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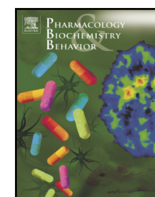

Neurobehavioral effects of L-carnitine and its ability to modulate genotoxicity and oxidative stress biomarkers in mice

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ABSTRACT

L-Carnitine, a natural vitamin-like compound supplied to the body by biosynthesis and dietary sources, has been shown to exert beneficial effects in disorders affecting cardiovascular, urinary, and nervous systems. However, the paucity of data on its effects does not guarantee the safe use of L-carnitine as a nutritional supplement, and further pre-clinical studies are required to assess toxicological aspects. The present study evaluated the effects of L-carnitine (10, 50 or, 100 mg/kg) in mice, in the open field test. Also, lipoperoxidation was assessed measuring thiobarbituric acid reactive substances (TBARS) and genotoxic/antigenotoxic activities were evaluated using the comet assay in several tissues. L-Carnitine 50 mg/kg impaired exploration, though with no effects on habituation to a novel environment. L-Carnitine increased TBARS in the brain and liver tissues, but it did not induce genotoxicity in any tissue. In *ex vivo* comet assay, a decrease in DNA damage in the blood and liver tissues was observed, while the opposite occurred in the brain tissue. In conclusion, L-carnitine may increase lipid peroxidation, though without inducing genotoxic effects, protect DNA against endogenous and induced oxidative damages in blood and liver; however, L-carnitine impaired exploratory behavior and increased the vulnerability of the brain tissue to oxidative stress, suggesting that the excessive consumption of L-carnitine may promote deleterious effects on the central nervous system.

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1. Introduction

L-Carnitine (LC) is a natural vitamin-like compound supplied to the body through dietary sources (*e.g.*, meat) and by endogenous biosynthesis mainly in the liver, kidney and brain, from lysine and methionine (Hoppel et al., 1980; Vaz and Wanders, 2002). It plays an essential role in human intermediary metabolism — primarily in lipid metabolism by transporting long-chain fatty acids into the mitochondrial matrix for β-oxidation and ATP production in peripheral tissues (Amin and Nagy, 2009; Zammit et al., 2009).

It is generally accepted that exogenous pharmacological amounts of LC can be beneficial in a number of physiopathological conditions. L-Carnitine protects the myocardium against ischemia (Reznick et al., 1992) and the skeletal muscle myopathy in heart failure (Vescovo et al., 2002). The dependence on LC uptake is evident from patients suffering from primary systemic carnitine deficiency (CDSP), an autosomal recessive disorder of fatty acid oxidation, caused by mutations in the *OCTN2* gene (Cruciani et al., 2009; Wang et al., 1999).

Based on its physiological functions, LC has to be taken as a nutritional supplement. The daily recommended dose may be as high as 1–6 g per day (Bloomer, 2007; Karlic and Lohninger, 2004; Zammit et al., 2009). It is consumed as a weight-loss agent as well as to increase exercise performance mainly by those who practice resistance training, but there is not enough evidence to prove LC efficacy and guarantee safe use in healthy humans (Karlic and Lohninger, 2004).

Since LC may accumulate in several tissues, including the brain (Burda et al., 2009; Haripriya et al., 2005), the aim of the present study was to evaluate the effects of LC on locomotion and habituation behavior in the open field test. Lipoperoxidation and genotoxicity were also evaluated, measuring TBARS and using comet assay, respectively, in young adult mice. In addition, antigenotoxic effects were evaluated using *ex vivo* comet assay in blood, liver, and brain cells. Hydrogen peroxide was used as inductor of DNA damage to assess the resistance of those tissues to oxidative stress after treatment with LC.

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2. Materials and methods

2.1. Animals

Forty *CF*-1 male mice weighing between 35 and 40 g (3 months old) were obtained from the Lutheran University of Brazil (ULBRA). Mice were housed in plastic cages, with *ad libitum* access to water and food, under a 12-h light/dark cycle (lights on at 7.00 AM) and at temperature of 23 ± 2 °C. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the consent of the Ethics Committee of the Lutheran University of Brazil.

2.2. Drug

L-Carnitine hydrochloride [(−)-β-Hydroxy-γ-(trimethylammonium) butyrate] (CAS registration number [6645-46-1]) was purchased from Sigma-Aldrich. All solutions were prepared immediately prior to administration.

2.3. Treatments and sampling schedule

For the behavioral procedures in the open field test, mice were divided in 4 groups with N = 10 animals, which were given an intra peritoneal (i.p.) injection of saline solution (NaCl 0.9%) or LC (10, 50, or 100 mg/kg) in a volume of 10 mL/kg body weight, 20 min before the training session. All training and testing were carried out between 8.00 AM and 12.00 PM in a bright room.

For genotoxic/antigenotoxic activities using comet assay, LC was administered daily for 3 days, following the recommendations described in Tice et al. (2000). Peripheral blood samples were collected 3 h after the 1st administration and 24 h after the 1st and 3rd administrations. On the 4th day the animals were sacrificed. The liver and brain were dissected and samples were used fresh for comet assay or stored at −80 °C for subsequent quantification of thiobarbituric acid reactive substances (TBARS). For the comet assay only 7 animals per group were employed.

2.4. Open field behavior

The mice were put in an open field, in other words, a compartment that is similar to a wooden box (50 × 50 × 30 cm), with a frontal glass wall and a brown floor with a pattern of twelve squares marked with black lines (3 × 4 arrays). Animals were placed in the rear left square, and allowed to freely explore the field for 5 min. The number of black lines crossings, of rearings performed, and latency to start locomotion were counted and used as measures of locomotion, exploration, and motivation (Kaefer et al., 2010).

2.5. Habituation

The animals used in the evaluation of open field behavior were re-exposed (test session) for 5 min to the open field 24 h after the 1st exposure (training session), and the number of rearings performed was recorded. The decrease in the number of rearings performed between the 1st and 2nd exploration sessions was considered as a measure of habituation.

2.6. Evaluation of thiobarbituric acid reactive substances (TBARS)

Protein was quantified using bovine albumin (SIGMA®) 1 mg/mL according to Lowry et al. (1951). The samples were measured spectrophotometrically at 625 nm.

Frozen liver and brain samples were homogenized in ice-cold phosphate buffer (KCl 140 mmol/L, phosphate 20 mmol/L, pH 7.4) and centrifuged at 4000 rpm (2150.4 *g*) for 10 min. The samples were incubated at 100 °C for 15 min. Subsequently 0.67% thiobarbituric acid was added to 10% trichloroacetic acid and centrifuged at 4 °C at 3000 rpm (1612.8 *g*) for 10 min. Absorbance was determined in the supernatants, using a spectrophotometer at 535 nm (Buege and Aust, 1978).

2.7. Comet assay

The alkaline comet assay was performed as described in the comet assay guideline (Tice et al., 2000). Each piece of forebrain and liver was placed in 0.5 mL cold phosphate-buffered saline (PBS) and finely minced in order to obtain a cellular suspension; blood samples were placed in 15 μL anti coagulant (heparin sodium 25,000 UI — Liquemine®). These suspension cells from the forebrain, liver and from the peripheral blood (5.0 μL) were embedded in 95 μL 0.75% low melting point agarose (Gibco BRL). The mixture (cell/agarose) was spread on a fully frosted microscope slide coated with a layer of 300 μL normal melting agarose (1%) (Gibco BRL). After solidification, slides were transferred to either PBS or 0.25 mM freshly prepared hydrogen peroxide (H_2O_2) solution (*ex vivo* treatment) for 5 min, at 4 °C as described by Flores et al. (2011). Slides were washed 3 times with PBS and then placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, freshly added 1% Triton X-100 (Sigma) and 10% DMSO, pH 10.0) for 48 h at 4 °C. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min, at 4 °C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to induce DNA electrophoresis. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver, and analyzed using a microscope. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal. Cells were also scored visually according to tail size into five classes, ranging from undamaged (0 = no tail), to maximally damaged (4 = largest tail), resulting in a single DNA damage score for each animal, and consequently for each group studied. Therefore, the damage index (DI) parameter was calculated ranging from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage, 100 cells × 4). The damage frequency (DF) was calculated based on the number of cells with tail *versus* those with no tails. The percentage of reduction in DI was calculated for: $R\% = [DI\ H_2O_2 - DI\ LC\ with\ H_2O_2] / [DI\ H_2O_2 - DI\ saline] \times 100$ (Flores et al., 2011).

2.8. Statistical analysis

These data were examined using the One-Way Analysis of Variance (ANOVA) followed by the Duncan's test. Comparisons between the number of rearings performed in training and test sessions within the same group in the habituation experiment were conducted using a paired *t*-test. TBARS and comet assay data were analyzed using ANOVA followed by the Dunnett's test. In all comparisons, $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Neurobehavioral parameters

The results of mice behavior for a 5-min exploration time in an open field are shown in Fig. 1. There were no significant differences among groups regarding the latency to start locomotion and crossings, suggesting that LC did not affect motivation and locomotion of the animals in this task. However, LC 50 mg/kg decreased the number of rearings performed, suggesting that exploration was inhibited.

Habituation was evaluated measuring the number of rearings performed in a test session carried out 24 h after the 1st exploration session (training session) in the open field task. The saline group showed a significantly lower number of rearings during the test session (Fig. 2). Similarly, the groups treated with LC 10 and 50 mg/kg

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