscopically and histopathologically. Moreover, hepatic GST-pi and Ki-67 expressions were observed to increase immunohistochemically as compared to controls. GST-pi is used as a marker of preneoplastic lesions [5]. However, Ki-67 is an indicator of cell proliferation [11]. Accordingly, our findings indicate that DEN+HFD treatment caused mild fibrosis together with precancerous lesions and a high rate of proliferation in hepatocytes of rats.

Oxidative stress is very important in development and progression of liver cirrhosis and HCC [5, 21, 23, 40]. In the present study, DEN+HFD treatment increased MDA and AOPP levels. No significant changes in liver antioxidant parameters were found, but there was a tendency towards increases in GSH levels and SOD, GSH-Px activities of DEN+HFD rats. This situation may prevent further increases in prooxidant mileu in the liver. Our results agree with previous studies showing that DEN+HFD treatment produces a prooxidant state in the liver of rats [5, 11].

In our study, we aimed to investigate the effects of ALA and NAC and their combination on DEN-initiated HFD-promoted hepatic lesions. ALA is an antioxidant with protective effects against various hepatic injuries [13, 14]. ALA supplementation was reported to reduce lipid accumulation, inflammation, and oxidative stress in the liver of MCD- and HFD-fed mice [13]. Moreover, ALA administration alleviated $CCl_4$-induced liver cirrhosis in rats [14]. Although ALA administration was reported to have an anti-carcinogenic effect in DEN-initiated and thioacetamide-promoted HCC [16], it showed a pro-carcinogenic effect in DEN-initiated MCD-promoted HCC [17]. However, in the present study, ALA supplementation decreased nodular structures in the liver, but this treatment did not affect the formation of preneoplastic lesions in DEN+HFD rats.

NAC is also an antioxidant and antiinflammatory compound [18]. There are some studies showing that NAC attenuates oxidative stress and liver pathology in rats with NASH promoted by HFD [19, 20]. NAC treatment was also reported to supress hepatic GST-pi expression and oxidative stress in DEN-initiated and indole-3-carbinol-promoted hepatocarcinogenesis model [21]. Moreover, it was found that NAC supplementation improved DEN-induced HCC in toll-like receptor 2 [TLR2]-deficient mice by suppressing oxidative/endoplasmic stress [22]. In addition, NAC treatment improved liver function, hepatic ROS levels and DNA damage in mice with DEN-initiated and high cholesterol-promoted hepatocarcinogenesis [23]. In the current study, according to histopathological observations, decreases in number of nodular structures was detected in the liver due to NAC supplementation in DEN+HFD-treated rats. However, fibrous bands and inflammatory response were not seen. Moreover, NAC supplementation decreased GST-pi and Ki-67 expresions were in DEN+HFD treated rats. In contrast, the results of liver histology and GST-pi and Ki-67 expressions in ALA+NAC-treated group were worse than NAC- and better than ALA-supplemented rats.

On the other hand, there were no changes in serum ALT and AST activities in DEN+HFD-treated rats due to ALA, NAC and ALA+NAC supplementations. However, these antioxidants significantly decreased serum LDH activity in DEN+HFD-treated rats. In addition, the effects of these antioxidants on hepatic prooxidant-antioxidant balance were observed to be similar and decrease prooxidant status in the liver.

In conclusion, NAC treatment decreased hepatic oxidative stress and formation of fibrosis and preneoplastic lesions in DEN+HFD-treated rats. However, ALA supplementation was found not to have a curative effect on these lesions and the co-administration of NAC and ALA does not produce a synergistic effect in the liver of DEN+HFD-treated rats.

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