Original Research

Alpha-lipoic acid affects the oxidative stress in various brain structures in mice with methionine and choline deficiency

Milena Veskovic[1], Dusan Mladenovic[1], Bojan Jorgacevic[1], Ivana Stevanovic[2], Silvio de Luka[1] and Tatjana Radosavljevic[1]

[1]Institute of Pathophysiology ''Ljubodrag Buba Mihailović'', Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia;
[2]Millitary Medical Academy, 11000 Belgrade, Serbia
Corresponding author: Tatjana Radosavljević. Email: tanjamm@med.bg.ac.rs

Abstract

Deficiency in methionine or choline can induce oxidative stress in various organs such as liver, kidney, heart, and brain. This study was to examine the effects of alpha-lipoic acid (LA) on oxidative stress induced by methionine and choline deficiency (MCD) in several brain structures. Male mice C57BL/6 ($n = 28$) were divided into four groups: (1) control – continuously fed with standard chow; (2) LA – fed with standard chow and receiving LA; (3) MCD2 – fed with MCD diet for two weeks, and (4) MCD2+LA – fed with MCD diet for two weeks and receiving LA (100 mg/kg/day intraperitonealy [i.p.]). Brain tissue (cortex, hypothalamus, striatum and hippocampus) was taken for determination of oxidative stress parameters. MCD diet induced a significant increase in malondialdehyde and NOx concentration in all brain regions, while LA restored their content to normal values. Similar to this, in MCD2 group, activity of total SOD, MnSOD, and Cu/ZnSOD was reduced by MCD diet, while LA treatment improved their activities in all brain structures. Besides, in MCD2 group a decrease in catalase activity in cortex and GSH content in hypothalamus was evident, while LA treatment induced an increase in catalase activity in cortex and striatum and GSH content in hypothalamus. LA treatment can significantly reduce lipid peroxidation and nitrosative stress, caused by MCD diet, in all brain regions by restoring antioxidant enzymes activities, predominantly total SOD, MnSOD, and Cu/ZnSOD, and to a lesser extent by modulating catalase activity and GSH content. LA supplementation may be used in order to prevent brain oxidative injury induced by methionine and choline deficiency.

Keywords: Methionine and choline deficient diet, oxidative stress, nitrosative stress, lipid peroxidation, lipoic acid

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Introduction

Methionine is a sulfur-containing essential amino acid which in form of *S*-adenosylmethionine (SAM) represents a major source of methyl-groups in organism,[1] and is particularly susceptible to oxidation induced by reactive oxygen species (ROS). As a result of oxidation processes, two forms of methionine-sulfoxide (MetO) are formed, which can be reduced back to methionine by methionine sulfoxide reductases. These enzymes play an important role in protection of proteins against oxidative stress and have been involved in regulation of the aging process.[2]

Choline is a dietary component essential for normal function of all cells, and is a part of vitamin B complex. It is needed for neurotransmitter synthesis (acetylcholine), cell-membrane signaling (phospholipids), lipid transport (lipoproteins), and methyl-group metabolism (homocysteine reduction) and is the major dietary source of methyl-groups via the synthesis of SAM.[3,4] Choline is required for synthesis of phospholipids, phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen, and sphingomyelin-essential components for all membranes.[5] Also, choline plays important roles in brain and memory development in the fetus and appears to decrease the risk of the development of neural tube defects.[6,7] In most mammals, prolonged (weeks to months) ingestion of a diet deficient in choline (and adequate though limited in methionine and folate content) induces hepatic, renal, pancreatic, memory, and growth disorders.[8] Various conditions are associated with deficiency in methionine or/and choline, such as alcoholism, pregnancy, lactation, gut bacterial infections, usage of some medicaments, malnutrition, malabsorption syndrome, Crohn's disease, folic acid and vitamin B12 deficiency, etc. and apathy, loss of pigmentation in hair, edema, lethargy, liver damage, muscle loss, fat loss, skin lesions, weakness, and slowed growth in children can occur. Indirect effect of methionine deficiency may be impaired homocysteine

status, which may affect cognitive functions via neurotoxicity.[9]

In recent studies, it has been shown that methionine and choline deficiency (MCD), both separately and in combination may induce oxidative stress in various tissues and organs such as liver, kidney, and brain.[10–13]

Alpha-lipoic acid (LA) is well know antioxidant, and has ROS scavenging capacity, the capacity to regenerate endogenous antioxidants such as glutathione, and vitamins E and C, and a metal chelating capacity. Besides, LA is both water and lipid soluble and therefore can cross biological membranes easily, such as blood–brain barrier, and performs its antioxidant action both in the cytosol and in the plasma membrane.[14] There are numerous studies that confirmed antioxidative effects of LA in many different organs and tissues,[15–18] and also neuroprotective effects.[19]

Since MCD can cause functional disorders in neuronal tissue by various pathogenetic mechanisms, including oxidative stress, the aim of this study was to examine the effects of LA supplementation on oxidative stress parameters and antioxidant enzymes activities in various brain regions – cortex (Cx), hypothalamus (H), striatum (S), and hippocampus (Hipp) in mice fed with MCD diet.

Methods and materials

Animals

The experiment was performed on male C57BL/6 mice, weighting on average 23 ± 3 g, raised at Military Medical Academy in Belgrade. Animals were housed in controlled laboratory environment (temperature $22 \pm 2^{\circ}$C, relative humidity $50 \pm 10\%$, 12/12 h light–dark cycle with lights turned on at 9:00 h a.m.) and had free access to water and standard chow before experiment. All experimental procedures were in full compliance with Directive of European Parliament and of the Council (2010/63EU) and approved by the Ethical Committee of University of Belgrade (No. 695/2).

All animals ($n = 28$) aged eight weeks were randomly divided into following groups: (1) control (C; $n = 7$) – continuously fed with standard chow; (2) (LA; $n = 7$) – group that was on standard chow and was treated with LA (100 mg/kg/day intraperitoneally [i.p.]); (3) (MCD2; $n = 7$) – group fed with MCD diet for two weeks; and (4) (MCD2+LA; $n = 7$) – group that was fed with MCD diet for two weeks and was treated with LA (100 mg/kg/day i.p.). MCD diet (02960439-MP Biomedicals, CA) composition is shown in Table 1. Control diet had the same composition as methionine/choline deficient diet except 2 g/kg choline and 3 g/kg DL-methionine were added at the expense of sucrose.

Before administration, LA (Sigma-Aldrich, Germany) 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.

On the day prior to sacrifice, mice were fasted overnight. After the treatment, animals were sacrificed by exsanguinations by cardiac puncture in ketamine (100 mg/kg i.p.) anesthesia. Brain samples were taken for determination of oxidative stress parameters and antioxidant enzyme activities in cortex, hypothalamus, striatum, and hippocampus.

Table 1 Methionine/Choline deficient diet composition (02960439-MP Biomedical, CA)[a]

Nutrient	g/kg
Sucrose	455
Corn oil	100
Corn starch	200
L-Amino acids	174
Alphacel non-nutritive bulk	30
AIN 76 mineral mix	35
Vitamin mix	6
Total	1000

[a]Control diet had the same composition as methionine/choline deficient diet except 2 g/kg choline and 3 g/kg DL-methionine were added at the expense of sucrose.

Sample preparation

Brain samples for biochemical analysis were homogenized on ice, in 0.25 mol/L cold-buffered sucrose medium (Serva, Feinbiochemica, Heidelberg, NY), 10 mol/L phosphate buffer (pH 7.0), and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich Chem. Co., St. Louis, USA). The homogenates were centrifuged at 2000*g* for 15 min at 40°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 40°C. Obtained sediments were dissolved in deionized water. After 1 h of incubation, the samples were centrifuged at 3000*g* for 15 min at 40°C and supernatants were stored at −700°C. Proteins were determined by the Lowry method using bovine serum albumin as the standard.

Biochemical analysis

Lipid peroxidation in brain regions homogenates was measured as malondialdehyde (MDA) production and assayed in the reaction with thiobarbituric acid as described by Girotti *et al*.[20] The results are expressed as units per milligram (U/mg protein) of proteins in brain homogenates. The concentration of nitrites and nitrates (NOx), markers of NO production, was determined by using Griess reagent. After reduction of nitrates, total nitrites react with sulfanilamide and *N*-(1-naphtyl)-ethylendiamine to produce an azo dye, which can be measured spectrophotometrically at 492 nm.[21] Activity of total superoxide dismutase (EC1.15.1.1.; tSOD) in the brain structures was measured spectrophotometrically, as an inhibition of epinephrine autooxidation at 480 nm.[22] After the addition of 10 mmol/L epinephrine (Sigma Aldrich Chem. Co.), analysis was performed in the sodium carbonate buffer (50 mmol/L, pH 10.2; Serva, Feinbiochemica, Heidelberg, NY) containing 0.1 mmol/L EDTA. Samples for manganese-SOD (MnSOD) were previously treated with 8 mmol/L potassium cyanide (KCN) (Sigma Aldrich Chem. Co.) and were then analyzed as described. Activity of copper/zinc-SOD (Cu/ZnSOD) was

calculated as a difference between the activities of total SOD and MnSOD.

Reduced glutathione (GSH) was determined using 5,5-dithiobis-2-nitrobenzoic acid (36.9 mg in 10 mL of methanol), which had reacted with aliphatic thiol 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 produced chemical compound.[23]

Catalase (CAT) activity 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.[24] One unit was defined as a reduction of 1 μmol/L H_2O_2 per minute at 25°C, pH 7.

Statistical analysis

Results are expressed as means ± standard deviation (SD). As 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 $P < 0.05$. Computer software SPSS 15.0 was used for the statistical analysis.

Results

Two weeks of MCD diet caused a significant increase in MDA concentrations in all brain structures (Cx: 631.98 ± 209.40 nmol/mg protein; H: 743.13 ± 111.36 nmol/mg protein; S: 520.39 ± 138.51 nmol/mg protein and Hipp: 607.00 ± 119.87 nmol/mg protein) in comparison with control (Cx: 368.64 ± 68.18 nmol/mg protein, $P < 0.05$; H: 489.71 ± 89.78 nmol/mg protein, $P < 0.01$; S: 369.61 ± 72.34 nmol/mg protein, $P < 0.01$ and Hipp: 364.96 ± 87.70 nmol/mg protein, $P < 0.01$). On the other hand, LA alone induced a significant decrease in MDA concentration in comparison to control group. However, LA treatment in MCD2 + LA group, significantly reduced MDA concentration in all brain regions with the most prominent decrease in hippocampus when compared to MCD2 (Figure 1a).

Brain NOx content was significantly increased by MCD diet in all brain regions (Cx: 46.45 ± 5.63 nmol/mg protein; H: 49.86 ± 5.73 nmol/mg protein; S: 48.56 ± 10.70 nmol/mg protein and Hipp: 60.30 ± 9.14 nmol/mg protein) in comparison with control ($P < 0.01$), while LA treatment in MCD diet fed mice induced a significant decrease in NOx content in all brain structures (Cx: 18.27 ± 3.66 nmol/mg protein; H: 24.24 ± 3.58 nmol/mg protein; S: 21.95 ± 3.11 nmol/mg protein and Hipp: 18.45 ± 4.39 nmol/mg protein) in comparison with MCD2 ($P < 0.01$). LA administered alone significantly reduced NOx content in all brain regions compared to control (Figure 1b).

CAT activity in brain tissue in group fed with MCD diet was significantly decreased in cortex (4.14 ± 2.86 U/mg protein) in comparison with control group (17.53 ± 7.11 U/mg protein, $P < 0.01$). In contrast to NOx, CAT activity in MCD2+LA group was significantly increased in two brain structures when compared to MCD2 group (Cx: 57.89 ± 8.02 U/mg protein, $P < 0.01$ and S: 18.39 ± 5.49 U/mg protein $P < 0.01$) (Figure 2a). However, LA alone significantly improved CAT activity only in cortex, while in other regions its activity remained unchanged when compared to control.

MCD diet did not cause changes in GSH content in brain regions except the significant decrease in hypothalamus (28.73 ± 4.60 nmol/mg protein) in comparison with control (41.18 ± 4.20 nmol/mg protein) ($P < 0.01$), while LA was found to increase GSH content in hypothalamus (46.58 ± 5.24 nmol/mg protein) when compared to MCD2 ($P < 0.01$). In contrast, LA in MCD2+LA group induced a significant decrease in GSH content in hippocampus (24.03 ± 5.24 nmol/mg protein) when compared to MCD2 group (42.80 ± 8.54 nmol/mg protein) and control (54.57 ± 10.33 nmol/mg protein) ($P < 0.01$) (Figure 2b).

MCD diet treatment for two weeks induced a significant decrease in total SOD and Cu/ZnSOD activity in all brain structures, while activity of MnSOD was decreased in all brain structures except hippocampus, when compared to control ($P < 0.01$). Total SOD and MnSOD activities were both significantly increased in all brain regions in MCD2 + LA group when compared with MCD2 group ($P < 0.01$). Additionally, Cu/ZnSOD activity was significantly increased in MCD2 + LA group in all brain regions, with the most prominent increase in cortex and striatum when compared to MCD2 group ($P < 0.01$). Besides, LA alone significantly improved total SOD and its isoenzymes activities in all brain regions in comparison with control (Table 2).

Discussion

When compared to other organs, the brain is likely to be especially susceptible to excessive oxidative stress. Since the brain is one of the most metabolically active organs it demands at least 20% of the body's available oxygen supply.[25] The brain might be more susceptible to ROS because it contains high levels of unsaturated fatty acids, which are the main targets for free radicals and cellular lipid peroxidation. Moreover, the brain contains only low to moderate levels of antioxidant enzyme activities, such as SOD, CAT, and glutathione peroxidase (GPx), compared with other organs.[26] Antioxidant effects of LA are predominantly manifested by ROS scavenging and stimulating GSH synthesis in astroglial cells. However, it remains unknown whether neuroprotective effects of LA may occur through other mechanisms, such as induction of endogenous antioxidants and phase 2 enzymes in neuronal cells.[27]

Various studies suggest that separately methionine or choline deficiency can induce lipid peroxidation in various organs.[28–30] It also has been shown that after i.p. administration of LA, antioxidant is primarily accumulated in liver, heart, and skeletal muscle after which it efficiently crosses the blood–brain barrier to accumulate in several brain regions, particularly striatum, hypothalamus, and cerebral cortex.[31]

Results of the present study indicate that combined MCD promotes oxidative stress by increasing lipid peroxidation and nitrosative stress in all examined brain regions. It has been shown that in animals fed with MCD diet,

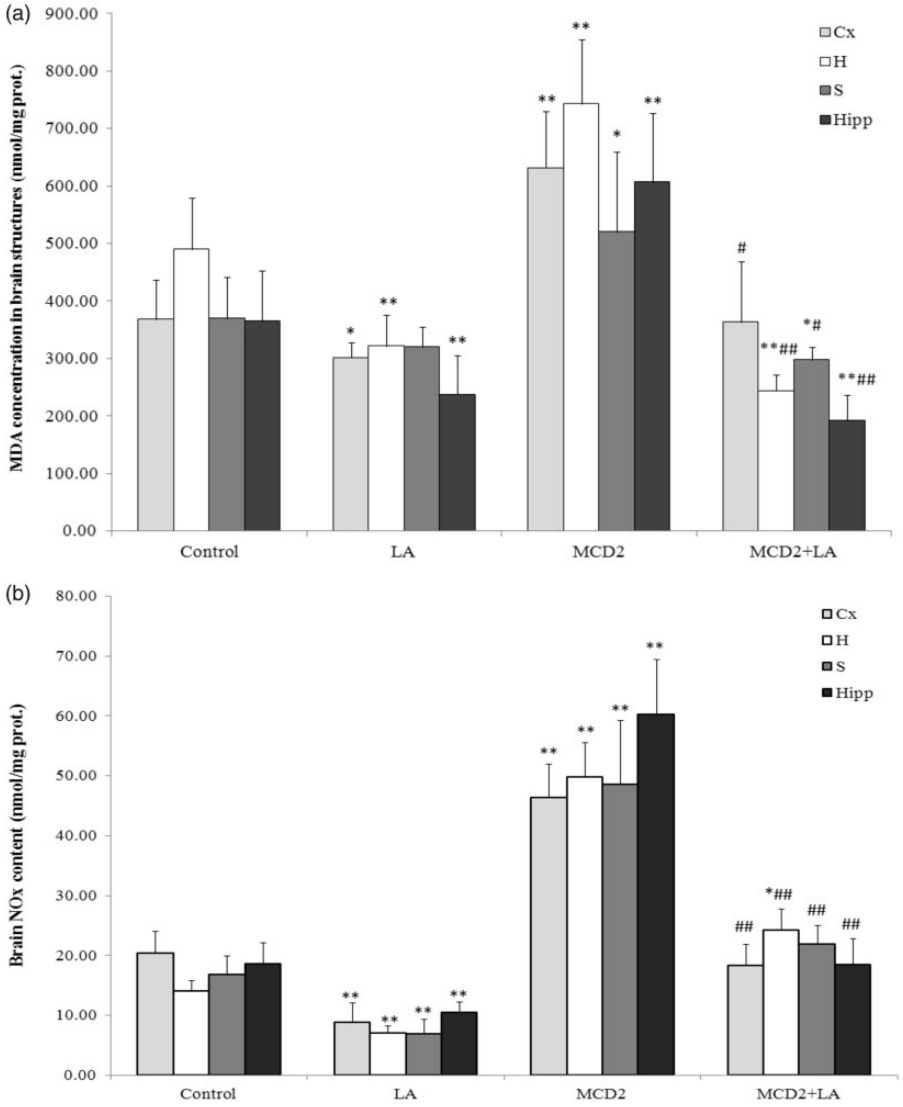

Figure 1 Concentration of MDA (a), and NOx (b) in various brain regions. Control group – continuously fed with standard chow; LA – group fed with standard chow and was receiving LA for two weeks; MCD2 group fed with MCD diet for two weeks, and MCD2 + LA – group that was fed with MCD diet for two weeks and was receiving LA. Significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test (*$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. MCD2 group). MDA: malondyaldehide; NOx: nitrates + nitrites; MCD: methionine–choline deficient; LA: lipoic acid; Cx: cortex; H: hypothalamus; S: striatum; Hipp: hippocampus

elevated oxidative stress is associated with increased mRNA and protein expression of the NADPH oxidase complex and with the induction of its components (Nox2, $p22^{phox}$, $p47^{phox}$, p67phox).[32] In research done with convulsive animals, LA has been shown to protect hippocampus against oxidative stress, by inhibiting the oxidation of lipids and proteins.[33] In accordance to this, in present study LA treatment significantly reduced lipid peroxidation – MDA level, with the lowest values in hippocampus. This is in agreement with previous report from our laboratory,[13] where LA treatment reduced oxidative and nitrosative stress in the liver. Observed significant increase in NOx levels could be a possible consequence of iNOS activation, especially in microglia, which is shown to promote nitrosative stress in various brain disorders. Study on neurodegenerative disorders has demonstrated that powerful pro-oxidant peroxynitrite is involved in pathogenesis, where nitrosative stress is probably derived from the reaction between NO and superoxide.[34] Study described by Liang and Akaike[35] demonstrated that LA could be a potent inhibitor of iNOS, without any proven cytotoxicity. Yamada *et al*. suggested that LA inhibits the induction of *iNOS* gene expression at a posttranscriptional step via iNOS mRNA stabilization, which results in decreased production of NOx.[36] According to these studies, this could be possible mechanism by which LA decreased NOx level in all examined brain regions in our study.

Antioxidant enzymes in nervous system, such as SOD and GPx are expressed in higher quantities than CAT, [37] and can be affected by methionine deficiency.[10] Spectrum of enzymatic defenses suggests that the brain may efficiently metabolize superoxide, but may have difficulty in eliminating the hydrogen peroxide produced by this reaction (i.e. superoxide dismutation). Hydrogen peroxide

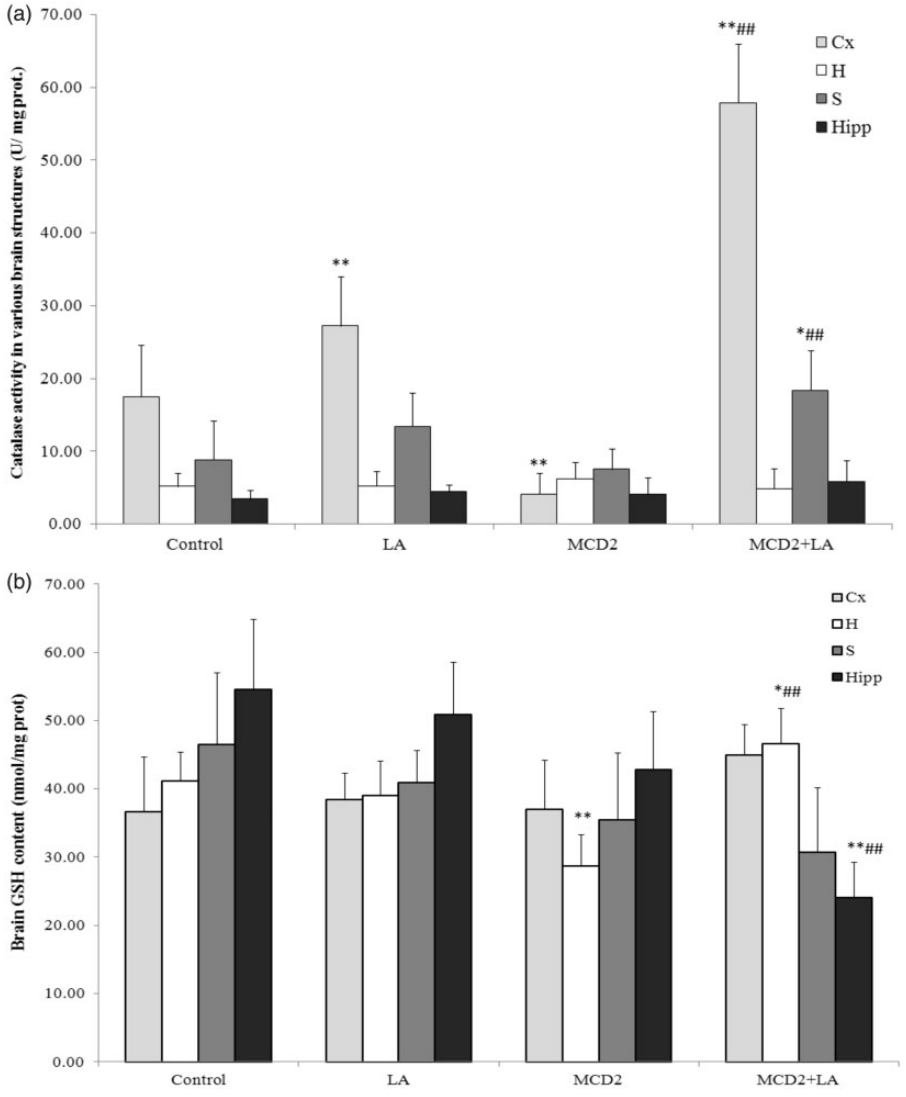

Figure 2 Brain CAT activity (a) and GSH content (b) in various brain regions. Control – continuously fed with standard chow; LA – group fed with standard chow and was receiving LA for two weeks; MCD2 group fed with MCD diet for two weeks, and MCD2 + LA – group that was fed with MCD diet for two weeks and was receiving LA. Significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. MCD2 group). CAT: catalase; GSH: glutathione; MCD: methionine-choline deficient; LA: lipoic acid; Cx: cortex; H: hypothalamus; S: striatum; Hipp: hippocampus

accumulation is of major concern, as the brain contains large quantities of iron and copper, which may catalyze the formation of hydroxyl radicals that can induce lipid peroxidation.[38] Enhanced hydrogen peroxide, in turn, is reduced to water by peroxidases, mostly GPx in the brain.[39] SOD catalyses the conversion of superoxide free radical to hydrogen peroxide and water, while GPx continues where SOD leaves off by catalyzing the reduction of hydrogen peroxide to water at the expense of glutathione.[40] It has been demonstrated that pretreatment with LA during acute phase of pilocarpine-induced seizures induces an increase in SOD and CAT activities and GPx in rat hippocampus.[41] Besides, LA was shown to reduce neurons damage caused by free radicals produced in neurodegenerative diseases.[42]

Results in our study suggest that MCD induced a significant decrease in total SOD activity as well as in its isoenzymes Cu/ZnSOD and MnSOD in all brain regions, whereas LA treatment restored the activities of total SOD and its isoenzymes even higher than in control group. This result probably can be explained by the effect of LA on nerve growth factor (NGF). NGF provides the expression of *SOD* gene and there is a possibility that LA enhances NGF-induced regulation of SOD gene.[40] This could be an important factor which leads to the increase of SOD activity in brain.

Methionine is a sulfur agent required for the production of GSH. Cysteine, formed by methionine, is necessary for GSH synthesis and activation. Therefore, two major homeostatic systems are coordinately regulated by methionine metabolism, cellular methylation, and the redox buffer system.[43] MCD diet caused a significant decrease in GSH content in hypothalamus, but not in other examined brain regions. This could be possibly explained by different effects of oxidative stress on CAT activity and GSH content in various brain regions, what was observed in many

Table 2 The effects of LA on total SOD, Mn SOD, and Cu/Zn SOD activity in mice various brain regions[a]

		Groups			
Enzyme activity(U/mg prot.)	**Brain region**	**Control**	**LA**	**MCD2**	**MCD2 + LA**
Total SOD	Cx	7.61 ± 2.78	20.54 ± 2.21**	2.03 ± 0.33**	24.20 ± 4.36**,##
	H	8.76 ± 3.27	19.21 ± 4.32**	2.38 ± 0.30**	21.86 ± 4.10**,##
	S	9.30 ± 2.03	16.11 ± 2.21**	2.54 ± 0.27**	15.11 ± 5.47*,##
	Hipp	8.28 ± 4.07	11.23 ± 3.11*	2.67 ± 0.66**	13.06 ± 5.49##
Mn SOD	Cx	2.64 ± 0.21	3.56 ± 0.32**	0.90 ± 0.16*	3.08 ± 1.11##
	H	4.13 ± 1.30	4.71 ± 0.21*	1.35 ± 0.10**	4.79 ± 0.10##
	S	4.72 ± 1.01	5.66 ± 1.00*	1.12 ± 0.11*	5.46 ± 1.20##
	Hipp	1.63 ± 0.65	4.12 ± 0.66**	1.47 ± 0.62	4.97 ± 0.87**,##
Cu/Zn SOD	Cx	4.97 ± 1.61	13.32 ± 2.45**	0.83 ± 0.24**	21.67 ± 7.75**,##
	H	4.85 ± 1.64	8.01 ± 0.76*	1.21 ± 0.41**	4.86 ± 1.37##
	S	4.86 ± 1.42	13.65 ± 1.76**	1.22 ± 0.49**	15.91 ± 4.26**,##
	Hipp	5.69 ± 2.39	6.88 ± 2.21	1.44 ± 0.54**	6.25 ± 2.49##

Cx: cortex; H: Hypothalamus; S: striatum; Hipp: hippocampus; MCD: methionine–choline deficient; LA: lipoic acid; SOD: superoxide dismutase; control: continuously fed with standard chow; LA: group fed with standard chow and was receiving LA for two weeks; MCD2: group fed with MCD diet for two weeks; MCD2 + LA: group that was fed with MCD diet for two weeks and was receiving LA.

[a]Significance of the difference was estimated by using one-way ANOVA with Tukey's *post hoc* test (*$P < 0.05$, **$P < 0.01$ vs. control; #$^{\#}P < 0.01$ vs. MCD2 group).

previous studies.[44,45] Study by Candelario-Jalil *et al*. showed that the hippocampus was the most vulnerable brain area to oxidative injury with respect to other areas in spite of showing a relatively high antioxidant capacity before kainate application.[44] In the same study, it was also shown that the levels of GSH and SOD may not correlate with an increase in lipid peroxidation. Similar to other studies,[46] this beneficial effects of LA can be explained by potential improvements in NADPH redox state and enhancement tissue GSH status by increasing cellular cysteine availability. *In vitro* studies showed that LA augmented cellular cysteine levels via dihydrolipoic acid (DHLA)-mediated reduction of extracellular cystine to cysteine, which facilitated rapid uptake of cysteine.[46,47]

CAT activity was significantly decreased by methionine–choline deficiency only in brain cortex, which was probably due to different sensitivity of various brain regions to oxidative damage, as mentioned before.[48–50] It has been shown that cortex and brainstem are more sensitive to lipid peroxidation induced by thioacetamide,[45] and CAT activity in those brain regions was significantly decreased, which is in accordance with results of the present study. However, it is not easy to compare those data to our study because of different stress conditions. On the other hand, LA improved CAT activity in cortex and striatum. This finding is in accordance with the study that examined effects of LA on cyanate-induced oxidative stress in brain regions, where LA improved CAT activity in the same regions.[51]

LA treatment significantly reduces lipid peroxidation and NOx level induced by MCD diet in brain cortex, hypothalamus, striatum, and hippocampus by restoring antioxidant enzymes activities, predominantly total SOD, MnSOD, and Cu/ZnSOD in all brain regions, and to a lesser extent by increasing CAT activity in cortex and striatum and GSH content in hypothalamus.

Accordingly, LA supplementation may be used in order to prevent brain oxidative damage induced by MCD.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; MV wrote the manuscript; DM and BJ conducted the experiment; LS and SI supplied reagents and determined all oxidative stress parameters in tissue samples. RT supervised the whole experiment and took part in research as well.

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