The effects of alpha lipoic acid on liver cells damages and apoptosis induced by polyunsaturated fatty acids

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We studied the effect of alpha-lipoic acid (ALA) on the liver cell damages and apoptosis by n-6 polyunsaturated fatty acids (PUFA) rich diet in young rats. 24 Wistar rats were divided into four groups. During the study, 12 of them (control) were fed with standard chow and other 12 (n-6) were fed with the food containing high-fat n-6 for 8 weeks. At the end of the fourth week, control and n-6 groups were randomly divided into two groups and then, 4 weeks, 35 mg/kg ALA are injected. Groups; control, control + ALA, n-6, n-6 + ALA. The liver tissue glutathione (GSH) activity was determined. Immunohistochemistry for caspase-3 and TUNEL method for apoptosis were performed. The GSH levels have significantly decreased ($p<0.001$) and vacuolization in the hepatocytes, infiltration and the collagen accumulation around the central vein, hepatic stellate cells in the sinusoids have increased in n-6 group compared with the other groups. TUNEL ($p<0.001$) and caspase-3 ($p<0.001$) positive cells increased in n-6 group whereas all degenerative observations decreased in n-6 + ALA group. Our results demonstrate that the feeding with n-6 PUFA causes fatty liver, fibrosis development, inflammations and apoptosis in the liver of young rats. ALA has a beneficial effects on these degenerative effects.

1. Introduction

In the world, there is an increasing prevalance of several chronic diseases such as diabetes mellitus, obesity, metabolic syndrome and non-alcoholic fatty liver disease (NAFLD) by high fatty diet and sedentary behavior in children.

Nutrition is an important factor in the pathogenesis of several liver diseases (Smith, 1993). The liver has a central role in lipid metabolism with its lipolysis, lipogenesis and fat storage feature (Adams et al., 2005). Increased intake of polyunsaturated fatty acids (PUFAs) in the diet induce fatty acid oxidation in the liver and increase lipid peroxidation and cause severe hepatic injury (Smith and Wills, 1981; Rukkumani et al., 2004). Imbalances in hepatic lipid metabolism causing accumulation of hepatic triglycerides, insulin resistance, inflammation, and apoptosis, are closely related to diseases of energy imbalance, such as obesity, diabetes, hyperlipidemia, and atherosclerosis (Yeh and Brunt, 2007; Adiels et al., 2008). n-6 Fatty acids are essential for growth and development. It has been suggested that high dietary n-6 PUFA and ratio of n-6/n-3 PUFA play a role in the high rates of chronic diseases. Numerous epidemiological and clinical studies suggest a relationship between excessive ratio of n-6/n-3 PUFA play and risk of developing several diseases such as metabolic syndrome, diabetes mellitus and NAFLD (Simopoulos, 2002; Gómez-Candela et al., 2011).

The increased fatty acid oxidation and oxidative stress, alteration of cellular membrane fatty acid, cholesterol content, phospholipid composition and ceramide signaling, and direct free fatty acid toxicity are possible mechanisms of liver injury (Malhi and Gores, 2008). Recent findings show that hepatocellular injury in some liver diseases is accompanied by activation of the apoptotic pathways (Bonni et al., 1999; Malhi and Gores, 2008).

Alpha-lipoic acid (ALA) (1,2-dithiolane-3-pentanoic acid) is a powerful antioxidant. It is synthesized by plants and animals. It is taken by cells and then reduced to dihydrolipoate. ALA is primarily metabolized in liver through mitochondrial oxidation. ALA works as a cofactor of key mitochondrial enzymes and thus controls the glucose oxidation. Liver is the main detoxifying organ for many toxic substances and drugs that contribute to oxidative stress (Görça et al., 2011; Stevens et al., 2000). The increased

Abbreviations: ALA, alpha lipoic acid; EM, electron microscopy; ECGs, extracellular matrix materials; GSH, glutathione; HSC, hepatic stellate cells; HSCORE, histological score; LM, light microscopy; MMP, matrix metalloproteinase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species.

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reactive oxygen species (ROS) production makes mitochondrial membranes highly susceptible to oxidative damage. ROS plays a critical role in the development of endothelial injury and hepatic fibrosis (Gorça et al., 2011). ALA may be effective in preventing the development of hepatic steatosis and hepatic fibrosis (Min et al., 2010; Park et al., 2008). It is also used for the treatment of diabetes-related neuropathy and liver diseases (Bustamante et al., 1998; Stevens et al., 2000; Gorça et al., 2011).

In this study, the effect of ALA treatment on the changes in the liver tissue caused by n6 group multi-fatty acid rich diet were examined both biochemically and morphologically.

2. Material and methods

2.1. Research design

The experimental protocol was approved by the Committee for the Ethics on Animal Care and Experiments, Istanbul University, Cerrahpasa Medical Faculty. Five weeks old 24 Wistar albino rats which are equal to human’s 8 years old (Quinn, 2000), were used and divided into four groups. During the study, 12 of them were fed with standard chow (control) and other 12 were fed with the food containing high-fat n-6 (n-6) for 8 weeks. The standard chow, contained as a percentage of calories, 65% carbohydrate, 12% fat and 23% protein. The high-fat diet rich in n-6 diet contained 60% fat from safflower oil, 20% kcal carbohydrate and 20% kcal protein. Fatty acid composition of the diets are shown in Table 1. At the end of the fourth week, control and n-6 groups were randomly divided into two groups and after 4 weeks 35 mg/kg DL-alpha lipoic acid (ALA, Sigma–Aldrich, St. Louis, MO, USA) was intraperitoneally injected. ALA dissolved in 1 N NaOH and given neutrality with 1 N HCl was administered daily (Bitar et al., 2004). Groups were designed as Control, Control + ALA, n6, n6 + ALA. At the end of the experiment, the animals were sacrificed. The liver tissues were fixed in 10% neutral buffered formalin for light microscopy and 4% glutaraldehyde for electron microscopy. For biochemical analyses, livers were thawed until the day of the analysis.

2.2. Biochemical assay

In this study, biochemical investigations were made on the liver tissue from all groups. The liver tissue was stored until biochemical analyses. Livers were thawed until the day of the analysis.

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Table 1 Fatty acid composition of the diets (as percentage of total fatty acid).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>n-6</th>
</tr>
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<tbody>
<tr>
<td>14:00</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>16:00</td>
<td>12.1</td>
<td>19.6</td>
</tr>
<tr>
<td>18:00</td>
<td>12.8</td>
<td>13.1</td>
</tr>
<tr>
<td>Σ Saturated</td>
<td>25.5</td>
<td>23.3</td>
</tr>
<tr>
<td>16:1-9</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>18:1-9</td>
<td>30.2</td>
<td>21.9</td>
</tr>
<tr>
<td>Σ n-9</td>
<td>31.2</td>
<td>22.4</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>34.3</td>
<td>51.2</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>4.8</td>
<td>–</td>
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<tr>
<td>Σ n-6</td>
<td>39.1</td>
<td>51.2</td>
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<tr>
<td>18:3 n-3</td>
<td>1.5</td>
<td>2.9</td>
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<tr>
<td>18:4 n-3</td>
<td>–</td>
<td>2.9</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>1.6</td>
<td>–</td>
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<td>22:6 n-3</td>
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<td>0.2</td>
</tr>
<tr>
<td>Σ n-3</td>
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</tr>
<tr>
<td>n6/n3</td>
<td>9.3</td>
<td>15.4</td>
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2.3. Histological evaluation

For histological examination, the liver tissues were dissected, fixed in 10% neutral buffered formalin for light microscopy and 4% glutaraldehyde for electron microscopy. For biochemical analysis, liver tissue samples were washed with physiological saline and kept at ~70°C until the day of the analysis.

2.4. Immunohistochemistry

For the detection of caspase-3 protein, immunohistochemical staining was performed. Histostain Plus Bulk Kit (Zymed Lab., CA, USA) was used for immunoperoxidase staining. Immunohistochemistry procedure was performed by using a combination of microwave oven heating for antigen retrieval and standard streptavidin–biotin-peroxidase method. Endogenous peroxidase activity was blocked by 3% H2O2. Each sections were then incubated for 15 min at room temperature with blocking solution. Sections were incubated with caspase-3 (CPS2 Ab-4) (dilution 1:100; LabVision Corp., CA, USA) or active (cleaved) caspase-3 antibodies (dilution 1:50; Millipore, USA) for 1 h at room temperature, then washed with PBS. Specific staining was performed with biotinylated universal secondary antibody, horseradish peroxidase streptavidin-complex, and amino-ethyl-carbazole as chromogen and then the sections were counterstained Mayer’s hematoxylin. As for negative control, normal rabbit IgG (Santa Cruz Biotecnology, Santa Cruz CA, USA) was used. In addition, distilled water was performed instead of primary antibody.

The intensity of liver immunostaining for pro-caspase-3 was semiquantitatively evaluated by the following categories: 0 (no staining), 1+(weak, but detectable staining), 2+(moderate or distinct staining), and 3+(intense staining). For each slide, a histological score (HSCORE) value was derived by summing the percentage of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the formula HSCORE = Σ Pi * Hi, where Pi represents the intensity score, and Hi is the corresponding percentage of the cells.

2.5. In situ DNA end labeling method (TUNEL)

TUNEL method was performed to detect apoptotic cells. Detection of DNA fragmentation in situ was visualized with the use of the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA). Deparaffinized tissue sections were incubated with proteinase K (20 μg/ml) Sigma–Aldrich, St. Louis, MO, USA). Tissue sections were subjected to 3% H2O2 for endogenous peroxidase inhibition and were incubated with 1+ equilibration buffer at room temperature for 30 min. The digoxigenin-labeled dNTP tail was incubated with TdT (terminal deoxynucleotidyl transferase) for 1 h at 37°C, and sections were washed in stop/wash buffer for 10 min at room temperature. Tissue sections were incubated with anti-digoxigenin-peroxidase antibody at room temperature for 30 min and were stained with diaminobenzidine (DAB) as a peroxidase substrate. Staining was evaluated by using a light microscope after counterstaining with methyl green.

2.5.1. Staining specificity controls

Thymus tissue sections from dexamethasone-treated rats were used as positive control (Kaya-Dagistani et al., 2005). For negative controls, distilled water was used instead of TdT enzyme.

2.5.2. Apoptotic index

Morphometric analysis of the positive cells in tissue stained by TUNEL method was performed under high power magnification (400×) in a blinded fashion. On each slide of the 24 rats, 15 fields were randomly selected. Apoptotic index was calculated according to the formula AI = (AC/AC + IC) × 100 (Apoptotic index; AC, Apoptotic cell number; IC, Intact cell number; %) (Barut et al., 2005).

2.6. Electron microscopy

The samples were dissected to 1 mm2 and fixed by 4% glutaraldehyde which is prepared by Soranssen’s phosphate buffer pH: 7.4 for 1 h. Samples were washed with phosphate buffer for 1 h and applied after fixation by osmium tetroxide which is prepared by Milloning’s buffer. The fixed samples were dehydrated in ethanol, embedded in araldite. Semithin sections were cut into 1 μm thickness and stained with Hematoxylin–Eosin (H + E) and Sirius red for basic histological evaluation using standard protocols. H + E is a staining method commonly used for detecting the general tissue morphology. Sirius red staining was used to demonstrate collagen fiber architecture. Fibrosis and fatty degenerations were graded by two independent researchers. The section was scored as described by Martinez-Hernandez (1985) and Senoo and Wake (1985) as follows; 0: Intact liver; 1: cirrhotic necrosis and fatty degeneration; 2: cirrhotic and midlobular fatty degeneration, perivascular fibrosis; 3: septal fibrosis, pseudolobule formation; and 4: regenerative nodule formation, cirrhosis.
with 1% toluidine blue (prepared with 1% borax in bidistilled water) and ultra-thin sections were cut into 500–700 μm. The cross-sections were then taken on copper grid, stained with uranyl acetate and Reynolds’s lead citrate, then investigated with Jeol JEM 1011. On each semithin section slides of all areas, the lipid droplets filled hepatic stellate cells were counted under high power magnification (400×) at different times by two researchers in a blind fashion.

2.7. Statistical analysis

Data was expressed as mean ± SEM and statistical analysis of the results were carried out by one-way ANOVA with post hoc Tukey test performed using GraphPad InStat version 3.00 for Windows 7® (GraphPad Software Inc., San Diego California USA). p < 0.05 was considered as statistically significant.

3. Results

3.1. Biochemistry

The biochemical results of liver tissue are illustrated in Fig. 1.

The liver tissue glutathione (GSH) levels of all groups. *p < 0.001 compare to control group, #p < 0.05 compare to n-6 group.

3.2. Histological results

Control group had a normal architecture with hepatocytes radially arranged around to centrilobular veins (Fig. 2A). In the n-6 treatment group, there were detectable cell infiltrations around central veins and the portal areas (Fig. 2B). There were severe vacuolization in the hepatocytes and fatty degeneration areas centrilobular liver (Fig. 2C, D and E). In n-6 + ALA treatment group, fatty degenerations, inflammation and cell vacuolization were decreased in the hepatocytes (Fig. 2F). Control + ALA group had a similar morphology to that of control group (Data not shown).

The collagen distribution was normal in the control (Fig. 3A and B) and control + ALA groups (Fig. 3F), whereas increased deposition of collagen was seen by Sirius red stainings in the livers of n-6 group (Fig. 3D). In addition to centrilobular and midlobular fatty degeneration, a gradually developed perivenular fibrosis and also remarkable collagen increasing around the venules and portal areas (Fig. 3C) was observed. But there was not a pseudolobule formation and septal fibrosis. In the n-6 + ALA treatment group, there was not a remarkable collagen increasing around the venules and portal areas and the morphology of the tissues was similar to that of the control groups (Fig. 3E). The fibrosis and fatty degeneration scoring belonging to all groups was statistically significant (Fig. 4).

3.3. Immunohistochemistry

3.3.1. Pro-caspase-3

The immunoreactivity of pro-caspase-3 of hepatocytes was analyzed within the livers (Fig. 5A–D). Pro-caspase-3 immunopositivity staining was mostly limited to cytoplasm. HSCORE analysis indicated that pro-caspase-3 immunostaining in liver cells in the n-6 group was significantly higher than in the n-6 + ALA group (p < 0.001) compared to the control and the control + ALA groups (Fig. 6).

3.3.2. Active (cleaved) caspase-3

The number of active caspase-3 positive cells for each group are shown in Fig. 7. Active caspase-3 positive signals were observed in the cytoplasm (Fig. 8C) and nucleus (Fig. 8D) of liver cells, and positive apoptotic bodies (Fig. 8E) similar to results of Eckle et al. (2004). The number of active caspase-3 positive cells was higher in n-6 group (8.43 ± 0.6) (Fig. 8B) than that of control (1.17 ± 0.09) (Fig. 8A) and control + ALA (1.19 ± 0.14) groups (p < 0.001). The number of cells expressing active caspase-3 was significantly decreased in the groups n-6 + ALA (3.13 ± 0.52) (p < 0.001) (Fig. 8F) in comparison with n-6 group.

3.4. Tunel

Apoptotic index for each group is shown in Fig. 9. Within the cell cords and the sinusoids, a number of TUNEL positive cells were observed. An increase in the number of TUNEL-positive cells was observed in the n-6 group (10.34 ± 1.25) (Fig. 10B and D) compared to the other groups (Fig. 10). A statistically significant reduction of hepatocyte apoptosis in n-6 + ALA group (4.98 ± 0.78) (Fig. 10E) was determined compared to the n-6 group (p < 0.001). It was also observed that control-ALA group (2.46 ± 0.30) (Fig. 10F) was similar to the control group (1.63 ± 0.56) (Fig. 10A) and also the control-ALA group exhibited no significant alterations in TUNEL-positive cells compared to the control group.

3.5. Electron microscopy

Ultrastructural morphology of the liver in all groups was examined. In the control group, hepatocytes and their organelles and membranes were intact and hepatocyte cords were regular and sinusoids included kupffer, HSC and endothelial cells as a normal state (Fig. 11A). In n-6 group, we observed that control-ALA group (2.46 ± 0.30) (Fig. 10F) was similar to the control group (1.63 ± 0.56) (Fig. 10A) and also the control-ALA group exhibited no significant alterations in TUNEL-positive cells compared to the control group.
dilated endoplasmic reticulum within the cell cords, apoptotic cells and focal necrotic areas were seen (Fig. 11B and C). In the sinusoids apoptotic cells, macrophages containing apoptotic bodies and a number of secondary lysosomes, hepatic stellate cells containing many lipid droplets (Fig. 12A) were observed. Collagen bundles localized mainly around the central veins, periportal area and also in some partial sinusoids in n-6 group (Fig. 11D). Decreased degenerative areas and fatty hepatocytes, increased peroxisomes in hepatocytes and also rarely collagen bundles were seen in n-6 + ALA group (Fig. 11E and F) compared to n-6 group. In semi-thin sections, a significant increase number of stellate cells filled by lipid droplets (Fig. 12B) were observed in the n-6 (5.76 ± 1.51) (p < 0.01) and n-6 + ALA (4.76 ± 1.34) (p < 0.05) groups compared to control group (2.52 ± 0.67). We observed that the number of HSCs and the lipid doplets within these cells in n-6 + ALA group compared to n-6 group were decreased but it is not statistically significant.

4. Discussion

In the present study, we determined for the first time that an n6-rich diet fed to young rats for an eight-week period of time
induced fatty liver changes and a high rate of hepatocellular apoptosis which was prevented by ALA treatment.

Fatty acid-induced lipotoxicity plays a crucial role in the pathogenesis of nonalcoholic liver disease. Saturated and unsaturated fatty acids have variety of effects on cell death and steatosis, but the mechanisms associated with those differences are uncertain (Malhi and Gores, 2008; Mei et al., 2011). n-6 PUFA belong to the essential fatty acids since the human body cannot synthesize n-6 fatty acids and they must be obtained by dietary intake. n-6 fatty acids are concentrated in animal products, vegetable oils, and trans-fatty acids in the modern Western diet (Gvozdjakova et al., 2008).

In hypercholesterolemic diets, the liver as the primary organ to metabolize the cholesterol ingested in excess, is affected by oxidative stress that results from an imbalance between the production of free radicals and effectiveness of antioxidant defense systems (Lum and Roebuck, 2001). Oxidative stress leads to cell damage related to free radicals and lipid peroxidation, including the destruction of the cell membrane (Halliwell and Gutteridge, 1990).

ALA is one of the most important naturally occurring antioxidants that is synthesized by plants and animals. ALA may increase intracellular glutathione levels (Islam, 2009). GSH plays a critical role in the detoxification of xenobiotic compounds and in the antioxidation of reactive oxygen species and free radicals. Low levels of GSH are observed in oxidative stress. (Bray and Taylor, 1993). It has been reported that ALA has a beneficial effect in patients with advanced cancer by means of increasing glutathione peroxidase activity and reducing oxidative stress (Mantovani et al., 2003). ALA is also used as a therapeutic agent in a wide variety of conditions related to liver disease, such as including alcohol-induced damage, mushroom poisoning, metal toxicity, and CCl4 poisoning (Bustamante et al., 1998; Goraca et al., 2011). Furthermore, several researchers have recently reported the protective effects of ALA on the liver which are induced by oxidative agents. In this

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**Fig. 4.** The comparison of the fibrosis and fatty degeneration scoring in all groups. *p < 0.001 compare to control and control + ALA groups, \( ^{b} \text{p} < 0.001 \) compare to n-6 group.

**Fig. 5.** Immunolocalization of pro-caspase-3 in liver of all groups (A) Control, (B) Increased caspase-3 immunopositive hepatocytes in the n-6 group compare to the other groups. (C) n-6 + ALA, (D) Control + ALA groups. Counterstaining: Hematoxylin. Magnification: 200×.
study, we have investigated the protective effects of ALA which is known as having anti-oxidative properties on the liver of rat fed with n-6 fatty diet. We observed that the high unsaturated fatty acid diet induced lipid peroxidation and cause a marked depletion in the GSH level of the liver similar to previous studies (Smith and Wills, 1981; Rukkumani et al., 2004; Yao et al., 2011) of the n-6 group of unsaturated fatty acids. After administration of ALA, the hepatic GSH levels increased in rats fed n-6 rich diet.

NAFLD involves an imbalance of n-6/n-3 PUFA ratio in the total lipids resulting in the accumulation of fat in the liver cells and inflammation of the liver. Ingestion of high-fat diet may lead to fatty acid accumulation and hepatic damage. Hepatic lipid accumulation in hepatocytes (hepatic steatosis) is the distinctive feature of NAFLD and a significant factor that can induce insulin resistance, lipid peroxidation, changes in energy metabolism, hepatic cell damage and inflammation (Araya et al., 2004; Videla et al., 2004). An increased fat intake with an excessive amount of n-6 fatty acids can be implicated in promoting necro-inflammation in human (Cortez-Pinto et al., 2006). In this study, the liver histology and ultrastructure showed centrilobular fatty degeneration, an increase of lipid droplets, mitochondrial degeneration and dilated endoplasmic reticulum in relation to the lipid droplets in the hepatocytes in the rat fed the n-6 fatty rich diet. In the ALA administered n-6 group, we observed a decrease in fatty degenerations, inflammation and cell vacuolization in hepatocyte. These results suggested that ALA can play a preventive role in this process.

Apoptosis, or programmed cell death, is a vital cellular process that helps to regulate tissue growth, embryonic development, hormone-dependent atrophy, immune response and aging. Apoptosis has been observed to play a role in a number of liver diseases such as NASH (Elmore, 2007). The examples for the characteristic of apoptosis are mitochondrial release of cytochrome c, DNA fragmentation, preservation of organelle structure and plasma membrane integrity, activation of caspase-3 and finally, cellular fragmentation generating the apoptotic bodies (Ziegler and Groscurth, 2004). Jiang et al. (2011) demonstrated that the numbers of apoptotic cells increased in the rats that were fed a high-fat diet for 12 and 16 weeks. Several researchers report the anti-apoptotic effects and anti-oxidative properties of ALA (Persson et al., 2001; Diesel et al., 2007; Duenschede et al., 2007). Duenschede et al. (2007) indicated that apoptotic hepatocyte numbers significantly decreased in ALA treatment of hepatic ischemia/reperfusion injury. Our data support Duenschede et al.’s results that ALA also reduces apoptosis by its antioxidative potential. In this study, we observed a significant decreasing of pro-caspase-3 (+), active caspase-3 and TUNEL (+) cell numbers in n-6 + ALA group compared to n-6 group.

Hepatic fibrosis is induced by the accumulation of extracellular matrix materials (ECMs) such as type I collagen in hepatic parenchyma. In a normal liver, hepatic stellate cells have a quiescent phenotype, store vitamin A, and secrete a small amount of basement membrane-like materials including laminin and type IV
Following hepatic damage, hepatic stellate cells turned their phenotype into myofibroblast-like cells (Friedman, 2000; Moreira, 2007), which secrete a large amount of ECM and growth factors (Kawada, 2011; Lee and Friedman, 2011). It was also reported that the amount of deposited collagen increased with the progression of liver fibrosis in several liver diseases (Washington et al., 2000; Standish et al., 2006). Aguilera et al. (2005) showed advanced hepatic fibrosis in hypercholesteolemic rabbits, which were fed by a diet rich in n-6 PUFA. Morini et al. (2005) suggested that the fibroelastic “activated” stellate cells were direct markers of the stage of liver fibrosis. In our study, we used Sirius red staining to indicate hepatic fibrosis. We indicated an increase in the deposition of collagen in liver perivenular and portal areas of n-6 group compared to control group. ALA treatment prevented the fibrosis in rats fed n-6 rich fatty diet. Stellate cells are located in the perisinusoidal space of Disse. In a healthy liver, the cells indicate a low proliferation rate but in the liver injury, the cells differentiate into myofibroblast-like cells with high proliferative capacity. In acute and chronic liver diseases, such as subacute massive hepatic necrosis, acute viral hepatitis and paracetamol-induced liver necrosis, the stellate cell population extends beyond the space of Disse in areas of liver injury (Hautekeete and Geerts, 1997; Moreira, 2007). In our study, an increase number of stellate cells filled by lipid droplets were observed in the n-6 group. A decrease was observed in stellate cells and the lipid droplets within these cells in n-6 + ALA group. These results were combined to ALA’s protective effects. There were numerous macrophages filled by apoptotic bodies and secondary lysosomes. Our data suggested that the n6-rich diet induces a marked degenerative effects in the liver. In the n6 group, HSCs have significantly increased compared to control group. In the n6/ALA group, the number of these cells

Fig. 8. Immunolocalization of active caspase-3 in liver of all groups. (A) Control, (B–E) n-6 group; increased active caspase-3 immunopositive liver cells, active caspase-3 (C) cytoplasmic and (D) nuclear staining in hepatocytes, and (E) positive apoptotic bodies (arrow). (F) n-6 + ALA group contains a few immunopositive cells. Counterstaining: Hematoxylin. Magnification: (A,B,D and F): 400×; (C and E): 1000×.

Fig. 9. The comparison of the apoptotic index in all groups. *p < 0.001 compare to control and control + ALA groups, †p < 0.001 compare to n-6 group.
was relatively high but it was not statistically significant. Our findings indicate that an increase in HSCs as a result of n6 rich high fatty diet, and that a number of HSCs could cause inflammation by secreting several cytokines (Atzori et al., 2009) and also fibrosis by transforming the stellate cells to myofibroblast like cells. ALA may inhibit the transdifferentiation of HSCs to myofibroblast-like cells or may prevent fibrosis by inducing matrix metalloproteinase (MMP) (Han, 2006) expression and degrading ECM. It is suggested that HSCs can behave as stem cells and also can differentiate into hepatocytes (Kordes et al., 2007). We suggest that the decreasing of liver degeneration by ALA treatment may be related to HSCs transformation to hepatocyte and also liver regeneration by inhibiting apoptosis via its antioxidant properties.

In conclusion, the present results demonstrate that the feeding of n-6 unsaturated fatty acids causes lipid accumulations in the hepatocytes, apoptosis in the liver cells, fibrosis development and inflammation in young rats liver, that ALA as an antioxidant agent prevents these degenerative. Further investigations are necessary for the role of ALA treatment on HSCs and liver regeneration in the lipotoxicity of the liver.
Conflict of Interest

The authors declare that there are no conflict of interest.

Acknowledgments

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Fig. 12. Hepatic stellate cells (HSC) filled lipid droplets are seen in n-6 group. (A) Electron micrograph of HSC. (B) HSCs in semithin sections by toluidin blue staining. Magnification: (A): 7500×; (B): 1000×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)


