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Short Communication

L-Carnitine supplementation decreases DNA damage in treated MSUD patients

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ABSTRACT

Maple syrup urine disease (MSUD) is an inherited disorder caused by severe deficient activity of the branched-chain α-keto acid dehydrogenase complex involved in the degradation pathway of branched-chain amino acids (BCAAs) and their α-ketoacid derivatives. MSUD patients generally present ketoacidosis, poor feeding, ataxia, coma, psychomotor delay, mental retardation and brain abnormalites. Treatment consists of dietary restriction of the BCAA (low protein intake) supplemented by a BCAA-free amino acid mixture. Although the mechanisms of brain damage in MSUD are poorly known, previous studies have shown that oxidative stress may be involved in the neuropathology of this disorder. In this regard, it was recently reported that MSUD patients have deficiency of L-carnitine (L-car), a compound with antioxidant properties that is used as adjuvant therapy in various inborn errors of metabolism. In this work, we investigated DNA damage determined by the alkaline comet assay in peripheral whole blood leukocytes of MSUD patients submitted to a BCAA-restricted diet supplemented or not with L-car. We observed a significant increase of DNA damage index (DI) in leukocytes from MSUD patients under BCAA-restricted diet as compared to controls and that L-car supplementation significantly decreased DNA DI levels. It was also found a positive correlation between DI and MDA content, a marker of lipid peroxidation, and an inverse correlation between DI and L-car levels. Taken together, our present results suggest a role for reactive species and the involvement of oxidative stress in DNA damage in this disorder. Since L-car reduced DNA damage, it is presumed that dietary supplementation of this compound may serve as an adjuvant therapeutic strategy for MSUD patients in addition to other therapies.

1. Introduction

Maple syrup urine disease (MSUD; branched-chain ketoaciduria) is an inherited metabolic disorder caused by mutations leading to deficient activity of in the mitochondrial branched-chain α-keto acid dehydrogenase (BCKDH) enzyme complex (E.C.1.2.4.4). This complex catalyses the irreversible oxidative decarboxylation of the branched-chain α-keto acids (BCKA) α-ketoisocaproate (KIC), α-keto-β-methylvalerate and α-ketoisovalerate, being also involved in the metabolism of the branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Ile), and valine (Val). The metabolic blockage at this step results in the accumulation of BCKA and BCAA in tissues and body fluids of the affected individuals [1–3].

Patients affected by classical MSUD usually present poor feeding, ketoacidosis, apnea, hypoglycemia, convulsions, coma, ataxia, failure to thrive, psychomotor delay and mental retardation.

Abbreviations: MSUD, maple syrup urine disease; BCAAs, branched-chain amino acids; L-car, L-carnitine; DI, damage index; MDA, malondialdehyde; BCKDH, branched-chain α-keto acid dehydrogenase; BCKA, branched-chain α-keto acids; KIC, α-ketoisocaproate; Leu, leucine; Ile, isoleucine; Val, valine.

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Central nervous system images reveal spongy degeneration of the white matter, reflecting hypomyelination/demyelination, edema and cerebral atrophy [1,4].

Although severe neurological damage occurs in most MSUD patients, the mechanisms involved in the neurotoxicity of this disease are still poorly understood. However, Leu and KIC have been considered the main neurotoxic metabolites in MSUD, since increasing concentrations of these metabolites are associated with the appearance and worsening of neurological symptoms [1,5].

Clinical management of MSUD comprises a lifelong strict and carefully adjusted semi-synthetic diet with restricted amounts of protein supplemented by vitamins, minerals and a mixture of essential amino acids [6]. Although patients improve significantly by the implementations of this therapy, protein restricted diet may lead to important nutritional deficiency and compromise of the antioxidant defense system [7,8].

L-Carnitine (L-car), a small quaternary amine highly polar and water soluble molecule obtained from dietary supply [9,10] has been used in the treatment of various organic aciduria [11,12]. Furthermore, L-car deficiency has been previously reported in treated MSUD patients with protein-restricted diet supplemented by a semisynthetic formula of essential amino acids without BCAAs [13]. Recent studies have observed that L-car has antioxidant properties and may protect cells from toxic reactive oxygen species in some metabolic disorders [14–17]. In this context, it was found that L-car administration prevents lipoperoxidation, protein damage and alterations on catalase and glutathione peroxidase activities in rat cerebral cortex in a chemically-induced acute model of MSUD [18]. Likewise, patients under BCAA-restricted diet plus L-car supplementation present a marked reduction of malondialdehyde content (lipid peroxidation) in relation to controls [13]. *In vitro* studies performed with human peripheral leukocytes have shown that L-car prevents DNA damage induced by the metabolites accumulating in MSUD [19]. However, to the best of our knowledge there are no reports in literature investigating DNA damage in MSUD patients and the role of antioxidant therapy in this process.

Thus, the aim of the present study was to evaluate DNA damage in leukocytes from MSUD patients and the role of dietary L-car dietary supplementation on this damage. The relationship between DNA damage, MDA levels and Leu concentrations in plasma from MSUD patients was also studied.

2. Material and methods

2.1. Patients and controls

Six MSUD patients (classic form) admitted at the Hospital de Clinicas de Porto Alegre, aged 5–12 years old (mean age 8.28 ± 2.87 years) were included in this study. The main clinical symptoms and laboratorial signals at diagnosis were seizures, convulsions, hypotonia, hypoglycemia, poor feeding, ketoacidosis and psychomotor delay. The length of treatment ranged from 15 days to 9.83 years and consisted of a natural protein restricted diet with low BCAA, supplemented with a semi-synthetic formula of essential amino acids, vitamins and minerals and not containing L-car (MSUD 2-Milupa). These patients were also supplemented for 2 months with dietary L-car (50 mg kg^{-1} day^{-1}) not exceeding 1.5 g day^{-1}. The control group was composed by six age-matched healthy individuals (mean age 6.0 ± 3.12 years). The comet assay, as well as malondialdehyde (MDA), Leu and free L-car concentrations were determined in blood of MSUD patients before (Group A) and after 1 month (Group B) or 2 months (Group C) of L-car supplementation and compared to control group. Blood Leu levels, expressed in μM, were measured by HPLC according to Joseph and Marsden [20] with slight modifications [21]. MDA was measured according to Karatepe [22] and expressed as μM of MDA. Free L-car levels were determined in blood spots by liquid chromatography electrospray tandem mass spectrometry (LC/MS/MS), using the multiple reaction monitoring (MRM) mode [23] and the results were reported as μM. The present study was approved by the Ethical Committee of Hospital de Clinicas de Porto Alegre, RS, Brazil. Parents from all patients included in the present study gave informed consent. Data are represented as mean ± SD for the various measurements. For the statistical analysis, comparison between means was analyzed by repeated measures of ANOVA followed by the Tukey's multiple range test when the F value was significant ($p < 0.05$). Correlations were carried out using the Pearson correlation coefficient.

2.2. Single-cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al. [24] in accordance with the general guidelines [25]. Isolated human leukocytes were suspended in agarose and spread onto a glass microscope slide pre-coated with agarose; the agarose was allowed to set at 4 °C for 5 min. The slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% Triton X-100 and 10% DMSO, pH 10) to remove the cellular proteins, leaving the DNA as histone-free nucleoids. After lysis, the slides were placed on a horizontal electrophoresis unit and covered with fresh electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). The slides were then neutralized, washed in bi-distilled water, and stained using a silver staining protocol [26]. After drying at room temperature overnight, the gels were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. The cells were visually scored and received scores from 0 (no migration) to 4 (maximal migration) according to the tail intensity. Therefore, the damage index (DI) of the cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). All slides were analyzed under blind conditions by two different individuals and the average scores were used for the calculations.

3. Results

Data (represented as mean ± SD) from MSUD patients are divided into Group A (without L-car supplementation), Group B (1 month of L-car therapy) and Group C (2 months of L-car supplementation). Leu levels were measured in plasma from controls and MSUD patients before and after treatment. We found the following values expressed as μM: controls = 130.8 ± 27.2, Group A = 178.2 ± 58.6, Group B = 264.6 ± 136.7, Group C = 278.0 ± 105.6. There were no significant differences between the groups.

On the other hand, patients from Group A had significantly decreased levels of L-car (20.9 ± 6.6 μM) as compared to the controls (43.8 ± 8.9 μM) that normalized after L-car supplementation [Group B (48.1 ± 9.1 μM) and Group C (54.8 ± 12.6 μM)] [$F(3,21) = 6.253$, $p < 0.05$]. Moreover, MDA levels were significantly elevated in Group A (0.087 ± 0.06 μM) as compared to the control group (0.029 ± 0.003 μM), indicating lipid peroxidation, that was reversed after 2 months (Group C) (0.036 ± 0.003 μM) of L-car therapy [$F(3,21) = 19.541$, $p < 0.05$]. With regard to DNA damage, we observed that only Group A had damage classes three and four (the highest damage class) before L-car supplementation and already in the first month of L-car administration this high damage was not more verified, being observed just zero, one and two damage classes (data not shown).

Fig. 1 shows a significantly higher DNA migration (damage class index = DI) in Groups A (DI = 72.3 ± 4.5), B (DI = 36.2 ± 5) and