

## Evidence that L-Carnitine and Selenium Supplementation Reduces Oxidative Stress in Phenylketonuric Patients

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**Abstract** It is well established that the involvement of reactive species in the pathophysiology of several neurological diseases, including phenylketonuria (PKU), a metabolic genetic disorder biochemically characterized by elevated levels of phenylalanine (Phe). In previous studies, we verified that PKU patients (treated with a protein-restricted diet supplemented with a special formula not containing L-carnitine and selenium) presented high lipid and protein oxidative damage as well as a reduction of antioxidants when compared to the healthy individuals. Our goal in the present study was to evaluate the effect of Phe-

restricted diet supplemented with L-carnitine and selenium, two well-known antioxidant compounds, on oxidative damage in PKU patients. We investigated various oxidative stress parameters in blood of 18 treated PKU patients before and after 6 months of supplementation with a special formula containing L-carnitine and selenium. It was verified that treatment with L-carnitine and selenium was capable of reverting the lipid peroxidation, measured by thiobarbituric acid-reactive species, and the protein oxidative damage, measured by sulfhydryl oxidation, to the levels of controls. Additionally, the reduced activity of glutathione peroxidase was normalized by the antioxidant supplementation. It was also verified a significant inverse correlation between lipid peroxidation and L-carnitine blood levels as well as a significant positive correlation between glutathione peroxidase activity and blood selenium concentration. In conclusion, our results suggest that supplementation of L-carnitine and selenium is important for PKU patients since it could help to correct the oxidative stress process which possibly contributes, at least in part, to the neurological symptoms found in phenylketonuric patients.

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### Introduction

The participation of reactive oxygen species (ROS) on the pathophysiology of a crescent number of pathologies, including cancer, neurodegenerative disorders, and inherited metabolic diseases is well established (Halliwell and Gutteridge 2007).

ROS are continuously produced during normal physiologic events and are capable of easily initiating deleterious

cascades inducing peroxidation of membrane lipids. In order to handle ROS production, organisms possess an efficient antioxidant system which includes non-enzymatic antioxidant defenses and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). However, when the balance between the generation of ROS and the inactivation of ROS by the antioxidant system is lost, oxidative stress occurs, leading to oxidative damage to cellular membrane or intracellular molecules, and causing pathological conditions (Halliwell and Gutteridge 2007; Chaudière 1994; German 1999).

Phenylketonuria (PKU) is an inborn error of phenylalanine (Phe) metabolism, caused by deficiency of the enzyme phenylalanine hydroxylase activity, which converts Phe to tyrosine (Tyr). Although Phe is thought to be neurotoxic in PKU, no single mechanism has been identified as being responsible for the central nervous system-related problems, and the pathophysiology of the disease still remains unclear (Scriver and Kaufman 2001; Van Spronsen et al. 2001).

In this context, there is strong evidence that oxidative stress contributes for the neurological damage in PKU, especially in untreated patients or in patients who do not comply with the low-Phe diet. It is presumed that Phe and/or its metabolites induce excessive production of free radicals and/or deplete the tissue antioxidant capacity (Colome et al. 2003; Schulpis et al. 2005; Sierra et al. 1998, 2006, 2009a; Sirtori et al. 2005). In addition, protein-restricted diet for PKU patients may lead to a decrease of the antioxidants intake, contributing to the oxidative damage verified in the disease (Artuch et al. 2004; van Backel et al. 2000).

We previously demonstrated that PKU patients submitted to a protein-restricted diet presented lipid and protein oxidative damage and a decrease in the antioxidant status (Sitta et al. 2006, 2009a). Additionally, we verified in PKU patients a positive correlation between the plasma antioxidant reactivity and L-carnitine (LC) concentrations and a negative correlation between malondialdehyde levels and LC levels, a substance with a potential antioxidant effect that is found to be reduced in PKU patients under strict diet (Sitta et al. 2009b). Therefore, in the present work we evaluated the effect of a long-term supplementation with LC and selenium (Se) on various oxidative stress parameters in PKU patients in order to test for the efficacy of this treatment on the antioxidant status of phenylketonuric patients.

## Methods

### Patients and Controls

Eighteen patients (mean age  $17.2 \pm 2.6$  years; range 15–22 years-old) with classical PKU under treatment were

studied. The average blood Phe levels calculated from the various measurements obtained at every 2 months was  $686 \pm 315$   $\mu\text{mol/l}$ . The dietary treatment consisted of a restricted protein diet supplemented with a special formula not containing LC and Se (PKU 3—Support<sup>®</sup>). The diet contained 220–450 mg/(kg day) Phe and 2.55–4.00 g/(kg day) Tyr according to patients' age. Oxidative stress parameters were analyzed in blood of PKU patients before and after at least 6 months of supplementation with Se and LC (PKU 3 Advanta—Support<sup>®</sup>—Se: 31.5 mcg/day; LC: 98 mg/day). Eighteen healthy children (mean age  $19.4 \pm 3.7$  years; range 18–23 years-old) were used as the control group.

The study was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre.

### Erythrocyte and Plasma Preparation

Erythrocytes and plasma were prepared from whole blood samples obtained from fasting individuals (controls and PKU patients) by venous puncture with heparinized vials. Fifty microliters of the whole blood was spotted onto specialized paper cards for posterior analysis of free L-carnitine. The additional whole blood was centrifuged at  $1,000 \times g$ , plasma was removed by aspiration and frozen at  $-80^\circ\text{C}$  until determinations. Erythrocytes were washed three times with cold saline solution (0.153 mol/l sodium chloride). Lysates were prepared by the addition of 1 ml of distilled water to 100  $\mu\text{l}$  of washed erythrocytes and frozen at  $-80^\circ\text{C}$  until analysis.

### Oxidative Stress Parameters

#### *Determination of Thiobarbituric Acid-Reactive Species (TBARS)*

Thiobarbituric acid-reactive species were determined according to the method described by Esterbauer and Cheeseman (1990). Briefly, 300  $\mu\text{l}$  of 10% trichloroacetic acid was added to 150  $\mu\text{l}$  of plasma and centrifuged at  $1,000 \times g$  for 10 min at  $4^\circ\text{C}$ . Three hundred microliters of the supernatant was transferred to a test tube and incubated with 300  $\mu\text{l}$  0.67% thiobarbituric acid (7.1% sodium sulfate) at  $100^\circ\text{C}$  for 1 h. The resulting pink stained TBARS were determined at 535 nm wavelength in a spectrophotometer. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that for the supernatants. TBARS were calculated as nanomoles per milligram protein.

#### *Glutathione Peroxidase (GSH-Px)*

GSH-Px activity was measured using the RANSEL kit (Randox Laboratories, UK). The method is based on Paglia

and Valentine (1967). Glutathione peroxidase catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, oxidized glutathione is immediately converted to its reduced form with a concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance at 340 nm is measured. One GSH-Px unit is defined as 1  $\mu$ mol of NADPH consumed per minute and the specific activity was represented as units per milligram protein.

#### *Determination of Protein Carbonyl Content*

Protein carbonyl formation was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of plasma was treated with 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 90 min. Samples were then precipitated with 500  $\mu$ l 20% TCA and centrifuged for 5 min at 10,000 $\times$ g. The pellet was then washed with 1 ml ethanol:ethyl acetate (1:1, V/V) and dissolved in 200  $\mu$ l 6 M guanidine prepared in 2.5 N HCl at 37°C for 5 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 370 nm. The results were calculated as nanomoles of carbonyl groups per milligram protein.

#### *Determination of Sulphydryl Content*

This assay is based on the reduction of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001). Thirty microliters of plasma was incubated with an equal volume of DTNB at room temperature for 30 min in a dark room. The sulphydryl content is inversely correlated to oxidative damage to proteins. Results were reported in nanomoles of TNB per milligrams of protein.

#### *Catalase Assay (CAT)*

CAT activity was assayed by the method of Aebi (1984) measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H<sub>2</sub>O<sub>2</sub>, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml. One unit of the enzyme is defined as 1  $\mu$ mol of H<sub>2</sub>O<sub>2</sub> consumed per minute and the specific activity was reported in units per milligram of protein.

#### *Superoxide Dismutase (SOD)*

SOD activity was determined using the RANSOD kit (Randox, United Kingdom). The method is based on the

formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in incubation medium from the xanthine–xanthine oxidase reaction system), which is assayed spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as one unit of SOD and the specific activity was represented in units per milligram protein.

#### *Free L-Carnitine Determination*

Free LC 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). Results were reported in micromoles/liter.

#### *Selenium Determination*

Atomic absorption spectrophotometry with hydride generation was used for plasma Se determination. Results were reported in micrograms/liter.

#### *Protein Determination*

Erythrocyte protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Plasmatic protein concentrations were determined by the Biuret method using a diagnostic kit (Labtest Diagnóstica, MG, Brazil).

#### *Statistical Analysis*

Data were expressed as mean  $\pm$  standard deviation, and were analyzed using repeated measures analysis of variance. Correlations between variables were calculated using the Spearman correlation coefficient. A *P* value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software in a PC-compatible computer.

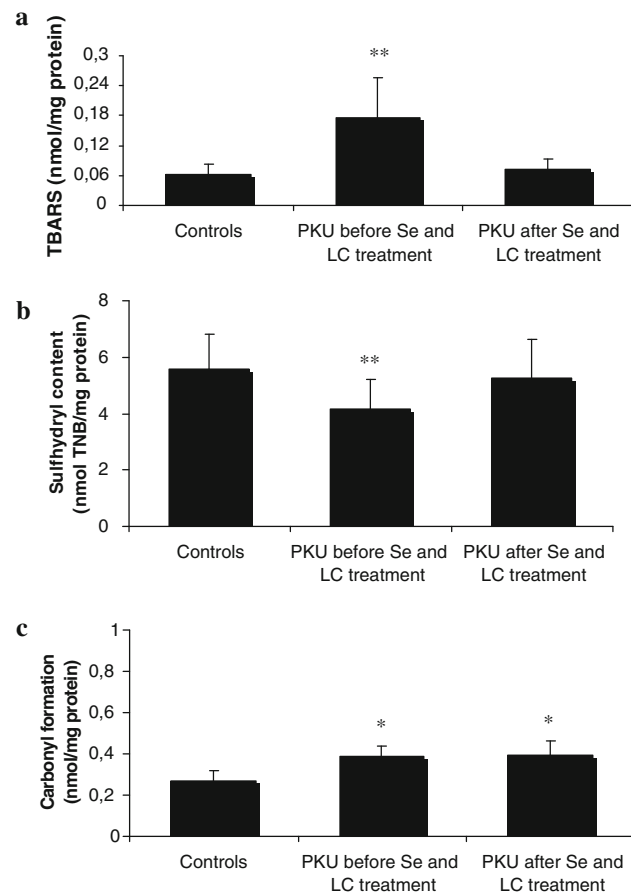
## **Results**

Table 1 shows blood Phe, free LC, and Se concentrations in PKU patients before and after supplementation with LC and Se and also in controls. Both LC and selenium levels were significantly reduced in PKU patients before supplementation when compared to controls. It can also be seen in the table that the antioxidant treatment was capable of reverting this deficiency and that Phe values were higher than expected.

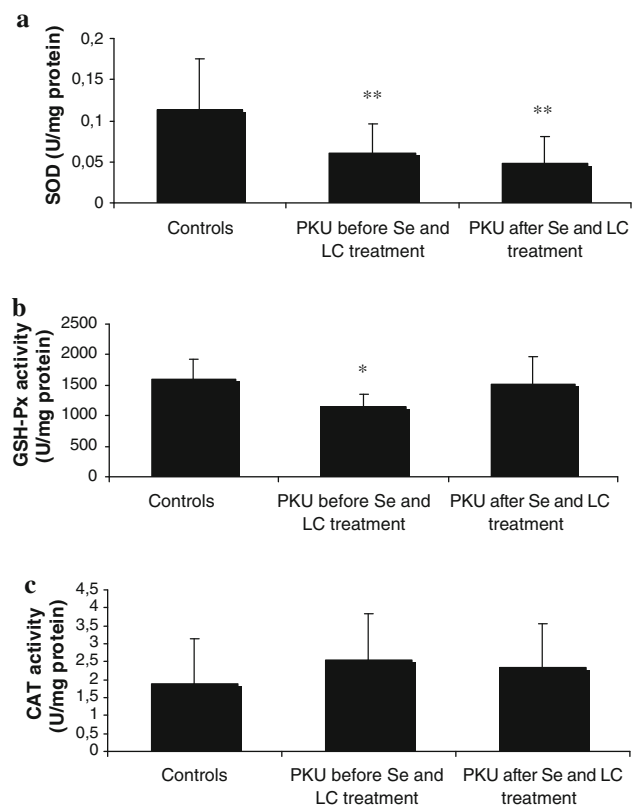
**Table 1** Blood phenylalanine, free L-carnitine, and selenium concentrations in controls and in PKU patients before and after supplementation with L-carnitine and selenium

	Controls (n = 18)	PKU patients before supplementation (n = 18)	PKU patients after supplementation (n = 18)
Phe ( $\mu\text{mol/l}$ )	34.2 $\pm$ 12.1	751.6 $\pm$ 239.3**	598.4 $\pm$ 298.2**
Se ( $\mu\text{g/l}$ )	33.9 $\pm$ 7.2	21.2 $\pm$ 5.5*	30.4 $\pm$ 3.8
Free L-carnitine ( $\mu\text{mol/l}$ )	44.4 $\pm$ 11.8	21.9 $\pm$ 5.5*	38.0 $\pm$ 9.8

\*  $P < 0.05$ , \*\*  $P < 0.01$ , different from controls

**Fig. 1** Lipid and protein oxidative damage in plasma from PKU patients ( $n = 18$ ). **a** Thiobarbituric acid-reactive species (TBARS). **b** Sulfhydryl content. **c** Carbonyl formation. Data represent mean  $\pm$  S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ , different from controls (Analysis of variance for repeated measures)

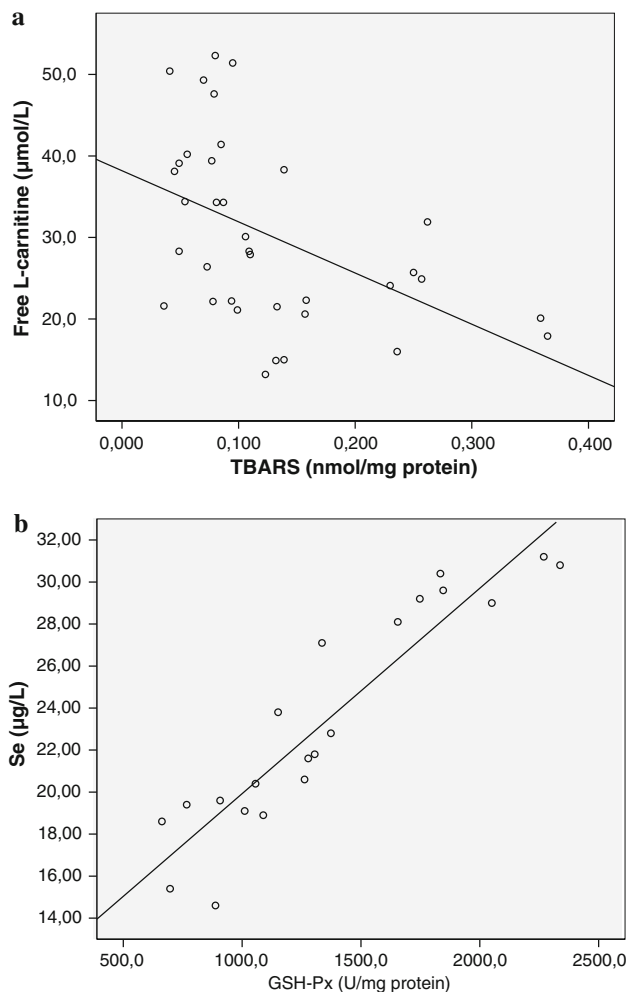
The evaluation of oxidative damage to lipids (TBARS) and proteins (sulfhydryl content and carbonyl formation) in PKU patients and controls is displayed in Fig. 1. TBARS were significantly increased in PKU patients before antioxidant supplementation, reflecting an elevated amount of malondialdehyde, an end product of membrane fatty acid

**Fig. 2** Enzymatic antioxidant activities in erythrocytes from PKU patients and controls ( $n = 10$ ). **a** Superoxide dismutase activity (SOD). **b** Glutathione peroxidase activity (GSH-Px) **c** Catalase activity (CAT). Data represent mean  $\pm$  S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ , different from controls (Analysis of variance for repeated measures)

peroxidation. The supplementation with Se and LC was capable of reverting this process. In addition, plasma protein sulfhydryl groups were significantly reduced while plasma carbonyl formation was significantly increased in PKU patients without supplementation of antioxidants when compared to controls. The administration of Se and LC corrected the oxidation of sulfhydryl groups but no effect was observed upon carbonyl formation.

Figure 2 shows the enzymatic antioxidant defenses (SOD, GSH-Px, and CAT) in erythrocytes from PKU patients and controls. It can be seen that GSH-Px and SOD activities were significantly reduced in PKU patients before antioxidant supplementation when compared to controls. The Se and LC treatment increased GSH-Px activity to the levels of controls, but did not alter SOD activity. On the other hand, CAT activity in PKU patients showed no significant difference from the controls.

As can be seen in Fig. 3, a significant negative correlation was verified between free L-carnitine levels and lipid peroxidation ( $r = -0.560$ ;  $P < 0.01$ ). On the other hand, a highly significant positive correlation between plasma selenium levels and erythrocyte glutathione peroxidase



**Fig. 3** Correlation between oxidative stress parameters and antioxidants in PKU patients. **a** Correlation between TBARS and free L-carnitine levels. **b** Correlation between GSH-Px activity and selenium levels

activity had been verified in treated PKU patients ( $r = 0.945$ ;  $P < 0.01$ ).

## Discussion

PKU is one of the most common inborn errors of metabolism (IEM) and is also considered the first successfully treated IEM (Bickel et al. 1953). Dietary therapy is the main treatment for PKU patients. To maintain plasma levels of phenylalanine within nearly normal levels, the recommended therapy is a low-protein diet poor in animal products including controlled amounts of cereal, fruit, and vegetables, in addition to a protein supplementation with phenylalanine-free synthetic formulas (Start 1998). Untreated PKU patients usually present with severe mental retardation, seizures, microcephaly, spasticity, and

developmental problems, whereas a well-controlled diet prevents these clinical manifestations (Huttenlocher 2000).

Although necessary to avoid mental retardation in PKU patients, the low ingestion of proteins with a high biologic value decreases the bioavailability of essential nutrients, including antioxidant compounds (Acosta 1996). Thus, it was reported that PKU patients under restricted diet present low levels of selenium, L-carnitine, and coenzyme Q10 substances that are necessary for a normal antioxidant capacity (Sitta et al. 2009b; Wilke et al. 1992; Artuch et al. 1999, 2001; Schulpis et al. 1990). In this context, recent studies carried out in animal models and also in PKU patients have emphasized the role of oxidative stress in the pathophysiology of PKU, which represent a disequilibrium between tissue antioxidant and reactive species formation in favor of the latter (Sierra et al. 1998; Sirtori et al. 2005; Sitta et al. 2006, 2009a, b; Ercal et al. 2002; Hagen et al. 2002; Martinez-Cruz et al. 2002). In this study, we evaluated the effect of supplementation with a special formula containing L-carnitine and selenium on various oxidative stress parameters in patients with classic PKU under treatment.

Initially, we verified that lipid peroxidation was significantly increased in PKU patients treated with low-protein diet and Phe-free synthetic formula not containing LC and Se. Furthermore, the supplementation of antioxidants LC and Se was capable of correcting this pathological process, reducing malondialdehyde levels, measured by the thio-barbituric acid-reactive species assay. Lipid peroxidation has received special attention since this process may damage cell structures by altering the integrity, fluidity, permeability, and functional loss of biomembranes, modifying low density lipoprotein and generating potentially toxic products (Greenberg et al. 2008) being, therefore, associated to a crescent number of pathological conditions, including neurological diseases (Adibhatla and Hatcher 2010). In this context, lipid oxidative damage has been reported in various neurodegenerative disorders including various inborn errors of metabolism (Deon et al. 2007; Ribas et al. 2010; Barschak et al. 2009; Mc Guire et al. 2009). This is probably because brain is particularly vulnerable to lipid oxidation since it contains high concentrations of polyunsaturated fatty acids and has relatively low antioxidant capacity compared to other organs (Smith et al. 2007; Markesbery and Lovell 2007).

Enhanced lipid peroxidation in PKU patients could be associated to high levels of the toxic metabolites accumulating in the disease, particularly Phe that could lead to an increased production of free radicals. The increase in plasma lipid peroxides could also result from a reduction in enzymatic and non-enzymatic antioxidant defenses, common in patients under restricted diets.

Our findings showing a marked diminution of plasma L-carnitine levels and significantly increased TBARS (lipid



oxidation) in PKU patients that were reestablished with L-carnitine supplementation, as well as a significant inverse correlation between L-carnitine and malondialdehyde (MDA) blood levels, indicate that lipid peroxidation in PKU patients was mainly due to shortage of L-carnitine. In particular, it is presumed that L-carnitine has a protective role against ROS by scavenging hydroxyl radicals, formed in the Fenton reaction system (Pietta 2000; Derin et al. 2004). L-Carnitine can also reduce MDA levels by facilitating fatty acid transport thereby lowering the availability of lipids for peroxidation (Rajasekar et al. 2005).

In addition to lipid peroxidation, high levels of protein oxidative damage (high levels of carbonyl formation and reduced levels of sulfhydryl groups) were also observed in treated PKU patients not receiving LC and Se. Oxidative damage to proteins, lipids, or DNA may all be deleterious, however, proteins are the most important targets for ROS and secondary by-products of oxidative stress when these are formed *in vivo*, as they are the major component of most biological systems and can scavenge 50–75% of reactive radicals such as hydroxyl (Davies et al. 1999). For this reason, in the last decade, there has been a considerable growth in the number of articles reporting increased levels of protein damage in various human diseases often correlating well with the progression of the disease (Dalle-Donne et al. 2003). Oxidative damage to proteins is induced either directly by reactive species or indirectly by reaction of secondary products of oxidative stress. Some ROS-induced protein modifications can result in unfolding or alteration of protein structure, and some are essentially harmless events. However, not all proteins are equally sensitive to oxidative damage, and oxidation susceptibility depends on the structure of the protein (Dalle-Donne et al. 2005).

Furthermore, L-carnitine and selenium treatment reversed oxidation of thiol groups but did not alter the increase of carbonyls in PKU patients. This is probably because thiol groups are easily oxidized and reduced pending on the redox status of the cell. These groups can be oxidized by reactive species, especially at protein cysteine residues that may mediate regulatory processes of protein, potentially leading to alterations of protein function. In this context, mild oxidation of cysteines can generate sulfenic acid, inter- or intra-molecular disulfides, protein-mixed disulfides with low molecular weight thiols, and S-nitrosothiols, all reversible modifications (Woo et al. 2003). On the other hand, carbonylation of proteins, a widespread indicator of severe oxidative damage and disease-derived protein dysfunction is irreversible since carbonyls tend to form high-molecular-weight aggregates that are resistant to degradation and accumulate as damaged or unfolded proteins (Dalle-Donne et al. 2006).

In our study, we found a decrease of the enzymatic antioxidant capacity in erythrocytes of PKU patients not

receiving Se and LC, as verified by a decrease of SOD and GSH-Px activities. As regard to GSH-Px activity, it is possible that Se deficiency found in PKU patients could be responsible for the decrease in this activity, which is dependent on this micronutrient. In fact, Se supplementation restored the activity of this enzyme and the concentrations of plasma selenium were strongly correlated with the GSH-Px activity in erythrocytes, which reinforces this presumption and is in agreement with previous studies (Sierra et al. 1998; van Backel et al. 2000). On the other hand, it may be speculated that because of the reduction of these antioxidant activities, these PKU patients might have a lower capacity to scavenge hydrogen peroxide and superoxide reactive species. On the other hand, LC and Se supplementation did not alter SOD activity, which does not depend on Se.

We used erythrocytes in the present study because selenium-dependent glutathione peroxidase is expressed in these cells and they are highly susceptible to oxidative stress because their membranes are rich in polyunsaturated fatty acids and they have a large content of oxygen and iron (Carmagnol et al. 1983). Furthermore, selenium has a higher affinity for erythrocyte glutathione peroxidase than for plasma glutathione peroxidase (Lombeck et al. 1996).

In conclusion, this report corroborates previous studies showing that PKU patients are susceptible to oxidative stress caused by an increase in free radical production and a depletion in antioxidant capacity. More importantly, to the best of our knowledge this is the first report describing that the supplementation of a mixture containing selenium and L-carnitine to PKU patients for a long period was capable of correcting lipid and protein oxidative damage and restoring the GSH-Px activity. For this reason, selenium and L-carnitine supplementation might be an adjuvant therapy for PKU patients consuming artificial low-protein diets.

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