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Urinary biomarkers of oxidative damage in Maple syrup urine disease: The L-carnitine role

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ABSTRACT

Maple syrup urine disease (MSUD) is a disorder of branched-chain amino acids (BCAA). The defect in the branched-chain α-keto acid dehydrogenase complex activity leads to an accumulation of these compounds and their corresponding α-keto-acids and α-hydroxy-acids. Studies have shown that oxidative stress may be involved in neuropathology of MSUD. L-carnitine (L-car), which has demonstrated an important role as antioxidant by reducing and scavenging free radicals formation and by enhancing the activity of antioxidant enzymes, have been used in the treatment of some metabolic rare disorders. This study evaluated the oxidative stress parameters, di-tyrosine, isoprostanes and antioxidant capacity, in urine of MSUD patients under protein-restricted diet supplemented or not with L-car capsules at a dose of 50 mg kg^{-1} day^{-1}. It was also determined urinary α-keto isocaproic acid levels as well as blood free L-car concentrations in blood. It was found a deficiency of carnitine in patients before the L-car supplementation. Significant increases of di-tyrosine and isoprostanes, as well as reduced antioxidant capacity, were observed before the treatment with L-car. The L-car supplementation induced beneficial effects on these parameters reducing the di-tyrosine and isoprostanes levels and increasing the antioxidant capacity. It was also showed a significant increase in urinary of α-ketoisocaproic acid after 2 months of L-car treatment, compared to control group. In conclusion, our results suggest that L-car may have beneficial effects in the treatment of MSUD by preventing oxidative damage to the cells and that urine can be used to monitorize oxidative damage in patients affected by this disease.

1. Introduction

Maple syrup urine disease (MSUD) is a metabolic disease caused by a severe deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity. The blockage of this pathway leads to the accumulation in tissues and body fluids of branched-chain amino acids (BCAA) leucine (Leu), isoleucine and valine and their respective α-keto-acids, α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid and α-ketoisovaleric acid, as well as the corresponding α-hydroxy acids, the α-hydroxyisocaproate, α-hydroxy-β-methylvalerate and the α-hydroxyisovalerate (Treacy et al., 1992; Chuang and Shih, 2001; Harris et al., 2004).

Based on the clinical presentation and biochemical responses to thiamine administration, MSUD patients can be divided into five phenotypes: classic, intermediate, intermittent, thiamine-responsive and dihydrolipoyl dehydrogenase (E3) deficient (Chuang and Shih, 2001). The main signs and symptoms presented by MSUD patients include psychomotor delay and mental retardation, coma, convulsions, poor feeding, apnea, ataxia, ketoacidosis, hypoglycemia, as well as generalized edema and

Abbreviations: ABTS, 2,2′-azino-di-3-ethylbenzthiazoline sulfonate; BCAA, branched-chain amino acids; BCKAD, branched-chain α-keto acid dehydrogenase; Di-tyr, di-tyrosine; ELISA, enzyme-linked immunoassay; GC/MS, gas chromatography–mass spectrometry; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase; KIC, α-ketoisocaproic acid; Leu, leucine; L-car, L-carnitine; LC/MS/MS, liquid chromatography electrospray tandem mass spectrometry; MRM, multiple reaction monitoring; MSUD, Maple syrup urine disease.

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hypomyelination/demyelination evidenced by magnetic resonance imaging studies of the central nervous system. The MSUD treatment consists of a low protein diet and a semi-synthetic formula poor in BCAA and supplemented by essential amino acids, vitamins and minerals (Chuang and Shih, 2001; Sitta et al., 2014) and the aim of the MSUD treatment is to keep the BCAA plasma concentrations in appropriate levels in order to minimize the brain damage found in MSUD patients without treatment or in metabolic crisis (Chuang and Shih, 2001; Wendel and Ogier de Baulny, 2006). The mechanisms involved in the neurological symptoms presented by MSUD patients are still poorly understood. However, several studies have demonstrated that Leu and/or KIC are the main neurotoxic metabolites in MSUD, and that high concentrations of these metabolites can be associated with the appearance of neurological symptoms (Chuang and Shih, 2001). The metabolites accumulated in this disease provoke convulsions (Coitinho et al., 2001), neuronal apoptosis (Jouvet et al., 2000), impairment of neurotransmitter synthesis (Zielke et al., 1997; Tavares et al., 2000), myelin alteration (Treacy et al., 1992) and affect energy metabolism in rat brain (Sgaravatti et al., 2003).

L-carnitine (L-car) is a highly polar quaternary amine that plays important metabolic functions in the organism, like transport of long-chain fatty acids across the inner mitochondrial membrane for utilization in β-oxidation. Furthermore, this compound has demonstrated antioxidant activity by reducing and scavenging free radicals and by enhancing the activity of enzymes involved in the defense against reactive species (Gulcin, 2006). It was verified that MSUD patients have L-car deficiency, since this compound is obtained mainly from protein food (Mescka et al., 2013; Sitta et al., 2014), which can cause antioxidant defenses impairment (Borglund et al., 1989; Barschak et al., 2006, 2007, 2008). Recent studies have demonstrated an increase of plasma antioxidant status in patients with inborn errors of metabolism supplemented with L-car (Ribas et al., 2010; Mescka et al., 2011, 2013; Sitta et al., 2011). There are no studies in literature focusing urinary amino acids, α-keto acids, α-hydroxy acids and oxidative stress parameters in MSUD treated patients. It is important to emphasize that BCAA metabolites are highly excreted in urine in this disease.

Thus, in this study, we extend our previous publication concerning the L-car effect in plasma from MSUD individuals (Mescka et al., 2013), evaluating oxidative stress parameters, as well as α-keto isocaproic acid concentration in urine of treated MSUD patients, supplemented or not with L-car. Our objective was to investigate a possible association between L-car and oxidative stress in urine, since this biological fluid could be used for monitoring oxidative biomarkers in this disease.

2. Material and methods

2.1. Patients, controls and biological samples

In this study it was studied seven patients (mean age, at the time of blood collection, 8.28 ± 2.87 years) with late diagnosis of classical MSUD under protein restricted diet following the treatment protocol from Medical Genetic Service of Hospital de Clínicas de Porto Alegre (HCPA), Brazil, which were diagnosed by elevated plasma BCAA levels. Whole blood on filter paper was used to evaluate free L-car levels, plasma was used to quantify amino acid and urine samples were used to determine oxidative stress the parameters and to measure KIC concentration.

Dietary treatment (median duration 0.95 year – range from 15 days to 9.83 years) consisted of protein-restricted diet supplemented with a semi-synthetic formula of essential amino acids (except leucine, isoleucine and valine), vitamins and minerals not containing L-car (MSUD 2-Milupa®). The diet contained the following amounts of Leu (before 12 months of age: 40–80 mg kg^{-1} day^{-1}; after 1 year of age: 275–535 mg day^{-1}), Ile (before 12 months of age: 20–50 mg kg^{-1} day^{-1}; after 1 year of age: 165–325 mg day^{-1}) and Val (before 12 months of age: 20–60 mg kg^{-1} day^{-1}; after 1 year of age: 190–375 mg day^{-1}). In addition, MSUD patients were supplemented for 2 months with L-car capsules, fractionated and mixed with the formula of amino acids, at a dose of 50 mg kg^{-1} day^{-1}, not exceeding 1.5 g day^{-1}. Oxidative stress parameters, amino acids, KIC and free L-car levels were analyzed in MSUD patients before (Group A) and after one (Group B) and 2 months (Group C) of L-car supplementation. The control group consisted of samples from six aged-matched healthy children (mean age at the time of blood collection, 6.0 ± 3.12 years). The study was approved by the Ethics Committee of HCPA, RS, Brazil. All parents of the patients included in the present study gave informed consent.

2.2. Amino acids determination

Amino acids were determined in plasma by high-pressure liquid chromatography (HPLC) method according to (Joseph and Marsden, 1986). The quantification was performed by relating the chromatographic peak area of each amino acid to those obtained from a known standard solution and to the peak area of the internal standard (homocysteic acid) with known concentration. Results were expressed in μmol/L.

2.3. Alfa-keto isocaproic acid determination

Alfa-keto isocaproic acid (KIC) was determined in urine by gas chromatography–mass spectrometry (GC/MS) according to (Sweetmann, 1995), using hexadecane and heptadecanoic acid as internal standards. The quantification was performed by relating the metabolites chromatographic peak area to those obtained from a known standard solution for each metabolite and to that of internal standard peak area. Results were expressed in μmol/L. The correction by creatinine was performed at the beginning of analysis, and the volume used for the quantification varied according to creatinine of each sample.

2.4. Free L-carnitine determination

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 (Chace et al., 1997) and the results were reported in μmol/L.

2.5. 15-F2t-isoprostane determination

15-F2t-isoprostane, a product of arachidonic acid metabolism and a biomarker of lipid peroxidation, was measured by a competitive enzyme-linked immunoassay (ELISA) (Oxford Biomed, EA 85), according the kit's instructions. First, the urine samples were mixed with dilution buffer. In this assay, the 15-F2t-isoprostane in the urine samples competes with the 15-F2t-isoprostane conjugated to horseradish peroxidase (HRP) for the binding to a specific antibody fixed on the microplate. The concentration of 15-F2t-isoprostane was determined by the intensity of color developed after addition of substrate (wavelength at 630 nm). Results were expressed as nanograms of isoprostanes per mg of urinary creatinine.

2.6. Di-tyrosine autofluorescence determination

Di-tyrosine (di-tyr) content, used to analyze in urine the levels of protein oxidation, was measured by autofluorescence, according to Kirschbaum (2002). For di-tyr fluorescence determination, 50 μL

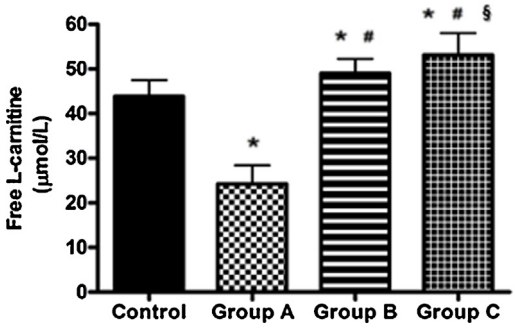

Fig. 1. Free L-carnitine measurement in blood spots from MSUD patients and controls by liquid chromatography electrospray tandem mass spectrometry (LC/MS/MS). Group A represents MSUD patients before treatment with L-car. Group B and C represent MSUD patients after 1 and 2 months of L-car supplementation, respectively. Data represent the mean ± SD. Number of MSUD patients = 7–4. Number of controls = 6. * $P < 0.05$ compared to controls, # $P < 0.05$ compared to Group A, § $P < 0.05$ compared to Group B.

of thawed urine was added to 950 μL of 6 mol/L urea in 20 mmol/L sodium phosphate buffer pH 7.4. After 30 min, the concentration was measured using a fluorometer (excitation 315 nm, emission 410 nm). Results were expressed as fluorescence units per mg urine creatinine (Kirschbaum, 2002).

2.7. Antioxidant capacity determination

The urinary antioxidant capacity was determined using a chemical assay (Antioxidant Assay Kit Cayman Chemical, 709001). This assay measures the capacity of antioxidants in the urine to inhibit the oxidation of 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) by metmyglobin. This reaction can be monitored by detecting the absorbance at 750 nm and the inhibition of the oxidation is proportional to antioxidants concentration. A standard curve using Trolox, a water-soluble tocopherol analogue, is used to calculate the capacity of the antioxidants in preventing the ABTS oxidation. Urinay antioxidant status was expressed as micromolar trolox equivalents.

2.8. Statistical analyzes

Comparison between means was analyzed by repeated measures ANOVA followed by the Tukey multiple range test for parametric data and Friedman test followed by Dunn's multiple comparison test for nonparametric values when the F value was significant ($P < 0.05$). Correlations were carried out using the Pearson correlation coefficient.

3. Results

Fig. 1 shows the measurement of free l-car in blood spots from MSUD patients, before and after the supplementation, and controls. The results demonstrated that MSUD patients had significantly reduced free L-car levels before the treatment (Group A) compared to control group. The supplementation with L-car was able to reverse this deficiency once it was observed a significant increase of L-car levels after 1 (Group B) and 2 (Group C) months of treatment [$F(3,19) = 6.253$, $P < 0.05$].

It were measured the levels of Leu in plasma from MSUD patients, before and after the treatment, and controls. We found the follow values, expressed in μmol/L: controls = 130.75 ± 27.16, Group A = 178.18 ± 58.59, Group B = 264.57 ± 136.74, Group C = 278 ± 105.55. Data are represented as mean ± SD. There was no significant difference between groups.

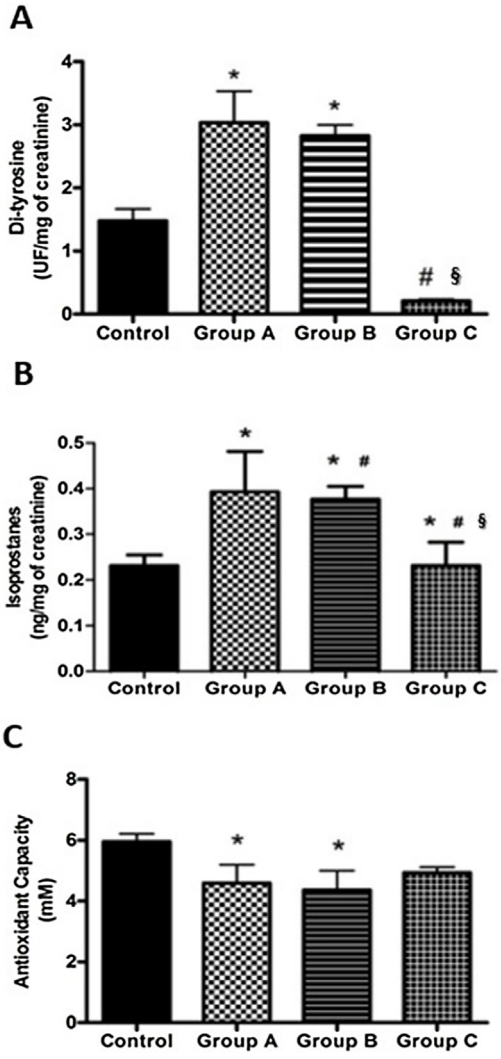

Fig. 2. (A) Di-tyrosine (di-tyr), (B) isoprostanes and (C) antioxidant capacity measurements in urine from MSUD patients and controls. Group A represents MSUD patients before treatment with L-car. Group B and C represent MSUD patients after 1 and 2 months of L-car supplementation, respectively. Data represent the mean ± SD. Number of MSUD patients = 7–4. Number of controls = 6. * $P < 0.05$ compared to controls, # $P < 0.05$ compared to Group A, § $P < 0.05$ compared to Group B.

Fig. 2 shows the evaluation of oxidative stress in urine from MSUD patients. The Fig. 2A exhibits di-tyrosine levels, a biomarker of oxidative damage to proteins. It was verified that di-tyr levels before the supplementation with L-car (Group A) was markedly increased compared to control group, as well as after 1 month of treatment (Group B). L-car therapy was able to reduce the di-tyr levels, only after 2 months of treatment (Group C). Group C was significantly different from groups A and B [$F(3,15) = 18.45$, $P < 0.05$]. It was found a weak significant negative correlation between free L-car levels and di-tyr levels ($r = -0.557$, $P < 0.05$) (Fig. 3).

Fig. 2B shows the isoprostanes levels, an end product of arachidonic acid peroxidation. It was observed a significant increase in isoprostanes levels before the treatment (Group A) compared to the control group and the supplementation with L-car was able to reduce the lipid peroxidation. Groups B and C show a significant decrease in the isoprostanes levels compared to the Group A [$F(3,16) = 4.184$, $P < 0.05$].

Fig. 2C shows the results of urinary antioxidant capacity in the groups. It was found that before the treatment (Group A) and after one month (Group B) with L-car supplementation, the patients had

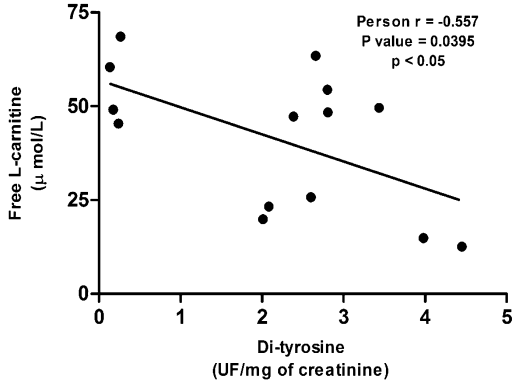

Fig. 3. Correlation between di-tyrosine vs. free L-carnitine levels in MSUD patients. Graphs show the Pearson correlation coefficient and probabilities.

a significant lower urinary antioxidant capacity compared to control group. However, 2 months of the L-car supplementation (Group C) was able to revert this process to control levels [$F(3,15) = 15.02$, $P < 0.05$].

It was measured the urinary KIC levels before and after the treatment in order to evaluate the effects of L-car on this ketoacid. Increased levels of KIC were found, mainly after 2 months of L-car supplementation (Group C) compared to control group. It was found the follow values, expressed in μmol/L: controls = 0.00 ± 0.00, Group A = 8.1 ± 8.1, Group B = 1.7 ± 2.5, Group C = 78 ± 53. Data are represented as mean ± SD. Group C was different from control and Group B ($P < 0.05$) [$F(3,18) = 1.018$, $P < 0.05$].

4. Discussion

In our study, we demonstrated that the levels of free L-car in MUSD patients before the treatment were significantly reduced and the supplementation with L-car during 1 and 2 months was able to restore to normal levels. Our results corroborate other studies, demonstrating that patients affected by inherited metabolic disorders and treated with protein restrict diet could have carnitine deficiency (Sitta et al., 2011, 2014).

Several studies have shown that some accumulated metabolites in inborn errors of metabolism induce excessive free radical production and reduce the tissue antioxidant defenses (Colome et al., 2000; Barschak et al., 2008; Wajner et al., 2004). In this context, *in vitro* and *in vivo* studies have been developed in MSUD (Bridi et al., 2005, 2006; Barschak et al., 2006, 2007, 2008; Mescka et al., 2011, 2013), but oxidative stress parameters in urine of MSUD patients have not been studied. In other hand, these parameters in urine have been investigated in some metabolic diseases. It has been reported that patients with propionic and methylmalonic acidurias had high urinary isoprostanes levels, which were reversed after the treatment with L-car. Furthermore, patients affected by these acidurias had low urinary antioxidant capacity (Ribas et al., 2012).

In this study, we demonstrated that MSUD patients treated with protein restricted diet, but without L-car supplementation, had significantly increased urinary di-tyr levels compared to control group. After 2 months of L-car supplementation, these patients presented di-tyr concentrations at normal levels. Furthermore, we found a weak significant negative correlation between urinary di-tyr and blood free L-car concentrations in MSUD patients, indicating that L-car treatment can be involved in the prevention of protein oxidative damage. Di-tyr is formed by the oxidation of adjacent protein tyrosine residues leading to the formation of a highly stable inter-phenolic bond that does undergo further metabolism (Kirschbaum, 2002).

Protein oxidation by reactive species can lead enzymes, receptors and transport proteins to malfunction and, eventually, induce alterations of cellular metabolism (Halliwell and Gutteridge, 2007). Our results are in agreement with other studies. It was demonstrated that MSUD patients present higher protein oxidative damage in plasma, measured by protein carbonylation (Mescka et al., 2013). Furthermore, studies in cortex of rats, evaluating carbonyls and sulfhydryl content as markers of protein damage, demonstrated that carbonyls content was significantly enhanced in cerebral cortex in MSUD group while sulfhydryl content was significantly reduced, indicating the occurrence of oxidized proteins. Treatment with L-car in this animal model prevented these effects, reducing this damage to control levels (Mescka et al., 2011). It is known that for other inborn errors of metabolism protein damage has already been verified since the patients with propionic and methylmalonic acidurias presented high levels of di-tyr in urine and the treatment with L-car was able to reduce this damage to control levels (Ribas et al., 2011).

We also demonstrated that treatment with L-car was able to reduce the isoprostanes levels progressively after 1 and 2 months of supplementation, indicating a decrease in the lipid oxidation. Recently, studies showed that L-car proved to be effective in reducing the lipid oxidation since malondialdehyde (Mescka et al., 2013) and thiobarbituric acid-reactive substances (Barschak et al., 2006, 2007) were markedly increased in MSUD patients and the therapy with L-car reversed oxidative damage to lipids in plasma of MSUD patients (Mescka et al., 2013). Furthermore, L-car prevented lipid peroxidation in cerebral cortex of rats in a chemically-induced acute model of MSUD (Mescka et al., 2011). L-car have been described in other studies as an antioxidant able to combat the lipid peroxidation (Abdul and Butterfield, 2007; Miguel-Carrasco et al., 2010).

Altered antioxidant capacity in plasma from MSUD patients was observed in other studies, as demonstrated by a decrease of the total antioxidant status, which represents the quantity of tissue antioxidants, and of the total antioxidant reactivity, which reflects the tissue capacity to react with free radicals (Barschak et al., 2006, 2008). Our results are also in agreement, showing a reduction of urinary antioxidant capacity in patients before L-car supplementation. Possibly, these alterations can occur because of the BCAA restrict diet, normally used in the MSUD therapy, is poor in micronutrients necessary for the antioxidant status (Barschak et al., 2007). Decreased urinary antioxidant capacity may reflect a reduction of antioxidants in the blood, such as uric acid and dietary antioxidants. Otherwise, it was verified in our study that after 2 months of L-car administration this process was reversed to control levels, reinforcing an important antioxidant effect of L-car in MSUD patients.

In this study, we quantified the major accumulated metabolite in urine from MSUD patients. We found an increase in KIC levels, compared to control group after 2 months of treatment. Leu and KIC are considered the main neurotoxic agents in MSUD (Chuang and Shih, 2001). The appearance of KIC in significantly higher concentrations after 2 months of L-car treatment suggests that this supplementation may influence the urinary excretion of this metabolite, decreasing its concentration in the blood and tissues. It should be emphasized that due to because the toxic effects of the accumulating metabolites in MSUD patients, the beneficial effects of L-car supplementation, such as correction of carnitine deficiency and restoration of intramitochondrial acyl-CoA/CoA ratios, may improve the metabolic status of these patients (Ribas et al., 2014). All these factors can be contributing to lower the levels of oxidative damage observed in MSUD patients during treatment with L-car.

It is important to emphasize that di-tyr and antioxidant capacity were reversed only 2 months after L-car treatment. Carnitine supplementation is frequently used in patients with organic

acidemias, as for example, propionic, methylmalonic, isovaleric, 3-hydroxy-3-methylglutaric and glutaric type I acidemias and beneficial effects are well described in these patients (Hoffmann, 1996; Bykov, 2004; Kölker, 2011; Ribas et al., 2014). In these diseases, therapy with L-car is used to promote the reduction of the acyl coenzyme A toxic accumulation, releasing coenzyme A for other essential oxidative pathways and restoring normal concentrations in cases where there is a deficiency (Chalmers et al., 1984).

In conclusion, our results demonstrated that there is damage to proteins and lipids in urine from MSUD patients, corroborating the findings published previously in plasma (Mescka et al., 2013), as well as a deficient antioxidant capacity in these patients. The supplementation with L-car could be involved in the prevention of oxidative damage to proteins and lipids in this disease since it was verified a decrease of di-tyrosine and isoprostanes levels in urine from patients treated with L-car, as well as an improvement on the urinary antioxidant capacity. Furthermore, we suggest that L-car could be useful in MSUD therapy representing a new approach to the current treatment, which consists of protein restrict diet. In addition, the use of urine, easily collected, can be an option for monitoring such patients by evaluating the parameters of oxidative stress.

Conflict of interest

The authors declare that there are no conflicts of interest.

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