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L-Carnitine prevents oxidative stress in striatum of glutaryl-CoA dehydrogenase deficient mice submitted to lysine overload

Gilian Guerreiro[a,d,*], Alexandre U. Amaral[c], Rafael Teixeira Ribeiro[c], Jéssica Faverzani[a,d], Ana Carolina Groehs[a], Angela Sitta[b], Marion Deon[b], Moacir Wajner[b,c], Carmen Regla Vargas[a,b,c,d,*]

[a] Faculdade de Farmácia, UFRGS, Av. Ipiranga 2752, 90610-000 Porto Alegre, RS, Brazil
[b] Serviço de Genética Médica, HCPA, UFRGS, Rua Ramiro Barcelos, 2350, 90035-903 Porto Alegre, RS, Brazil
[c] Programa de Pós-Graduação em CB:Bioquímica, UFRGS, Rua Ramiro Barcelos, 2600, 90035 000 Porto Alegre, RS, Brazil
[d] Programa de Pós-Graduação em Ciências Farmacêuticas, UFRGS, Av. Ipiranga, 2752, 90610-000 Porto Alegre, RS, Brazil

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ABSTRACT

The deficiency of the enzyme glutaryl-CoA dehydrogenase leads to predominant accumulation of glutaric acid (GA) in the organism and is known as glutaric acidemia type I (GA1). Despite the mechanisms of brain damage involved in GA1 are not fully understood, oxidative stress may be involved in this process. Treatment is based on protein/lysine (Lys) restriction and L-carnitine (L-car) supplementation. L-car was recently shown to have an important antioxidant role. A knockout mice model (*Gcdh*$^{-/-}$) submitted to a dietary overload of Lys was developed to better understand the GA1 pathogenesis. In this study, we evaluated L-car and glutarylcarnitine levels, the lipid and protein damage, reactive oxygen species (ROS) production and antioxidant enzymes activities in striatum of *Gcdh*$^{-/-}$ and wild-type (*WT*) mice. We also determined the effect of the L-car treatment on these parameters. Thirty-day-old *Gcdh*$^{-/-}$ and *WT* mice were fed a normal chow (0.9% Lys) or submitted to a high Lys diet (4.7%) for 72 h. Additionally, these animals were administered with three intraperitoneal injections of saline or L-car in different times. *Gcdh*$^{-/-}$ mice were deficient in L-car and presented a higher glutarylcarnitine levels. They also presented lipid and protein damage, an increased ROS production and altered antioxidant enzymes compared to WT mice. Additionally, mice exposed to Lys overload presented higher alterations in these parameters than mice under normal diet, which were significantly decreased or normalized in those receiving L-car. Thus, we demonstrated a new beneficial effect of the L-car treatment attenuating or abolishing the oxidative stress process in *Gcdh*$^{-/-}$ mice.

1. Introduction

Glutaric acidemia type 1 (GA1) is a neurometabolic inherited disease with a worldwide prevalence estimated of 1:30,000–1:100,000 newborns [1,2]. It is caused by a severe deficiency of glutaryl-CoA dehydrogenase (*GCDH*, EC 1.3.99.7) activity, leading to a blockage in the metabolic pathway of the amino acids lysine (Lys), hydroxylysine and tryptophan [3] and resulting in an accumulation of glutaric acid (GA) and 3-hydroxy-glutaric (3-HG) in brain, blood, urine, CSF and other tissues [4,5]. Affected patients present mainly neurological symptomatology, without significant systemic manifestations, so that GA1 is considered a cerebral organic aciduria [4,6]. At birth, patients present macrocephaly and cortical atrophy [7]. Later, acute striatal degeneration develops during encephalopathic crises usually precipitated by situations of high catabolism, as infections or vaccinations. After these episodes, symptoms become worse being manifested as dystonia, dyskinesia, seizures, muscle stiffness and spasticity [5,6,8,9].

Urinary organic acids analysis by gas chromatography coupled to mass spectrometry (GC/MS) is the golden standard test for diagnosis of GA1, being characterized by high amounts of GA and 3-HG. The blood concentrations of glutarylcarnitine (C5DC), measured by liquid chromatography electrospray tandem mass spectrometry (LC/MS/MS), is used for its diagnosis at the newborn screening [10]. Rapid and aggressive treatment during crises of metabolic decompensation based on severe protein/lysine restriction, correction of hypoglycemia and dehydration, allied to L-carnitine (L-car) supplementation is essential for the survival of the affected individuals [11,12]. Treatment maintenance

* Corresponding authors at: Serviço de Genética Médica, HCPA, Rua Ramiro Barcelos, 2350, CEP 90035-903 Porto Alegre, RS, Brazil.
E-mail addresses: gilianguerreiro@gmail.com (G. Guerreiro), crvargas@hcpa.ufrgs.br (C.R. Vargas).

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is achieved by restriction of lysine intake and L-car supplementation in order to increase the excretion of the accumulated metabolites and to correct the secondary deficiency of this substance [13,14]. L-car is also critical to transport long-chain fatty acids across the inner mitochondrial membrane for utilization in β-oxidation, with subsequent ATP generation by the respiratory chain [15]. Moreover, more recently an anti-inflammatory and antioxidant effect have been attributed to L-car due its role decreasing free radical formation and increasing antioxidant enzyme activity [16,17], but this effect was not yet investigated in GA1.

A great deal of data in the literature indicates that GA and 3-HG are neurotoxic and crucial in the pathogenesis of the major symptoms presenting by GA1 patients. In this context, in vitro and in vivo animal studies have demonstrated deleterious effects of mainly GA causing excitotoxicity [18], bioenergetics impairment and oxidative stress [5,19–26]. Recently it was demonstrated that GA1 patients have a pro-oxidant and pro-inflammatory status and that L-car presents a protective role with antioxidant beneficial effects [19].

A knockout model was developed for GA1 in mice knocking out the *GCDH* gene ($Gcdh^{-/-}$) in the hopes to better elucidate the mechanisms of brain damage and helps understanding the pathogenesis of the disease. However, striatal degeneration characteristic of GA1, generally found in these patients were not satisfactorily reproduced [27]. For the purpose of improvement, an adaptation was performed with oral administration of a Lys overload to the $Gcdh^{-/-}$ mice [28]. It was then verified that GA concentrations in the brain of $Gcdh^{-/-}$ mice increased significantly and that they presented striatal lesion similar to GA1 patients [28].

In the present work we evaluated brain oxidative damage to biomolecules and more important the potential protective effects of L-car treatment upon this process in striatum of $Gcdh^{-/-}$ and wild type (WT) mice submitted to a normal diet (0.9% Lys) or to Lys overload (4.7% Lys).

2. Material and methods

2.1. Animals

Thirty-day-old wild type (WT) and $Gcdh^{-/-}$ mice with 30 days of age, of both sexes (52% males and 48% females), and of the 129SvEv background were used in the experiments and maintained at Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (UEA/HCPA). The animals were kept on a 12:12 h light/dark cycle in constant temperature (22 ± 1 °C) colony room. They had free access to water and food. Diets composed by a standard feed (0.9% Lys) with 20% protein (normoproteic diet) or a high Lys overload (4.7% Lys), with the same amount of protein were given to WT and $Gcdh^{-/-}$ mice for 72 h. In addition, these animals were submitted to three intraperitoneal injections of saline or L-car (100 $mg^{-1}\,kg^{-1}\,day^{-1}$, saline was used as solvent) on three different times. The first injection was performed 2 h prior to the beginning of the normal or high Lys diet. The second injection of saline or L-car administration occurred after 24 h and the third after 48 h after diet beginning. Animals were killed 24 h after the last dose of saline or L-car, i.e. after 72 h of exposure to the normal or high Lys diet, a period when $Gcdh^{-/-}$ animals reach very high brain concentrations of GA and 3-HG [28]. Striatum and blood samples were immediately separated for posterior analyses. All analyses were performed in triplicate.

2.2. Ethical statement

All animal procedures were performed in accordance with current Brazilian legislation and the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH (publication no. 85-23, revised in 2011). This study was approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre (16-0412). All efforts were made to minimize suffering, discomfort, stress and the number of animals necessary to produce reliable scientific data.

2.3. Experimental groups

$Gcdh^{-/-}$ mice were separated in Group KA (0.9% Lys + saline), Group KB (4.7% Lys + saline) and Group KC (4.7% Lys + L-car). WT mice were separated in Group WA (0.9% Lys + saline), Group WB (4.7% Lys + saline) and Group WC (4.7% Lys + L-car). Number of animals used was 5–7 per group. All groups of animals were treated simultaneously in parallel.

2.4. Tissue preparation

Mice were killed by decapitation with anesthesia by inhalation (isoflurane) after treatments. The brain was immediately removed and placed on a Petri dish on ice. Brain structures that did not interest to the study were discarded and the striatum was dissected and weighted. Next, the striatum was homogenized 1:15 (w/v) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The obtained homogenate was centrifuged at 750*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was used to measure the oxidative stress parameters. The obtained supernatant is constituted by a suspension of mixed and preserved organelles, including mitochondria and the enzymes quantified in this study. All procedures were performed in ice. Samples were stored immediately in a freezer, at −80 °C. After being frozen, thawing was only carried out at the time of the analysis and no refreezing was allowed.

2.5. Blood spot samples preparation

After decapitation, an appropriated paper filter was impregnated with a drop of whole blood (approximately 25 μL). This sample was used to determine free-carnitine (C0) and C5DC levels, as described in Section 2.12.

2.6. Thiobarbituric acid-reactive substances (TBARS)

Malondialdehyde levels were measured by the method of thiobarbituric acid-reactive substances (TBARS) according to Ohkawa et al. [29]. Briefly, in a glass tube were added 100 μL of homogenate, 50 μL of SDS 8.1%, 375 μL of 20% acetic acid and 375 μL of 0.8% thiobarbituric. The mixture was incubated at 100 °C for 30 min. After the incubation, it was made the extraction with butanol. A calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that of the samples. The color pink is proportional to the concentration of TBARS. The results were expressed as nmol TBARS/mg protein.

2.7. Sulfhydryl content

The sulfhydryl content was measured according to Aksenov and Markesbery [30]. This method is based on the oxidation of free thiols present in the sample that leads to the formation of disulfide bonds. The 5,5′ dithio-bis (2-nitrobenzoic acid) (DTNB) is reduced by sulfhydryl groups, generating a yellow derivate TNB, that can be read spectrophotometrically. The oxidative damage to proteins is inversely correlated with the sulfhydryl content. The results were expressed as nmol TNB/mg protein.

2.8. Carbonyl content

Determination of the carbonyl protein content, a marker of protein oxidative damage, was performed in 100 μL of supernatant, according Levine et al. [31]. The method is based on the combination of groups

carbonyl with dinitrophenylhydrazine (DNPH) and resolubilization in guanidine-HCl, after precipitation and centrifugation. The resulting staining was measured in a spectrophotometer at 370 nm and the results were expressed as nmol of carbonyls/mg of protein.

2.9. 2-7-Dihydrodichlorofluorescein (DCFH) oxidation

This assay is used to estimate intracellular generation of reactive oxygen species (ROS) [32], DCF-DA is oxidized by the ROS present in the cells to form a non-fluorescent DCFH, which is rapidly converted to the DCF, a higher fluorescent molecule. The fluorescence intensity is read at an excitation (488 nm) and emission (530 nm) wavelength. Briefly, 250 µL of DCF-DA stock solution, stored at −20 °C was added to 1 mL of 0.1 M NaOH and incubated at room temperature for 30 min. To stop the reaction, a neutralizing solution with phosphate buffered saline (PBS) was added, which converts DCF-DA to DCFH. Results were expressed by units of fluorescence (UF)/mg of protein.

2.10. Superoxide dismutase (SOD) activity

Total SOD activity was measured according to Marklund et al. [33]. This assay is based on the capacity of pyrogallol to autooxidize in a superoxide highly dependent process. In the presence of SOD, occurs an inhibition of pyrogallol autoxidation and the activity can be indirectly measured spectrophotometrically at 420 nm. Tissue supernatant was added to a reaction medium containing 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase and 0.38 mM pyrogallol. Results were expressed as unit (U)/milligram protein.

2.11. Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel et al. [34], using tertbutylhydroperoxide as substrate. The reaction medium of this assay comprised, besides the tissue supernatant, 100 mM potassium phosphate buffer containing 1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. NADPH disappearance in this reaction medium monitored at 340 nm was used to determine GPx activity. Results were expressed as unit (U)/milligram protein.

2.12. Free carnitine (C0) and glutarylcarnitine (C5DC) quantification

C0 and C5DC levels were determined in blood spots by liquid chromatography electrospray tandem mass spectrometry (LC/MS/MS) using the multiple reaction monitoring (MRM) mode, according to Chace et al. [35]. Punches of blood spot, in a size of 3 mm of diameter and 100 µL of standard solution (deuterium–labeled acylcarnitines in methanol) were mixed. After this step, the samples were shaken and the supernatant was evaporated with nitrogen in a temperature of 50 °C. The next step was the derivatization with 60 µL of 3 N butanolic-HCl followed by an incubation at 65 °C for 15 min. After this time, the samples were dried with nitrogen at 50 °C. Immediately before the injection in LC/MS/MS, 100 µL of an acetonitrile/water/formic acid (50/50/0.1%) mixture was added to the samples and centrifuged for 5 min. 30 µL of this mixture were injected in the LC/MS/MS using as mobile phase acetonitrile/water/formic acid (50/50/0.025%) with a gradient flow, without column. Other parameters such as entrance energy, collision energy and exit energy were 3 V, 16 eV and 2 V, respectively. The transition masses detected in MRM for C0 and C5DC were 218 ➔ 85 *m/z* and 388 ➔ 85 *m/z*, respectively. The cone voltage for both analytes was 29 V. Results were expressed as µM.

2.13. Lysine determination

Plasma Lys levels were determined by high-pressure liquid chromatography (HPLC) according to Joseph and Marsden [36]. The quantification was performed by relating the chromatographic peak areas of this amino acid in plasma to the known concentration areas of the amino acid in the mixture standard solution and of the internal standard (homocysteic acid). Results were expressed in µM.

2.14. Protein determination

Protein concentrations were measured by the method of Lowry et al. [37] using bovine serum albumin as standard.

2.15. Statistical analyses

All the analyses were performed using the GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA – version 5.0) software. Comparison between means was analyzed by one-way ANOVA followed by the Tukey multiple range test. Correlations were carried out using the Pearson correlation coefficient. A $P < 0.05$ was considered significant. The sample size was calculated using the Minitab® 16 program. A significance level $P < 0.05$ and a power of 80% were assigned for the calculations.

3. Results

3.1. L-car treatment attenuated L-carnitine deficiency and decreased glutarylcarnitine levels in Gcdh$^{-/-}$ mice

Firstly, we determined the blood concentrations of free carnitine (C0) and glutarylcarnitine (C5DC) in both *Gcdh*$^{-/-}$ and WT mice. Fig. 1A shows that *Gcdh*$^{-/-}$ mice have significantly decreased C0 levels in comparison to normal mice in all tested groups. Furthermore, *Gcdh*$^{-/-}$ animals fed a high Lys chow (KB) had a greater decrease of CO that

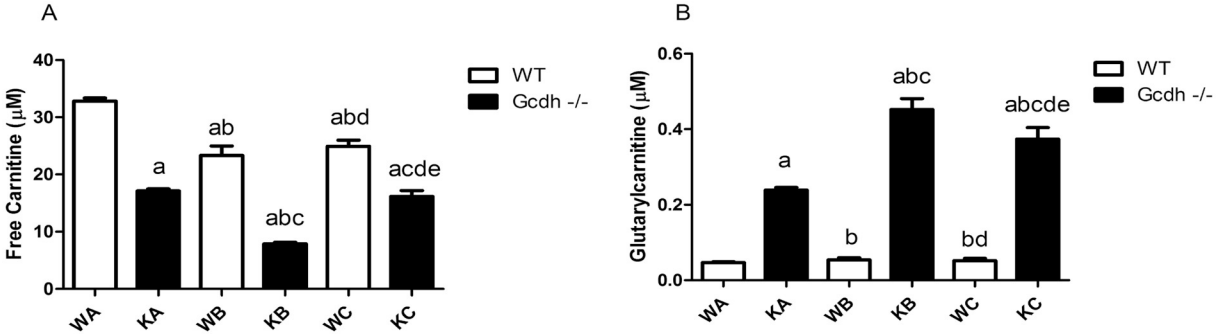

Fig. 1. (A) Free carnitine in blood from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice (B) Glutarylcarnitine measurement in blood from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice. Number of animals = 5–7 per group. Data represent the mean ± SD. $^{a}P < 0.05$ compared to *WA*. $^{b}P < 0.05$ compared to *KA*. $^{c}P < 0.05$ compared to *WB*. $^{d}P < 0.05$ compared to *KB*. $^{e}P < 0.05$ compared to *WC*. One-way ANOVA followed by the Tukey multiple range test.

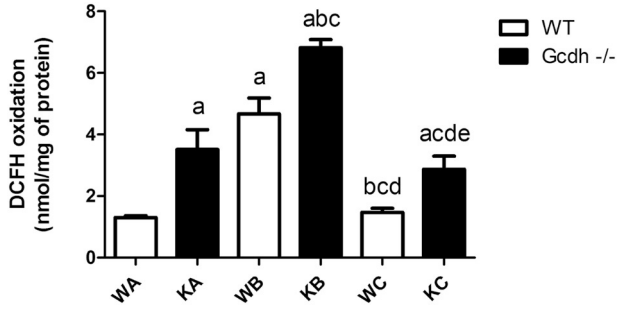

Fig. 2. DCFH oxidation in striatum from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice. Number of animals = 5–7 per group. Data represent the mean ± SD. $^{a}P < 0.05$ compared to *WA*. $^{b}P < 0.05$ compared to *KA*. $^{c}P < 0.05$ compared to *WB*. $^{d}P < 0.05$ compared to *KB*. $^{e}P < 0.05$ compared to *WC*. One-way ANOVA followed by the Tukey multiple range test.

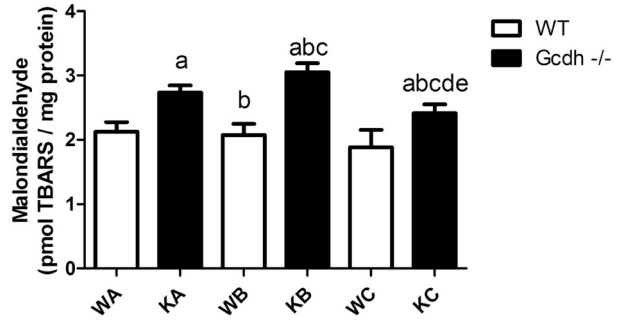

Fig. 3. Malondialdehyde (TBARS) in striatum from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice. Number of animals = 5–7 per group. Data represent the mean ± SD. $^{a}P < 0.05$ compared to *WA*. $^{b}P < 0.05$ compared to *KA*. $^{c}P < 0.05$ compared to *WB*. $^{d}P < 0.05$ compared to *KB*. $^{e}P < 0.05$ compared to *WC*. One-way ANOVA followed by the Tukey multiple range test.

was partially recovered by L-car treatment (KC) [$F(5,31) = 73.66$, $P < 0.05$], whereas there was no difference between WC and WB. Fig. 1B shows that *Gcdh*$^{-/-}$ mice have highly increased concentrations of C5DC than WT animals, especially when submitted to Lys overload (KB), and L-car administration reduced these levels by 21%, (KC) [$F(5,32) = 104.7$, $P < 0.05$].

3.2. *Gcdh*$^{-/-}$ mice presented higher ROS levels than WT animals. After L-car treatment, these levels were reduced in the *Gcdh*$^{-/-}$ group

To evaluate ROS production, DCFH levels were measured in both groups of animals, as showed in Fig. 2. *Gcdh*$^{-/-}$ animals presented higher DCFH levels than WT mice in all groups, indicating increased free radicals production in the knockout animals. Furthermore, the reactive species formation was higher in both groups of animals submitted to high Lys diet (KB and WB). It was also found a decrease in this parameter after L-car supplementation, mainly in *Gcdh*$^{-/-}$ mice, reducing at KA levels, a group that did not receive any treatment or Lys overload. By comparing groups KC and KB, it was verified that L-car induced a marked reduction in DCFH levels (around 134%). L-car treatment was effective not only in *Gcdh*$^{-/-}$ mice but also in WT animals since DCFH levels in WC group was significantly reduced when compared to WB group [$F(5,35) = 27.17$, $P < 0.05$].

3.3. *Gcdh*$^{-/-}$ mice presented higher lipid peroxidation than WT mice. L-car treatment decreased TBARS content

In order to evaluate lipid peroxidation, we determined the malondialdehyde (TBARS) content in striatum. Fig. 3 shows a marked increase of malondialdehyde levels in *Gcdh* $^{-/-}$ mice compared to WT animals in each tested group. It was also observed that lipid peroxidation was significantly enhanced in KB group (Lys overload group) and decreased in animals that received L-car treatment (KC group), reducing by 25% the malondialdehyde levels [$F(5,35) = 61.19$, $P < 0.05$]. Furthermore, there was no difference between WT groups.

3.4. *Gcdh*$^{-/-}$ mice presented protein oxidative damage compared to WT mice. L-car treatment was able to decrease protein oxidation

Sulfhydryl and carbonyl content were determined to evaluate protein oxidative damage in the striatum (Fig. 4A and B, respectively). Sulfhydryl content, which is inversely proportional to oxidative damage, presented significantly lower levels in *Gcdh*$^{-/-}$ mice compared to WT animals. The carbonyl levels were significantly higher in *Gcdh*$^{-/-}$ than in WT mice. It was also found a beneficial effect of the L-car treatment on sulfhydryl and carbonyl levels, reducing the carbonyl content (by 104% in KC group compared to KB) and enhancing the sulfhydryl levels (by 35% in KC group compared to KB) and L-car restore this parameter to control levels. It was also observed that Lys overload was able to increase protein damage. Sulfhydryl content was 34% lower and carbonyl content 200% higher in KB than WB groups [$F(5,35) = 10.58$, $P < 0.05$] (sulfhydryl content) and [$F(5,36) = 79.24$, $P < 0.05$] (carbonyl content).

3.5. Antioxidant enzyme activities SOD and GPx were increased in *Gcdh*$^{-/-}$ in relation to WT mice. L-car treatment was able to reduce these activities in knockout mice

We found significant differences between WT and *Gcdh*$^{-/-}$ mice in

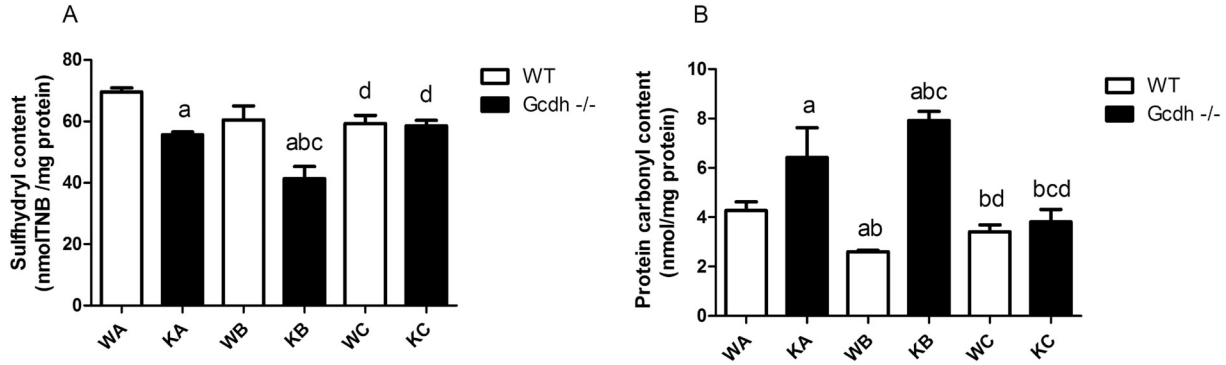

Fig. 4. (A) Sulfhydryl content in striatum from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice. (B) Protein carbonyl content in striatum from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice. Number of animals = 5–7 per group. Data represent the mean ± SD. $^{a}P < 0.05$ compared to *WA*. $^{b}P < 0.05$ compared to *KA*. $^{c}P < 0.05$ compared to *WB*. $^{d}P < 0.05$ compared to *KB*. One-way ANOVA followed by the Tukey multiple range test.

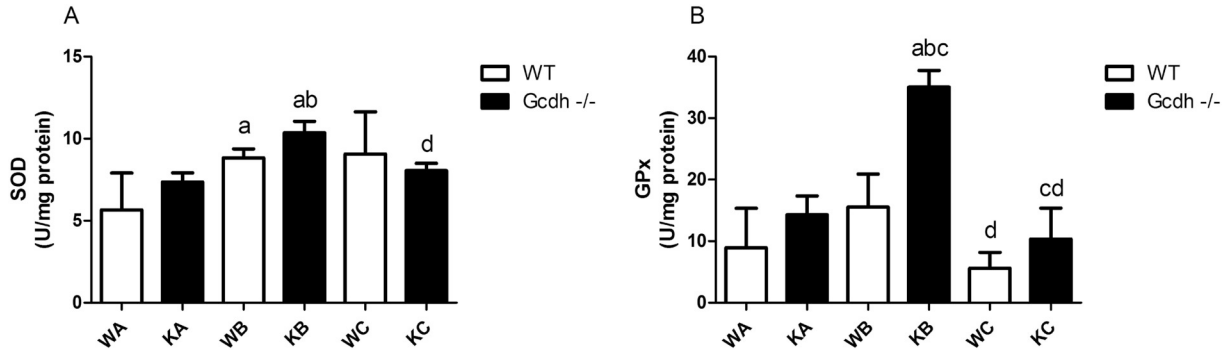

Fig. 5. (A) Superoxide dismutase (SOD) activity in striatum from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice. (B) Glutathione peroxidase (GPx) activity in striatum from *Gcdh*$^{-/-}$ (black bar) and *WT* (white bar) mice. Number of animals = 5–7 per group. Data represent the mean ± SD. $^{a}P < 0.05$ compared to *WA*. $^{b}P < 0.05$ compared to *KA*. $^{c}P < 0.05$ compared to *WB*. $^{d}P < 0.05$ compared to *KB*. One-way ANOVA followed by the Tukey multiple range test.

the activities of the antioxidant enzymes SOD and GPx, as shown in Fig. 5A and B. Animals that were submitted to a Lys enriched diet (KB group) presented a significantly increase of these activities, compared to the KA group. Furthermore, L-car treatment (KC group) reduced these activities to normal levels similar to the control KA group [$F(5,26) = 6.027$, $P < 0.05$] (SOD) and [$F(5,24) = 22.91$, $P < 0.05$] (GPx).

3.6. ROS was positively correlated with malondialdehyde and negatively with sulfhydryl content and free L-carnitine (C0)

We tested possible correlations between ROS (DCFH) and malondialdehyde (TBARS) levels. A positive correlation between these parameters was observed (Fig. 6A; $r = 0.8029$, $P < 0.05$). It was also verified negative correlations between DCFH and sulfhydryl content as well as between DCFH and C0, as shown in Fig. 6B ($r = -0.7537$, $P < 0.05$) and 5C ($r = -0.7350$, $P < 0.05$), respectively.

3.7. Free L-carnitine (C0) was negatively correlated with C5DC

It was also found a negative correlation between C0 and C5DC ($r = -0.6846$, $P < 0.05$) (Fig. 6D).

3.8. Plasma concentration of Lys was increased in Gcdh$^{-/-}$ mice receiving the dietary lysine overload

Table 1 describes the values found for the measurement of Lys in the plasma of *Gcdh*$^{-/-}$ and WT mice. Animals from groups KB and KC, that were submitted to a Lys enriched diet, presented a significantly increase, around 349% and 389% of this amino acid in relation those that

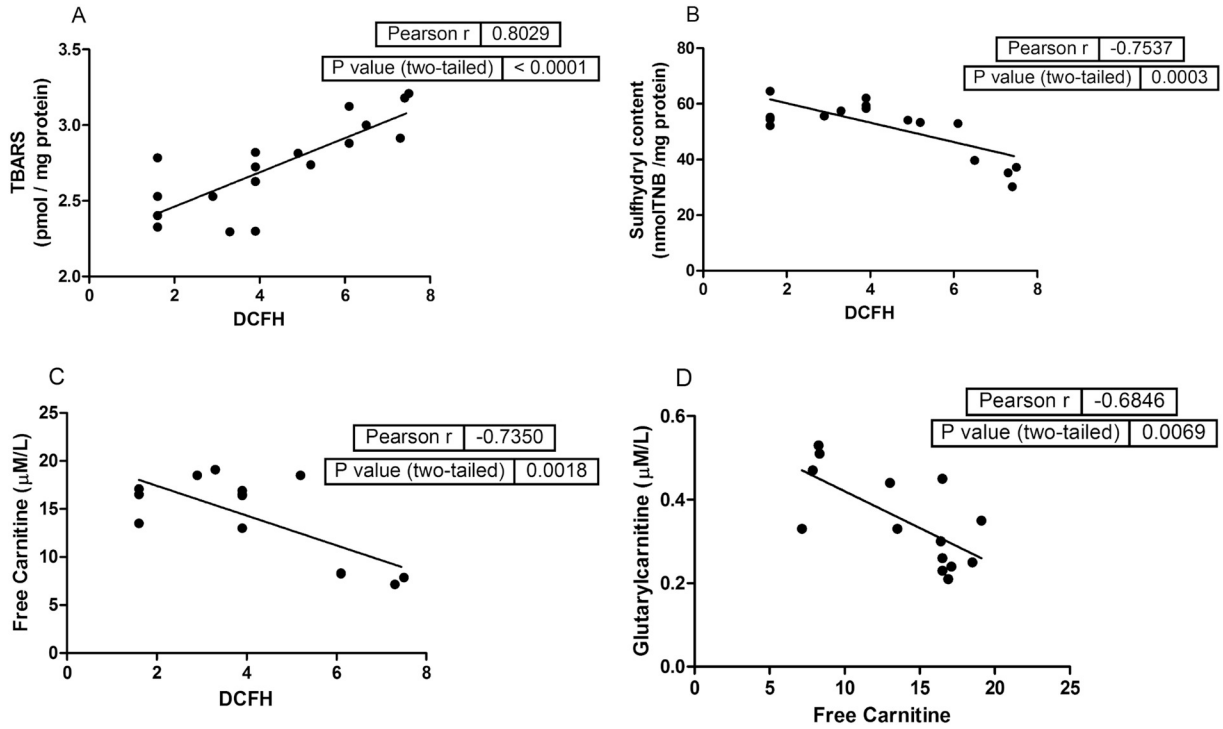

Fig. 6. Correlation between DCHF vs. TBARS values (A), DCFH vs. sulfhydryl content (B), DCFH vs. free carnitine values (C) and free carnitine vs. C5DC (D) in striatum from *Gcdh*$^{-/-}$ mice. Graphs show the Pearson correlation coefficient.

Table 1
Plasma concentrations of Lysine in *Gcdh*$^{-/-}$ and WT mice.

Group	Lysine μM
KA	223.9 ± 74.7
KB	782.83 ± 120.4[a]
KC	873.2 ± 50.6[a]
WA	311 ± 16.01[b,c]
WB	442.83 ± 58.23[a,b,c]
WC	323.2 ± 54.65[b,c]

Values represents mean ± SD. One-way ANOVA followed by the Tukey multiple range test.
[a] $P < 0.05$ compared to KA.
[b] $P < 0.05$ compared to KB.
[c] $P < 0.05$ compared to KC.

were fed with normal diet (KA). The lysine concentration in the animals of groups KB and KC were also higher than that presented by the WT animals. In addition, no significant differences were found between WT animals that received and that not receiving dietary lysine overload.

4. Discussion

GA1 is a severe neurometabolic disease starting early in life usually during catabolic states with acute encephalopathy that may lead to death and are associated with irreversible striatal damage, as well as with progressive cortical atrophy, although there are late onset insidious variants without apparent metabolic crises that also result in relevant neurological symptoms [4,38,39]. A great deal of studies have been developed to uncover the underlying mechanisms causing the brain damage and, consequently, explain the main cerebral signs and symptoms of this disease [25,40]. In this context, dietary Lys restriction and L-car supplementation were shown in the last decades to prevent approximately two third of the encephalopathic crises that affect GA1 patients in the first three years of live [2,4]. Recently, Guerreiro et al. have described beneficial effects of L-car on oxidative damage to proteins, lipids and DNA in blood of GA1 patients [19]. These are interesting findings and may be related to previous findings demonstrating oxidative and nitrative damage, involving ROS and RNS, in brain of the genetic mice model of GA1 [41–43], so that this deleterious process may possibly underlie the acute striatum degeneration and the progressive cortical damage in GA1 and L-car a protective role.

Our present study tested the effects of L-car as a protective antioxidant agent on oxidative damage in striatum of the genetic animal model of GA1, since to the best of our knowledge there are no studies in the literature evaluating L-car effect at the central level in this GA1 model.

Therefore, the levels of C0 and C5DC were initially measured in *Gcdh*$^{-/-}$ and WT animals. Our results showed that *Gcdh*$^{-/-}$ animals were deficient in free L-car compared to WT mice, presenting a similar pattern to GA1 patients [4,19]. For animals that underwent Lys overload, this deficiency was even more severe and was corrected by L-car treatment. These data reinforce the important role of the use of L-car since it corrects the secondary deficiency of this compound with important roles on cellular survival [3,4].

Since the brain is a tissue that particularly requires high oxygen consumption and presents a low antioxidant defense compared to other organs, it has a greater susceptibility of brain damage in biomolecules such as lipids and proteins [44]. The damage to these biomolecules has already been evaluated in the GA1 genetic animal model, as well as in blood of GA1 patients. Seminotti et al. verified that *Gcdh*$^{-/-}$ submitted to Lys overload showed increased levels of MDA and decreased sulfhydryl content in brain [40,45]. These results were attributed to the increase in GA and 3-HG production caused by dietary Lys overload. In line with these findings, Latini et al. have verified that acute and chronic administration of GA in rats lead to an increase in lipid peroxidation, indicating that this organic acid s involved in oxidative damage [22,46].

The oxidation of proteins by free radicals can lead to a malfunction of enzymes, receptors and transport proteins. In the present study, we evaluated the sulfhydryl and carbonyls content, which reflects, respectively, the reversible oxidation caused by reactive species to proteins containing amino acid thiol groups and the secondary reactions that lead to protein carbonylation [30,31,47]. Another situation that can lead to loss of function and, often, cell death is the high levels of peroxidated lipids, which may be harmful to cell membranes, and has been evaluated by malondialdehyde determination by the TBARS method [29].

Corroborating with the literature, we verified that striatum from *Gcdh*$^{-/-}$ animals presented greater damage to lipids (increased malondialdehyde levels) and proteins (elevated sulfhydryl oxidation and carbonyl content) when compared to WT mice. Besides, this damage was higher in mice submitted to Lys overload, whereas L-car treatment attenuated lipid and protein oxidation.

Furthermore, ROS are known to induce damage to biomolecules, severely disturbing cell homeostasis [44] and the organic acids accumulated in GA1 were shown to stimulate ROS production and reduce the antioxidant defenses, such as glutathione levels, as well as to cause changes in the activity of various antioxidant enzymes [43,45,48] and probably explain why dietary Lys overload that lead to increased GA and 3-HG concentrations accelerate these processes [40,41,49].

Thus, we determined striatum DCFH levels as an indicator of reactive species formation and evaluated enzymatic antioxidant defenses by analyzing SOD and GPx activities. It was found that striatum of *Gcdh*$^{-/-}$ mice have increased DCFH levels, as well as SOD and GPx activities compared to normal animals, and these parameters were further enhanced by high intake of Lys by the animals. These enzymatic antioxidant defense activities have different mechanisms to detoxify and protect the cell against an imbalance in redox homeostasis [50]. Therefore, it seems reasonable to postulate that the increased ROS production may be related to the changes in the activities of SOD and GPx. Moreover, L-car again showed beneficial effects since animals treated with this substance had lower values for reactive species formation and lower enzymatic activities. Alterations in antioxidant defenses and a protective role of L-car have been already observed in MSUD and GA1 patients [19]. In this context, L-car is widely used in the treatment of organic acidemias in general, including GA1, since this compound is able to correct its secondary deficiency, besides helping in the removal of the accumulated organic acids in the form of carnitine esters [15]. This process is mediated through the action of the enzyme carnitine acyltransferase, which catalyzes the formation of glutarylcarnitine and other carnitine esters, and this process is also able to restore the concentration of free intramitochondrial CoA and consequently the free acyl CoA/CoA mitochondrial ratio [51]. The doses of L-car used in patients for the treatment of these diseases range from 100 to 200 $mg^{-1}\,kg^{-1}\,day^{-1}$, depending on the needs and clinical status of the patients [52].

More recently potential antioxidant and anti-inflammatory effects of L-car have been observed in various neurometabolic diseases, including GA1 [19,53]. In this context, Mescka et al. quantified L-car levels in the brain tissue of rats with a chemically-induced animal model of maple syrup urine disease, demonstrating that this molecule cross the blood brain-barrier and prevents the brain oxidative alterations caused by the toxic accumulating metabolites [54]. Part of this antioxidant potential of L-car is due to its capacity to act in the Fenton reaction and to chelate Fe^{2+} ions. This helps to prevent the formation of reactive species. In addition, this compound has been shown to act as a free radicals scavenger and reduce their formation, as the superoxide radical, which is involved in the formation of other reactive species [55].

The relationship between ROS formation and lipid and protein oxidation was defined by the correlations between DCFH and TBARS

and sulfhydryl content, since the marker reflecting high reactive species concentration (DCFH) was positively correlated with a marker of lipid oxidative damage (TBARS) and negatively correlated with a marker of protein oxidative damage (sulfhydryl content). It should be remembered that the animals with the highest values of these markers were those that had a higher concentration of Lys in their diets and higher concentrations of C5DC, indicating that Lys overload leads to an increase in the concentrations of GA and of reactive species leading to oxidative damage, which is very probably involved in the pathophysiology of this disease. However, it is important to note that even animals that were not submitted to Lys overload presented higher values of these parameters of oxidative stress as compared to WT mice, due to the inherent motifs of the knockout genetic condition.

On the other hand, the negatively correlation between C0 and DCFH, as well as between C0 and C5DC in *Gcdh*$^{-/-}$ striatum found in this study reinforces the beneficial effects of L-car in GA1 treatment. Its fundamental role in GA1, as described earlier, is based in the understanding that L-car also induces the elimination of the toxic metabolites and indirectly aids in the decrease of the reactive species production, probably by also removing the toxic acyl-CoA clusters, or through its antioxidant benefits, or possibly by the combination of these mechanisms.

In conclusion, we found that *Gcdh*$^{-/-}$ mice have increased markers of oxidative damage in striatum in relation to WT animals, which is even more evident when under Lys overload. We also demonstrated for the first time in literature that L-car treatment is able to attenuate these processes in brain tissue. Our results are promising and help to better understand the pathophysiology of GA1, since there may be a high contribution of oxidative stress in the mechanisms responsible for brain damage found in GA1 patients. Nevertheless, more studies are needed in order to better understand the mechanism by which GA acts and by which L-car promotes these beneficial effects on the oxidative damage found in this disease.

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Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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