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The Effects of α-Lipoic Acid on Liver Oxidative Stress and Free Fatty Acid Composition in Methionine–Choline Deficient Diet-Induced NAFLD

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ABSTRACT Development of nonalcoholic fatty liver disease (NAFLD) occurs through initial steatosis and subsequent oxidative stress. The aim of this study was to examine the effects of α-lipoic acid (LA) on methionine–choline deficient (MCD) diet-induced NAFLD in mice. Male C57BL/6 mice ($n=21$) were divided into three groups ($n=7$ per group): (1) control fed with standard chow, (2) MCD2 group—fed with MCD diet for 2 weeks, and (3) MCD2+LA group—2 weeks on MCD receiving LA i.p. 100 mg/kg/day. After the treatment, liver samples were taken for pathohistology, oxidative stress parameters, antioxidative enzymes, and liver free fatty acid (FFA) composition. Mild microvesicular hepatic steatosis was found in MCD2 group, while it was reduced to single fat droplets evident in MCD2+LA group. Lipid peroxidation and nitrosative stress were increased by MCD diet, while LA administration induced a decrease in liver malondialdehyde and nitrates+nitrites level. Similary, LA improved liver antioxidative capacity by increasing total superoxide dismutase (tSOD), manganese SOD (MnSOD), and copper/zinc-SOD (Cu/ZnSOD) activity as well as glutathione (GSH) content. Liver FFA profile has shown a significant decrease in saturated acids, arachidonic, and docosahexaenoic acid (DHA), while LA treatment increased their proportions. It can be concluded that LA ameliorates lipid peroxidation and nitrosative stress in MCD diet-induced hepatic steatosis through an increase in SOD activity and GSH level. In addition, LA increases the proportion of palmitic, stearic, arachidonic, and DHA in the fatty liver. An increase in DHA may be a potential mechanism of anti-inflammatory and antioxidant effects of LA in MCD diet-induced NAFLD.

KEY WORDS: • ***antioxidant*** • ***DHA*** • ***FFA*** • ***lipid peroxidation*** • ***lipotoxicity*** • ***liver*** • ***mice*** • ***steatosis***

INTRODUCTION

METHIONINE–CHOLINE DEFICIENT (MCD) diet is widely used for the development of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) animal model.[1] NAFLD includes a wide spectrum of liver diseases ranging from simple steatosis to steatohepatitis and cirrhosis, which often progress to hepatocellular carcinoma (HCC).[2] The earliest stage of NAFLD is simple hepatic steatosis, which is characterized by deposition of triglycerides in more than 5% of hepatocytes. Hepatic steatosis can progress to NASH and about 20% of patients with NASH develop cirrhosis, while about 28% of patients with NASH-induced cirrhosis develop hepatocellular carcinoma.[3] Nowadays, the prevalence of NAFLD increases rapidly parallel with obesity and diabetes.

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Mechanisms involved in the progression of steatosis to NASH are complex and not completely understood. Insulin resistance, oxidative stress, inflammation, and innate immune system activation contribute to the development of NAFLD through steatosis and inflammation in the liver.[4] Although still poorly understood, the pathogenesis of NAFLD is widely explained by a two-hit model theory in which the first hit is an initial metabolic disturbance that increases the inflow of free fatty acids (FFAs) and *de novo* lipogenesis, leading to steatosis. Triglyceride accumulation in hepatocytes increases the sensitivity of the liver to secondary insults (the ''second hit''), which include oxidative stress, decreased hepatic ATP production, and induction of proinflammatory cytokines.[5,6] Many studies on animals and humans have shown correlation between severity of NASH and oxidative stress degree.[7,8]

Various animal models of NAFLD/NASH were used in previous studies, and they may be classified into genetic models, nutritional models, and their combinations. Although numerous animal models are used, neither completely reflects pathophysiology and liver histopathology of

human NAFLD/NASH.[9] One commonly used model is treatment with CCL_4 (carbon-tetrachloride). CCl_4-induced toxicity and its mechanisms have been extensively investigated after oral administration to rodents. A large number of rat models have been reported to develop both liver cirrhosis and HCC using CCl_4 or diethylnitrosamine.[10] CCl4 is a well-known compound for the production of chemical hepatic injury which is mediated by metabolites that react with antioxidant enzymes, such as glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD), and increase the level of inflammatory cytokines,[11] which make this model of liver injury suitable for investigation of antioxidant activity *in vivo*.

Recently, the role of lipotoxicity was emphasized in the pathogenesis of NAFLD. Lipotoxicity is known to promote hepatocyte death, which in the context of NAFLD is termed lipoapoptosis.[12] Lipoperoxides generated by the reaction of free radicals with membrane fatty acids can induce hepatocyte membrane damage. The fatty acid profile may influence the formation of lipoperoxides, as it is known that unsaturated fatty acids are more susceptible to oxidative modification than are saturated fatty acids.

α-Lipoic acid (LA) is a natural compound, chemically named 5-(1,2-dithiolan-3-yl) pentanoic acid, that is also known as thioctic acid. In humans, LA is synthesized in the liver and other tissues with high metabolic activity, such as the heart and kidney. It is also synthesized in small amounts by plants.[13] Previous studies have suggested that LA can improve liver antioxidant defense in rats.[14] LA has reactive oxygen species scavenging capacity, the capacity to regenerate endogenous antioxidants, such as GSH, and vitamins E and C, and a metal-chelating capacity.[15] Unlike other antioxidants, LA is both water and lipid soluble and, therefore, can cross biological membranes easily and has its antioxidant action both in the cytosol and in the plasma membrane.[16]

Although the direct antioxidative role of LA was confirmed, its indirect effects on the course of NAFLD through changes in liver fatty acid profile are not completely clear. A possible link between fatty acid profile and antioxidative properties of LA may have potential beneficial effects in the prevention of steatosis progression into NASH. Therefore, the aim of this study was to examine the effects of LA treatment on liver oxidative stress parameters, FFA composition, and pathohistology in MCD diet-induced NAFLD in mice.

MATERIALS AND METHODS

Animals

The experiment was performed on male C57BL/6 mice, weighing on average 23 ± 3 g, raised at Military Medical Academy in Belgrade. Animals were housed individually in a controlled laboratory environment (temperature 22°C ± 2°C, relative humidity 50% ± 10%, 12/12 h light-dark cycle with lights turned on at 9:00 h a.m.). All experimental procedures were in full compliance with Directive of European Parliament and of the Council (2010/63EU) and approved by the ethics committee of the University of Belgrade (no. 695/2).

All animals ($n = 21$) were on standard chow before the experiment. At the age of 8 weeks, they were randomly divided into the following groups: (1) control (C; $n = 7$)—continuously fed with standard chow; (2) MCD2 group ($n = 7$)—fed with MCD diet for 2 weeks, and (3) MCD2 + LA group ($n = 7$)—fed with MCD diet and simultaneously treated with α-LA (100 mg/kg daily i.p.) for 2 weeks. Control diet contained 2 g/kg choline chloride and 3 g/kg methionine, while MCD diet (MP Biomedicals) had the same composition as control standard chow without methionine and choline with the expense of sucrose (22.76%). Before administration, LA (Sigma-Aldrich) was dissolved in saline in a concentration of 10 mg/mL (injection volume 0.01 mL/g body weight). All animals had free access to adequate food and water during the experiment. During the diet, daily food intake was measured, while body weight was measured weekly and compared with liver weight after sacrifice.

After the treatment, animals were sacrificed by exsanguinations in ketamine (100 mg/kg i.p.) anesthesia. On the day before sacrifice, mice were fasted overnight. Liver samples were collected for pathohistological analysis, FFA level measurement, determination of oxidative stress parameters, and antioxidative enzymes activity.

Sample preparation

Liver samples for biochemical analysis were homogenized on ice in cold buffered 0.25 M sucrose medium (Serva, Feinbiochemica) that contained 10 M phosphate buffer (pH 7.0) and 1 mM EDTA (Sigma Chem. Co.). The homogenates were centrifuged at 2000 *g* for 15 min at 4°C. Crude sediments were dissolved in a sucrose medium and centrifuged. The supernatants were transferred into the tubes and centrifuged at 3200 *g* for 30 min at 4°C. Obtained sediments were dissolved in deionized water. After 1 h of incubation, the samples were centrifuged at 3000 *g* for 15 min at 4°C, and supernatants were stored at −70°C. Supernatants obtained were combined after three rounds of centrifugation. Proteins were determined by the Lowry method using bovine serum albumin as the standard.[17]

Biochemical analysis

Total SOD (tSOD; EC1.15.1.1) activity in the liver was measured spectrophotometrically, as an inhibition of epinephrine autooxidation at 480 nm. After the addition of 10 mM epinephrine (Sigma), an analysis was performed in the sodium carbonate buffer (50 mM, pH 10.2; Serva, Feinbiochemica) containing 0.1 mM EDTA (Sigma). Samples for manganese SOD (MnSOD) were previously treated with 8 mM KCN (Sigma) and then analyzed as previously described.[18] Activity of copper/zinc-SOD (Cu/ZnSOD) was calculated as a difference between the activities of total SOD and MnSOD.

Lipid peroxidation in the plasma and liver homogenates was measured as malondialdehyde (MDA) production,

assayed in the thiobarbituric acid.[19] The results are expressed as nM/mg protein in liver homogenates.

The concentration of nitrites + nitrates (NOx) as a measure of nitric oxide (NO) production was determined by using Griess reagent. With nitrites, Griess reagent forms a purple azo dye, which can be measured spectrophotometrically at 492 nm.[20]

Reduced GSH was determined using 5,5-dithiobis-2-nitrobenzoic acid (36.9 mg in 10 mL of methanol), which had reacted with aliphatic tiol compounds in Tris-HCl buffer (0.4 mol, pH 8.9), thus making yellow-colored *p*-nitrophenol anion. Spectrophotometric measurement of GSH concentration at 412 nm was proportioned to color intensity of the chemical compound produced.[21]

CAT activity in the liver was determined spectrophotometrically at 240 nm on the basis of decline of hydrogen peorxide absorbance and was expressed as units per mg of proteins in the sample.[21,22] One unit was defined as a reduction of 1 μM H_2O_2 per minute at 25°C, pH 7.

Pathohistological analysis

Liver tissue was sectioned and incubated in 10% formalin solution at room temperature. After the fixation, the liver samples were processed by the standard method. Tissues were incorporated in paraffin, sectioned at 5 μm, and then stained with hematoxylin–eosin. Then, they were prepared for light microscopy analysis. All samples were evaluated by an experienced pathohistologist who was blinded to the experiment. Preparations were analyzed and photographed using a combined photobinocular light microscope Olympus BX51-equipped Artcore 500 MI Artray, Co. Ltd., Japan Camera.

FFA analysis

Lipids for further fatty acid analysis were extracted according to the Bligh and Dyer method.[23] The methyl esters of the fatty acids (FAMEs) from the lipid extract were transesterified with HCl in methanol according to the method described by Ichihara and Fukubayashi.[24] FAMEs were quantified using an Agilent Technologies 7890A Gas Chromatograph with a flame ionization detector. Separation of the FAMEs was performed on a 112-88A7, HP-88 capillary column (100 m × 0.25 mm × 0.2 μm) using He as a carrier gas at a flow rate of 105 mL/min. The samples were injected at a starting oven temperature of 175°C, injector temperature was 250°C, and detector temperature was 280°C. The oven temperature was programmed to increase from 175°C to 220°C at 5°C/min. Fatty acids were identified by their retention time with reference fatty acid standards (Supelco™ FAME Mix) and were expressed as a percentage of total fatty acids in the liver.

Statistical analysis

Results are expressed as means ± SD. Since the normal distribution of parameters was confirmed by Kolmogorov–Smirnov test, one-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used for testing the difference among groups. The difference was considered statistically significant if $P < .05$. Computer software SPSS 15.0 was used for the statistical analysis.

RESULTS

During the experiment, no significant difference ($P > .05$) in animal weight and daily food intake between groups was found (data not shown).

Oxidative stress

Our results have shown significantly increased liver MDA level in MCD2 group (398.02 ± 78.43 nmol/mg protein) as compared with control (239.78 ± 41.09 nmol/mg protein) ($P < .01$). In contrast, MCD diet with LA treatment induced a significant decrease in liver MDA level (93.56 ± 22.06 nmol/mg protein) as compared with control ($P < .01$) (Fig. 1A).

Similar to liver MDA level, MCD diet caused a significant increase in liver NOx concentration (35.23 ± 4.78 nmol/mg protein) when compared with control group (19.45 ± 6.02 nmol/mg protein) ($P < .01$). On the other hand, NOx concentration was not significantly different between MCD2 + LA group and control ($P > .05$) (Fig. 1B).

CAT activity in the liver was significantly decreased in both MCD2 (63.77 ± 13.85 U/mg protein) and MCD2 + LA (56.36 ± 15.00 U/mg protein) groups as compared with control (133.10 ± 40.54 U/mg protein) ($P < .01$), with no significant difference ($P > .05$) between experimental groups (Fig. 2).

The liver GSH content was significantly decreased 2 weeks after the initiation of MCD diet (102.44 ± 10.12 nmol/mg protein) as compared with control (123.23 ± 12.32 nmol/mg protein) ($P < .05$). In contrast to liver CAT activity, LA treatment induced a significant increase in liver GSH content (154.87 ± 18.66 nmol/mg protein) when compared with control and MCD2 group ($P < .01$) (Fig. 3).

MCD diet alone caused a significant decrease in liver total SOD activity (2.95 ± 1.40 U/mg protein) as compared with control (27.96 ± 5.98 U/mg protein). However, in the group treated with LA and MCD diet, total SOD activity was significantly increased (55.08 ± 8.04 U/mg protein) when compared with control and MCD2 group ($P < .01$).

Changes in total SOD activity were accompanied by the same changes in MnSOD and Cu/ZnSOD activity. While MCD diet alone provoked a significant decline in the activity of both izoenzymes (1.62 ± 0.92 U/mg protein, and 1.33 ± 0.69 U/mg protein, respectively) as compared with control (16.09 ± 2.49 U/mg protein and 13.16 ± 2.46 U/mg protein, respectively) ($P < .01$), the activities of MnSOD and Cu/ZnSOD were significantly increased in MCD2 + LA versus control group (29.60 ± 9.06 U/mg protein, and 33.56 ± 8.97 U/mg protein, respectively; $P < .01$) (Fig. 4).

Pathohistological analysis

No pathological changes were found in control group (Fig. 5A). Mild hepatic steatosis with focal fatty change in

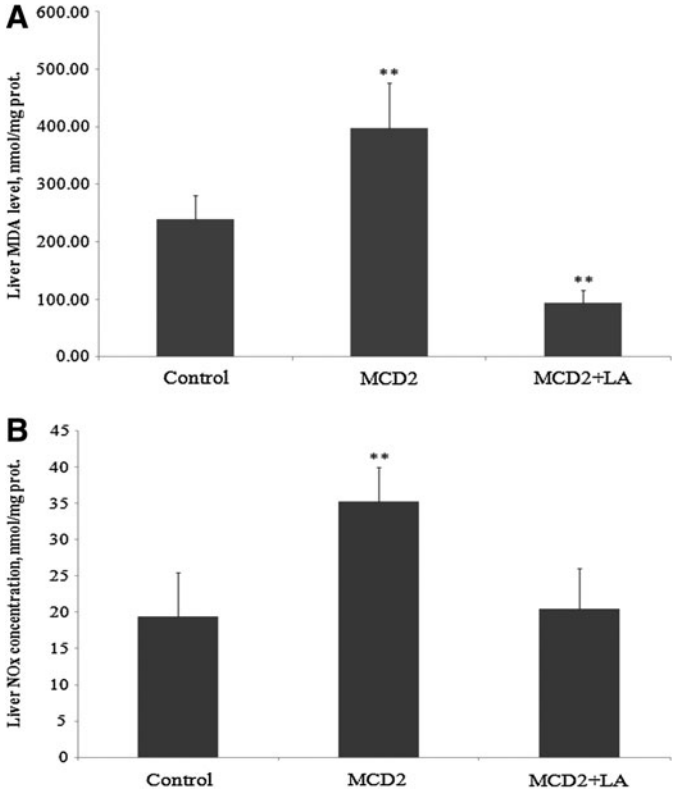

FIG. 1. Liver MDA level **(A)** and NOx concentration **(B)** in MCD diet-treated mice and mice on MCD diet with LA administration. (MCD2 and MCD2 + LA respectively). Since the normal distribution of parameters was confirmed by Kolmogorov–Smirnov test, significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test (**$P < .01$ vs. control). MCD, methionine–choline deficient; LA, α-lipoic acid; MDA, malondialdehyde; NOx, nitrates + nitrites; ANOVA, analysis of variance.

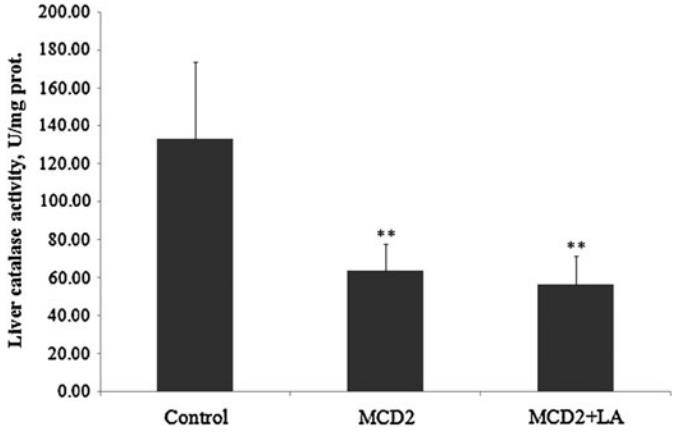

FIG. 2. Liver CAT activity after MCD diet treatment and MCD diet with LA administration. Since the normal distribution of parameters was confirmed by Kolmogorov–Smirnov test, significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test (**$P < .01$ vs. control). For further information, see Figure 1. CAT, catalase.

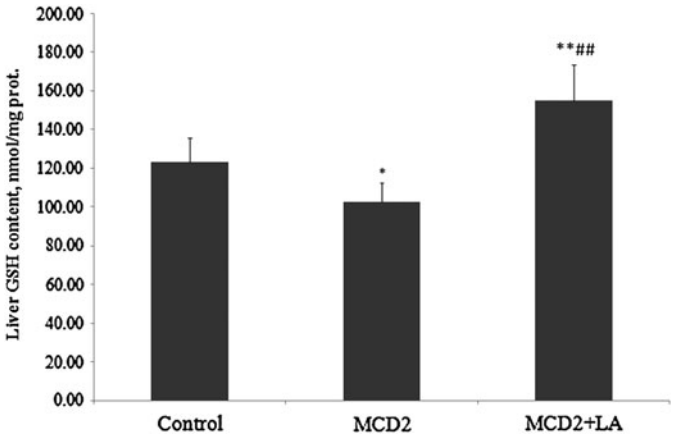

FIG. 3. Liver GSH content in mice treated with MCD diet with or without LA administration. Since the normal distribution of parameters was confirmed by Kolmogorov–Smirnov test, significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test (*$P < .05$, **$P < .01$ vs. control; ##$P < .01$ vs. MCD2 group). For further information, see Figure 1. GSH, glutathione.

the form of microvesicular steatosis was found in MCD2 group, accompanied with mild perivascular inflammation (Fig. 5B). In MCD2 + LA group, steatosis and inflammation were less pronounced than in MCD2 group with a few fat droplets occasionally seen in hepatocytes (Fig. 5C).

FFA analysis

MCD diet induced a significant decrease in liver palmitic (C16:0) and stearic acid (C18:0) proportion after 2 weeks as compared with control ($P < .01$). Palmitic acid proportion was, also, significantly lower in MCD2 + LA group versus control, but to a significantly lesser extent than in MCD2 group ($P < .01$). On the other hand, stearic acid proportion was significantly higher in MCD2 + LA versus. control group ($P < .05$). While the proportion of oleic acid (C18:1 n9) was significantly higher, the proportion of oleic acid isomer (C18:1 n7) was significantly lower in MCD2 group when compared with control ($P < .01$). The combination of LA and MCD diet induced no significant changes in liver oleic acid isomer versus control group ($P > .05$). The proportion of linoleic acid (C18:2 n6) was significantly higher in MCD2 group versus control ($P < .01$), while in MCD2 + LA group, the proportion of linoleic acid did not significantly differ from the control ($P > .05$). When compared with control group, arachidonic acid (C20:4 n6) was significantly lower ($P < .05$) and docosapentaenoic acid (DPA, C22:5 n3) was significantly higher ($P < .01$) in MCD2 group. Similar to linoleic and oleic acid, in MCD2 + LA group, no significant difference in liver arachidonic acid and DPA was found when compared with control ($P > .05$). In contrast, the proportion of liver docosahexaenoic acid (DHA, C22:6 n3) was not significantly changed in MCD2 group as compared with control ($P > .05$), while LA treatment in combination with MCD diet induced a significant increase in DHA when compared with control ($P < .05$) (Table 1).

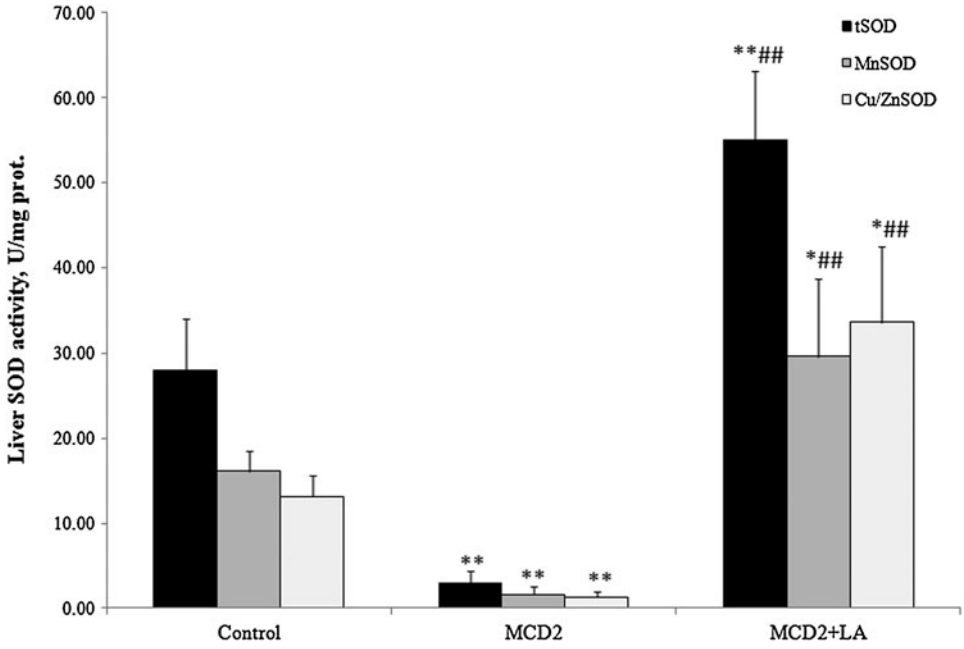

FIG. 4. The effects of MCD diet and LA treatment on tSOD, MnSOD, and Cu/ZnSOD activity in mice liver. Since the normal distribution of parameters was confirmed by Kolmogorov–Smirnov test, significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test ($*P < .05$, $**P < .01$ vs. control; $^{\#\#}P < .01$ vs. MCD2). For further information, see Figure 1. tSOD, total superoxide dismutase; MnSOD, manganese SOD; Cu/ZnSOD, cuprum/zinc SOD.

DISCUSSION

It is well known that oxidative stress contributes to liver injury regardless of its etiology. Recently, many studies have pointed to the antioxidant effects of LA.[25] In accordance with other studies of NAFLD,[26] lipid peroxidation and nitrosative stress were also accompanied with mild steatosis in our study after MCD diet (Fig. 1). NO promotes oxidative stress-induced cell injury by formation of peroxynitrite anion, a potent prooxidant that causes protein nitration and tissue injury.[27] Oxidative injury in MCD diet model of NAFLD may be due to GSH depletion, as well as to decline in CAT and SOD activity (Figs. 2–4). Poor activity of CAT in both experimental groups may be potentially due to secondary activated alternative GSH-pathways, so the CAT could be more active in the early phase of the model. The role of GSH depletion in the pathogenesis of NAFLD is supported by many other studies.[8,26] A decrease in GSH/GSSG ratio reflects the shift of liver redox balance toward pro-oxidants as well as decreased synthesis of GSH due to methionine deficiency in the food. In addition, increased NO production may also contribute to a decline in intracellular GSH level and aggravation of cellular injury.[28]

Studies in experimental models and in the human population suggest that FFAs may play an important role in the pathogenesis of NAFLD.[29] Recent data suggested that saturated fatty acids have more severe lipotoxic and proapoptotic effects in the liver than do unsaturated fatty acids.[30,31]

However, changes in liver FFA profile in NAFLD still remain controversial. Some studies on patients with NAFLD and NASH have shown that hepatic FFAs were unchanged across the spectrum of liver injury,[32] while plasma FFA levels were significantly increased in NAFLD and they were suggested to be the primary source for triglyceride (TAG) synthesis in hepatocytes.[33] On the other hand, a high fat diet was found to induce an increase in liver DHA level in mice.[34] Our study has shown a decrease in hepatic saturated (palmitic and stearic acid) and arachidonic acid proportions in the group treated with MCD diet for 2 weeks.[29,35]

LA was found to have antioxidant effects, as well as to affect lipid metabolism in the liver. It has been shown in cultured rat hepatocytes that LA treatment significantly

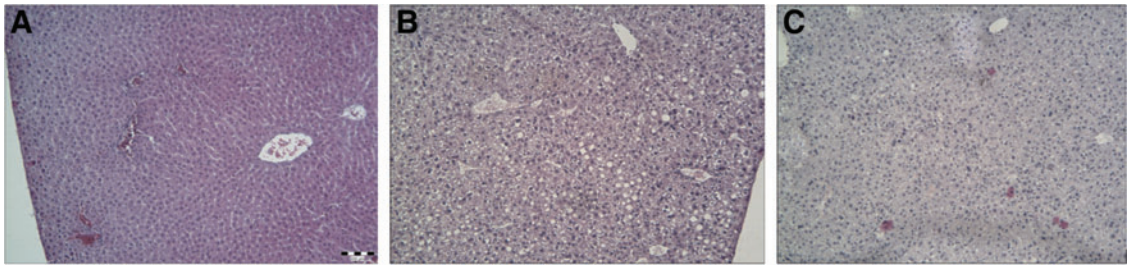

FIG. 5. Histological findings of liver tissue (hematoxylin and eosin, ×10). Control **(A)** group shows normal morphology of the liver. In MCD2 group **(B),** microvesicular hepatic steatosis with focal fatty change was observed with mild perivascular inflammation. In MCD2 + LA group **(C),** reduced steatosis with single fat droplets was evident with reduced inflammatory infiltrate. Color images available online at www.liebertpub.com/jmf

Table 1. The Effect of Methionine-Choline Deficient Diet and α-Lipoic Acid Treatment on Liver Free Fatty Acid Profile

	Hepatic free fatty acid (%)							
Group	*C16:0*	*C18:0*	*C18:1 n9*	*C18:1 n7*	*C18:2 n6*	*C20:4 n6*	*C22:5 n3*	*C22:6 n3*
Control	22 ± 0.73	13.11 ± 0.67	10.25 ± 0.57	2.40 ± 0.32	18.40 ± 0.74	15.45 ± 0.59	0.55 ± 0.05	10.04 ± 0.43
MCD2	16.66 ± 1.17**	9.00 ± 1.65**	14.44 ± 1.94**	0.91 ± 0.19**	26.07 ± 2.54**	12.20 ± 2.21*	1.23 ± 0.24**	9.13 ± 0.76
MCD2 + LA	19.81 ± 0.81*$^{\#\#}$	15.27 ± 0.60*	9.87 ± 1.01	0.84 ± 0.08**	19.44 ± 1.36	15.64 ± 1.13	0.59 ± 0.23	11.56 ± 1.15*

Animals were treated with MCD diet and MCD + LA (100 mg/kg daily i.p.) for 2 weeks (MCD2 and MCD2 + LA groups, respectively). Since the normal distribution of parameters was confirmed by Kolmogorov–Smirnov test, significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test (*$P < .05$, **$P < .01$ vs. control; $^{\#\#}P < .01$ vs. MCD2 group).

C16:0, palmitic acid; C18:0, stearic acid; C18:1 n9, oleic acid; C18:1 n7, oleic acid isomer; C18:2 n6, linoleic acid; C20:4 n6, arachidonic acid; C22:5 n3, docosapentaenoic acid (DPA); C22:6 n3, docosahexaenoic acid (DHA); LA, α-lipoic acid; MCD, methionine–choline deficient; ANOVA, analysis of variance.

inhibited FFA oxidation or reduced it even by 82%. In the same study, it was also suggested that LA at therapeutic concentrations increased pyruvate oxidation by activation of the pyruvate dehydrogenase complex and decreased gluconeogenesis.[36] In addition, LA treatment of insulin-resistant rats for 14 weeks decreased plasma FFA concentrations and this effect appears to be mediated, in part, by increased hepatic PPAR-α expression, which may beneficially affect insulin resistance.[37]

In our study, LA diminished MCD diet-induced lipid peroxidation and nitrosative stress, probably due to increased GSH level and SOD activity (Figs. 3 and 4). Similar effects of LA were found in heart and kidney tissues,[27] but the effects on GSH level was found to be most marked in the liver because of high activity of GSH-synthesizing enzymes in this organ.[38,39] However, the antioxidant effects of the LA in NAFLD may be potentially due to its direct inhibition of the free radicals (in the case of peroxinitrites), rather than increased expression of the antioxidant enzymes. The antioxidative effects of LA may be potentially mediated by changes in fatty acid profile. *n*-3 Polyunsaturated fatty acids may reduce oxidative damage and restore free radical homeostasis by incompletely understood mechanisms.[11] It has been shown that linoleic, eicosapentaenoic acid (EPA), and DHA have beneficial effects on oxidative stress in the kidneys by reducing MDA level, and increasing SOD and CAT activity.[40,41] However, our study suggests only the role of DHA, and not of linoleic acid as a mediator of antioxidative effects of LA in the liver, as only the proportion of DHA increases after LA administration (Table 1). This possibly indicates that supplementation of DHA, but not all polyunsaturated acids may potentiate the hepatoprotective effects of LA in NAFLD.

Other studies have also shown that supplementation with DHA significantly reduced NO content and increased SOD and CAT activity in the liver and brain. DHA supplementation improves cognitive functions and delays the onset of pentylenetetrazole-induced seizures, at least partly through attenuation of oxidative stress. In addition, DHA positively modulates phosphatidylserine biosynthesis and accumulation in neuronal cells, increases cell membrane fluidity, and inhibits apoptosis in a phosphatidylserine-dependent manner.[42] The effects of DHA on membrane lipid composition in the liver has to be further investigated, but these effects may potentially contribute to modulation of oxidative stress in NAFLD after LA treatment found in our study.

Arachidonic acid was, also, found to cause an increase in MnSOD, Cu/ZnSOD, and CAT activities in rat hippocampal slices.[43] Since LA prevented MCD diet-induced decline in arachidonic acid level in the liver, this can possibly be an additional indirect mechanism of LA antioxidant effect. However, this effect has to be further investigated by supplementation with arachidonic acid using various models of NAFLD.

Apart from influence on polyunsaturated fatty acids, antioxidant effects of LA may be potentially further mediated by an elevation of stearic acid proportion in the liver (Table 1). This may be surprising, as saturated fatty acids were found to contribute to the apoptosis of hepatocytes and to the progression of NAFLD, but stearic acid treatment has been shown to increase the activity of SOD, especially Cu/ZnSOD izoenzyme, CAT, and GSH peroxidase.[44] This can be considered one of the potentially beneficial effects of stearic acid in NAFLD.

The effects of LA on liver fatty acid profile may, also, be responsible for the anti-inflammatory effects of LA in NAFLD found in the present study. *n*-3 Polyunsaturated fatty acids reduce the inflammation in the liver through stimulation of resolvin and protectin synthesis, as well as through inhibition of phospholipase A2, an initial enzyme in the synthesis of prostaglandins and leukotrienes. In addition, palmitic acid increases the hepatocyte sensitivity to the cytotoxic effects of TNF-α.[45] DHA also exerts anti-inflammatory effect in the brain through reduction of microglial secretion of proinflammatory cytokines such as IL-6 and TNF-α.[46] A recent study has shown that α-linoleic acid, EPA, and DHA reduce inflammation in both the heart and the liver and prevent cardiac fibrosis.[40] The present study suggests that increased liver DHA proportion may be a potential mechanism of anti-inflammatory effects of LA in MCD-induced model of NAFLD (Table 1).

Based on our results, it can be concluded that LA ameliorates lipid peroxidation and nitrosative stress in MCD diet-induced hepatic steatosis through an increase in SOD activity and GSH level. In addition, LA increases the proportion of palmitic, stearic, arachidonic, and DHA in the

fatty liver. Increase in DHA may be considered a potential mechanism of anti-inflammatory and antioxidant effects of LA in MCD diet-induced NAFLD.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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